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

4

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22

23 **Highlights**

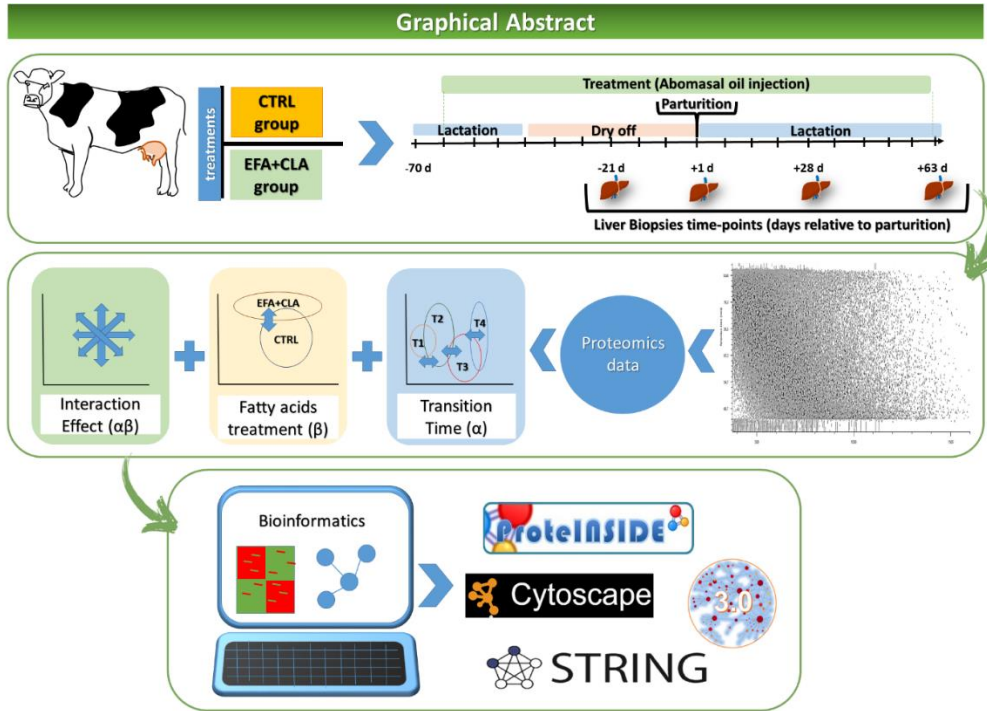
24 1- ANOVA-simultaneous component analysis applied to time course experimental design

25 2- Oxidation capacity as a signature of hepatic metabolic adaptation to lactation

26 3- Fatty acid (FA) supplementation amplified hepatic FA oxidation

27 4- Ligand-activated nuclear receptors primary regulate hepatic FA oxidation

28 **Graphical abstract**



29

30

31 **Abstract**

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

45

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

47 **Significance**

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

58 **1. Introduction**

59 A state of negative energy balance (NEB) during the transition from late gestation to early lactation initiates a series
60 of profound metabolic and physiological adaptations in dairy cows to meet the energy demands for milk production
61 [1]. During NEB, fatty acids (FA) are mobilised from adipose tissue to be oxidized in the liver for supplying energy
62 [2]. Therefore, the major adaptive mechanism is shifting towards the use of non-esterified fatty acids (NEFA) by
63 hepatocytes, where they are further metabolized via various pathways [3]. Numerous transcription factors and
64 coactivators, such as peroxisome proliferator-activated receptors (PPAR) and sterol regulatory element-
65 binding proteins (SREBPs), control these regulatory mechanisms [4]. Also, various nutritional treatments, e.g., FA
66 that are not only substrates for generating energy but also natural ligands for nuclear receptors [5], may interact and
67 impact metabolic adaptations [6, 7].

68 Over the past decades, mass spectrometry (MS)-based proteomics technology has emerged and matured as a powerful
69 approach to discern the key factors contributing to systemic metabolic homeostasis and health in many species,
70 including ruminants [8-11]. With a growing interest that has been paid to proteomics studies, it is not uncommon to
71 have an intricate experimental design with different time points (or ‘longitudinal’), treatments (multi-group, e.g.
72 different dose groups), and multi-subject (containing data of multiple animals) [12]. For instance, *in vivo* longitudinal
73 animal studies frequently deal with random physiological states (such as lactation, pregnancy, and growth), which
74 could stand as the primary source of variation, especially if the treatment effect is negligible. Such intricate
75 experimental designs call for specific multivariate analysis with predefined matrices of additive effects.

76 One approach would be the principal component analysis (PCA) which is designed for reducing the dimensionality of
77 large datasets while increasing interpretability [13, 14]. However, the straightforward use of PCA without predefining
78 the factors may come up with clusters in which the primary sources of variation may be due to the longitudinal effect
79 instead of the treatment effect [15]. Combining the analysis of variance with PCA led to the development of the
80 ANOVA-simultaneous component analysis (ASCA) method (developed by Smilde [12]). This method is particularly
81 suited for time-resolved studies and has the advantages of decomposing variability separately within the ‘treatment’
82 and ‘time’ and between ‘time and treatment’ (interaction of time and treatment). Subsequently, PCA is performed on
83 each defined source of variation independently (for review [16]).

84 The ASCA design has been previously reported in some studies, including a metabolomics intervention study, in
85 which guinea pigs were treated with varying doses of vitamin C, and their urine metabolite profiles were analyzed
86 using NMR spectroscopy at several points in time [12]. The application of this design is not limited to metabolomics
87 [17], but there is no report on other omics-based datasets yet.

88 Previously, we have investigated in detail the effect of supplementation with essential FA (EFA) and conjugated
89 linoleic acids (CLA) on the liver proteome of dairy cows in several time points from the ante (AP) to the postpartum
90 (PP) period without considering time as a fixed effect (since it was not the main focus of our study [60]). This routine
91 procedure had pointed out and emphasized on the treatment effect, and was complemented by our specific longitudinal
92 design for its potential for revealing yet undiscovered aspects: i.e., exploring the shift of proteins within the transition

93 from gestation to lactation could be particularly informative in understanding the physiology of adaptation and
94 lactation as a secondary purpose of a study. Moreover, relatively little is known about hepatic metabolic adaptation in
95 transition dairy cows in response to supplemented FA (interaction effect) at the proteome level.

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

102

103 **2. Material and methods**

104 **2.1. Experimental design, sampling, and peptide preparation**

105 The study used raw LC-MS/MS results from our previously reported liver proteomics study [60]. Briefly, 16
106 multiparous Holstein dairy cows ($11,101 \pm 1,118$ kg milk/305 d in second lactation and BW of 662 ± 56 kg; means \pm
107 SD) were abomasally injected with either a control fat (coconut oil; CTRL, n = 8; Bio-Kokosöl #665, Kräuterhaus
108 Sanct Bernhard, KG, Bad Ditzgenbach, Germany) or EFA+CLA supplement, containing a combination of linseed oil
109 (DERBY® Leinöl #4026921003087, DERBY Spezialfutter GmbH, Münster, Germany), safflower oil (GEFRO
110 Distelöl, GEFRO Reformversand Frommlet KG, Memmingen, Germany) and Lutalin® (CLA, n = 8; 10 g/d cis-9,
111 trans-11, trans- 10, cis-12 CLA, BASF SE, Ludwigshafen, Germany) from d 63 AP until d 63 post PP (Figure 1 A).
112 The experimental procedures were carried out at the Research Institute for Farm Animal Biology (FBN),
113 Dummerstorf, Germany and approved by German Animal Welfare Act (Landesamt für Landwirtschaft,
114 Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, Germany; LALLF M-V/TSD/7221.3-1-038/15).
115 Liver tissues were obtained from each animal on d -21 AP, and d 1, 28, (by biopsy) and 63 PP after slaughtering the
116 cows (Figure 1 A). More information regarding diet ingredients, chemical composition, performance, and plasma
117 metabolite data can be found elsewhere [18].

118

119 **2.2. Liver sample preparation and proteomics analysis**

120 The preparation steps were previously explained in more detail in [60]. Briefly, extracted liver proteins were subjected
121 to in-gel digestion and the peptide mixtures were then analyzed using high-resolution nano-liquid chromatography
122 (Ultimate 3000 RSLC nano-system (Dionex)) coupled to an Orbitrap Q Exactive HF-X mass spectrometer (Thermo
123 Fisher Scientific) [60] (Figure 1 B). It is important to point out that some steps were considered to reduce between-
124 group variability and increase the power of analysis. In this regard, LC-MS/MS was performed on all 64 samples
125 consecutively but randomly without any order related to time or treatment using the same setting and unique analytical
126 columns. Before and after MS analysis, LC-MS/MS efficiency (quality of liquid chromatography separation and mass

127 spectrometry performance) was checked using the Pierce™ HeLa Protein Degradation Standard (catalogue number:
128 88328 Thermo Scientific™). For more details, see [60].

129

130 **2.3. Data processing**

131 Peptides MS/MS spectra were aligned to the reference sample automatically defined by Progenesis QI software
132 (version 4.2, Nonlinear Dynamics, Newcastle upon Tyne, UK). It has to be highlighted that the reference sample is
133 defined regardless of time or treatment, and alignment is done to all samples to obtain a set of comparable peaks. After
134 peptide quantification, the identified/quantified peptide ions were searched against a *Bos taurus* decoy database
135 (Uniprot, download date: 2019/11/07, a total of 37,513 entries) using MASCOT (version 2.5.1) interrogation engine.
136 The specific validated peptides were then inferred to corresponding proteins and their intensities in Progenesis QI
137 software (Figure 1B). A total of 1681 proteins were maintained for analysis after applying strict exclusion criteria
138 (deamidated, carbamidomethyl, and oxidation contaminant proteins, having at least two peptides and two unique
139 peptides, and presence in at least 50% of the samples in each treatment group/timepoint) [19].

140

141 **2.4. Decomposing matrices for ASCA and statistical analysis**

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

145 The ASCA method was described in detail previously [12, 20]; here, we have briefly illustrated its design for our
146 proteomics dataset. In this study, a balanced experiment was structured by ASCA. Our proteomics dataset comprised
147 64 distinct proteomes that were organized as described below (Figure 1C):

148

149 Individuals: 16 cows were included in the experiments.

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

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

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

155

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

158

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

159

160 Equation (1) indicates a series of j ANOVAs where μ is an overall offset, α_i the effect of the first factor (transition
161 from gestation to lactation), β_j the effect of treatment (supplemented FA), $(\alpha\beta)_{ij}$ the interaction between them, $S_k(j)$
162 is the random effect of the k^{th} subject and ϵ_{kij} the residuals.

163

164 Then, applying PCA to each score sub-matrix in (1) (indicated by T_α , T_β , and $T_{\alpha\beta}$) and submodel loadings (are
165 given by matrices P_α , P_β , and $P_{\alpha\beta}$) and examining estimated effects for all variables simultaneously by

$$(2) X = X_m + T_\alpha P_\alpha T_\alpha + T_\beta P_\beta T_\beta + T_{\alpha\beta} P_{\alpha\beta} T_{\alpha\beta} + X_e,$$

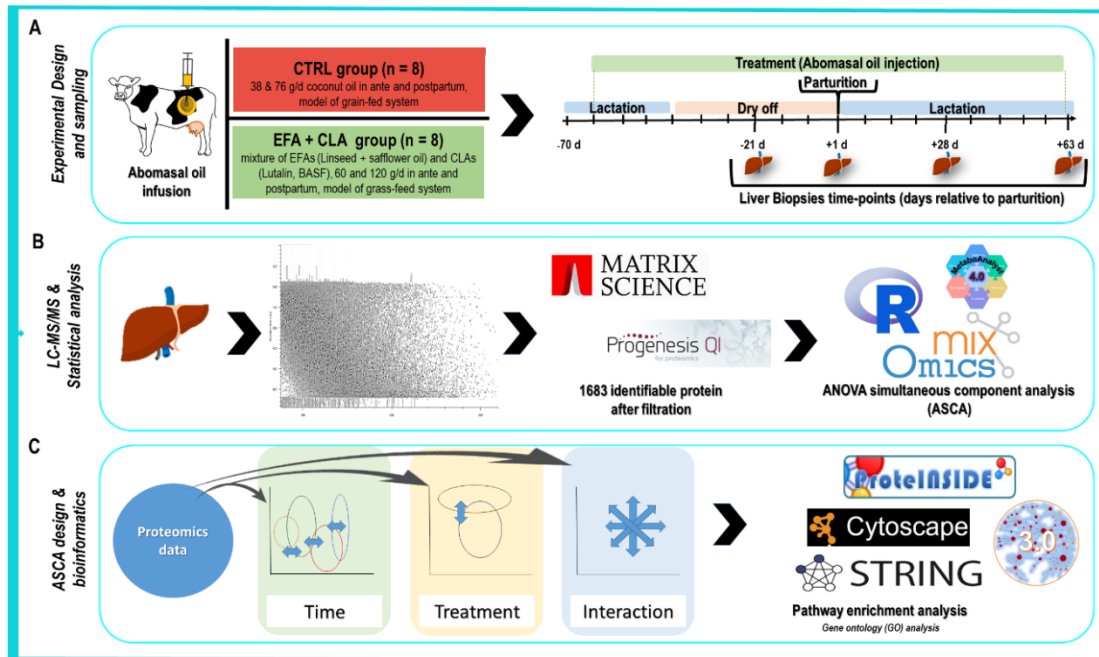
167
168 where T and P - as mentioned before- represent the scores and loadings matrices for each corresponding factor or
169 interaction, respectively, and X_e defines the residual or deviations of each individual replicate from the average
170 effects. The performed operation (2) was a Simultaneous Component Analysis (given the name ASCA) or repeated
171 PCA on a common set of measured variables allocated to predefined matrices. The following criteria were set to
172 decompose the ASCA model in R: Leverage threshold= 0.9, and alpha threshold= 0.05.

173

174 **2.5. Bioinformatics analysis of differentially abundant proteins**

175 Before bioinformatics analysis, proteins' accession was converted into Gene ID by the UniProt (retrieve/ID mapping)
176 database conversion tool, and undefined proteins were blasted and replaced with their Gene ID in other species. The
177 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis of DAP
178 were performed with STRING (version 11.0) and proteINSIDE (version 1.0) setting *B. Taurus* genome as background,
179 and only pathways enriched with P-value < 0.05, corrected to FDR with Benjamini-Hochberg method (p-adjust <
180 0.05) and at least two hits in each term were considered significantly enriched (Figure 1 C). Protein-protein interaction
181 networks were constructed and visualized by inputting the DAP identified on main effects (time, treatments, and their
182 interaction) to Cytoscape version 3.8.2 software, in which nodes and edges represent proteins and their interactions,
183 respectively [19].

184



185

186 Figure 1) Schematic diagram of the (A) study design, (B) proteomics workflow and peptide identification, and
 187 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
 188 +63 d relative to parturition). Bold lines indicate liver biopsy sampling timepoints. (B) High-resolution LC-MS/MS analysis, peptide alignment
 189 (progenesis), and protein identification (mascot) procedure were performed by Progenesis software coupled with the Mascot search engine,
 190 statistical analysis was based on Multivariate Analysis of variance – simultaneous component analysis (ASCA), and (C) ASCA design and
 191 bioinformatics analysis.

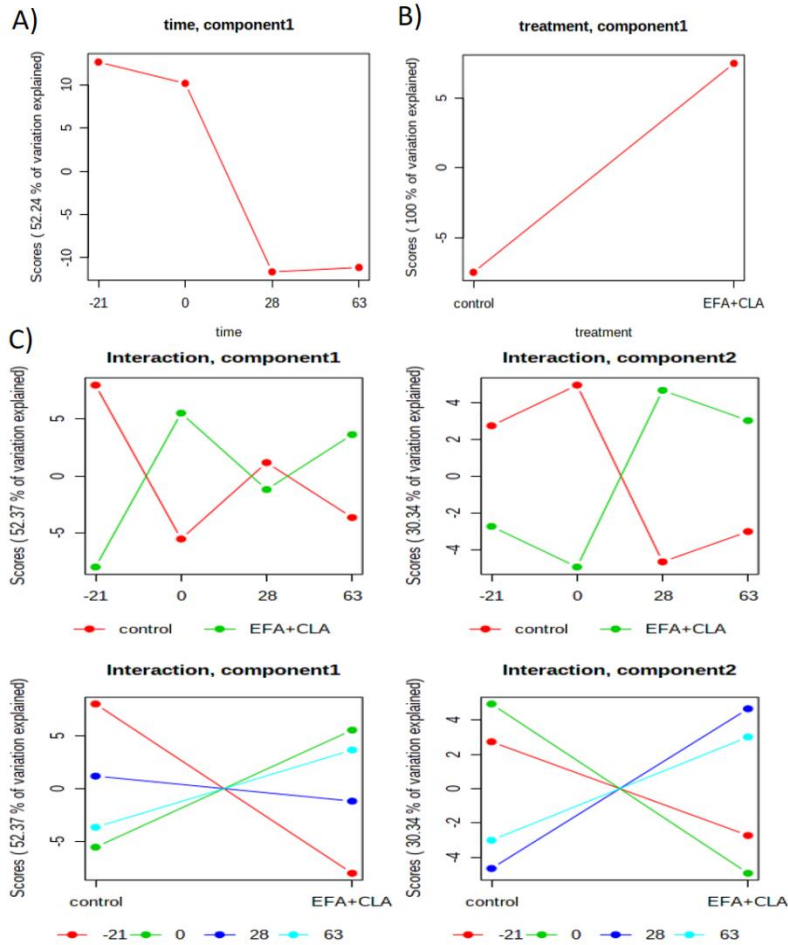
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193 3. Results and discussion

194

195 3.1. Differential proteomic analysis: repeated measurements analysis of variance – simultaneous 196 component analysis

197 From a total of 1681 proteins, 116 proteins during the transition period, 43 proteins between treatments, and 97 proteins
 198 in the interaction of them were identified as differentially abundant (Table 1, more details are provided [19]). Figure
 199 2 represents the major pattern described by the ASCA model associated with transition time, FA treatment, and their
 200 interaction, respectively. Figure 2 A, is a time score plot based on component 1 (52.24% of variation explained) and
 201 demonstrated that there is a considerable difference (elbow break) between days 0 and 28. Figure 2 B showed that the
 202 groups differed in their principal component (PC) 1 scores (100% of variation explained), with the CTRL and
 203 EFA+CLA groups exhibiting the lowest and highest scores, respectively. Figure 2 C visualizes the major pattern
 204 assessed for the interaction effect on PC1 (more than 50% of variation explained) and PC2 (more than 30% of variation
 205 explained). Leverage/SPE scatter plots, scree plots, and the permutation tests are provided in Supplementary Figure
 206 S1.



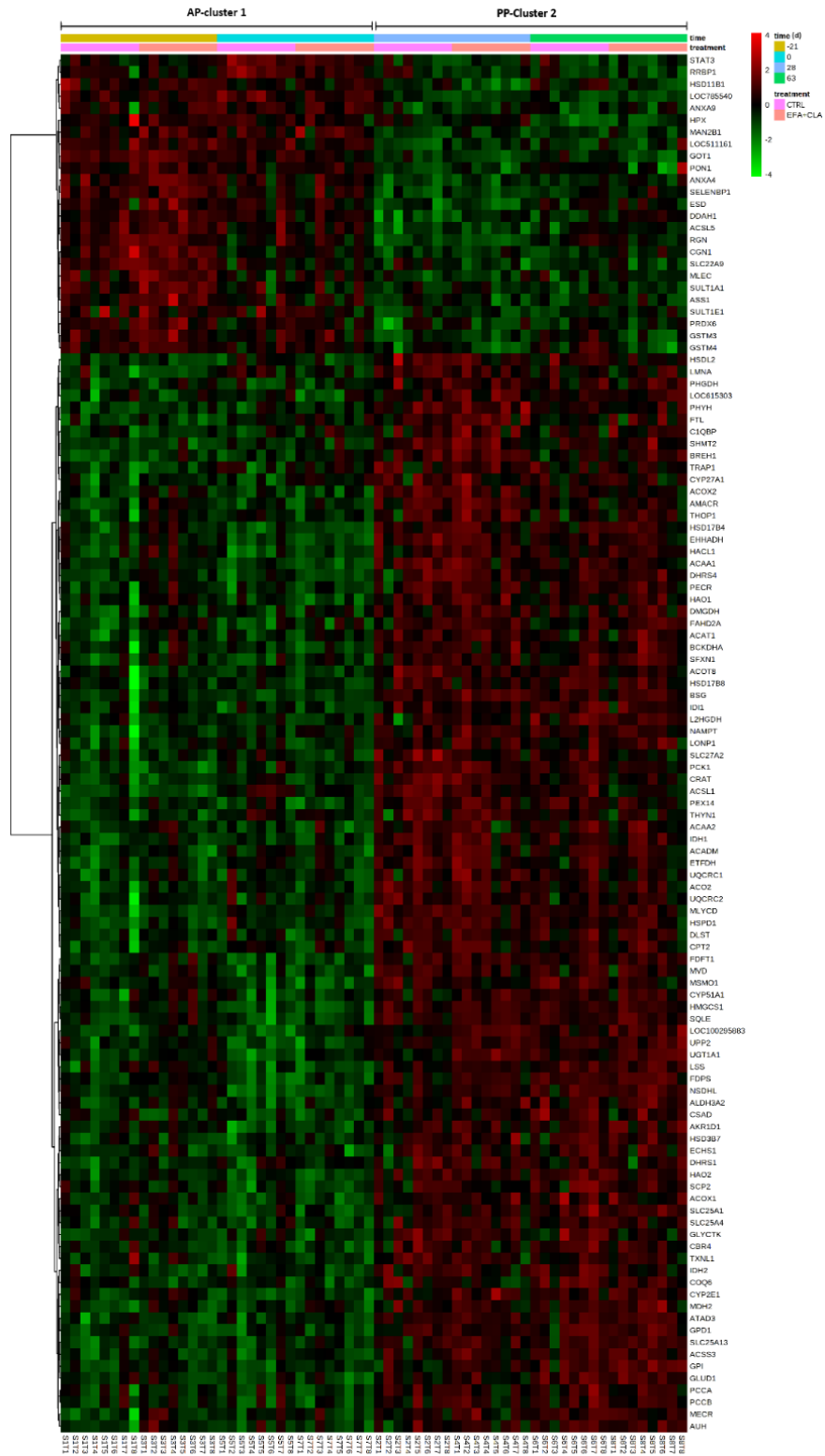
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208 Figure 2) Major patterns associated with transition time (A), FA treatment (B) and their interaction (C) calculated by analysis of variance -
 209 simultaneous component analysis (ASCA), in dairy cows supplemented with or without EFA+CLA in 4 time-points (-21, +1, +28, and +63 d
 210 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
 211 interaction of them (ab).

212

213 3.2. Gene ontology and functional enrichment analyses of differentially abundant proteins during the 214 transition period

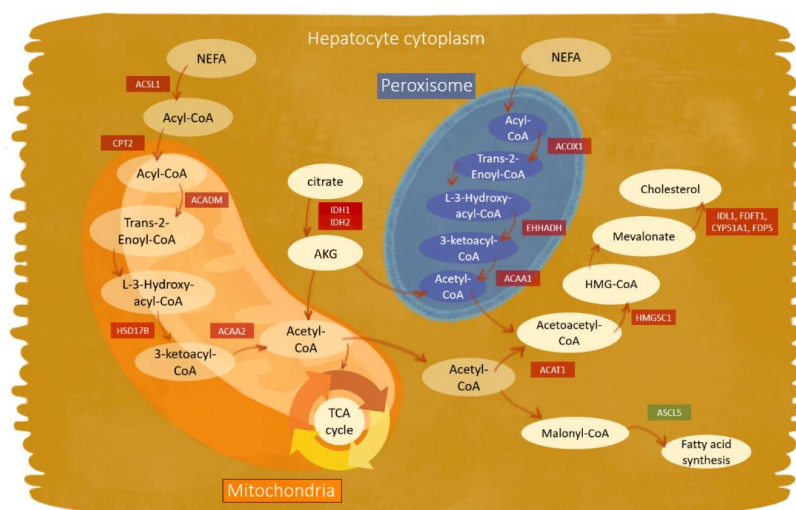
215 The relative abundance of DAP during the transition period is illustrated in a Heatmap (fold changes ranged from -4
 216 to +4) in Figure 3. The protein abundance patterns within time points are graphed in the score plot (Figure 2 a), in
 217 which the only considerable difference among them was observed between d +1 and d +28, that was also seen by two
 218 separate clusters containing d -21 AP and +1 PP as the first cluster (AP-cluster1) and d +28 and +63 PP as the second
 219 one (PP-cluster2). The first cluster (AP-cluster1) was representative of AP; vice versa, PP-cluster2 represented the PP
 220 period. Out of the 116 DAP obtained during the transition period, the relative abundance of 93 proteins increased, and
 221 23 proteins decreased in PP-cluster2 compared with AP-cluster1 [19].



222

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

250 These findings were consistent with previous liver proteome [21], and transcriptome [22] studies that have reported
 251 enrichment of carbohydrates, lipids, and protein metabolism-related pathways in the early and/or peak of lactation
 252 compared to the dry period to support milk synthesis. Since none of the cows in any treatment group showed any signs
 253 of metabolic disorders, all these massively enriched pathways could be considered as conventional metabolic
 254 adaptations to preserve whole-body metabolic homeostasis during the NEB period. Indeed, we have previously
 255 reported [18] the elevated plasma concentrations of NEFA and β -hydroxybutyrate (BHB) during the transition from
 256 late pregnancy to early lactation, implying that dairy cows from the present study were in a classical physiological
 257 NEB state. Thus, within the liver increased gluconeogenesis and ketogenesis are expected to interconvert and
 258 metabolize nutrients to support pregnancy and lactation. Consistent with this very general view of liver metabolic
 259 adaptations, we have identified proteins involved in ketogenesis, gluconeogenesis, and oxidative capacity through
 260 both the TCA cycle and the cytosolic organelles synthesis.
 261 Indeed, once taken up by the liver, NEFA are oxidized either via Acetyl-CoA through the TCA cycle or in ketone
 262 bodies from ketogenesis. The over-abundance of ACO2, DLST, IDH1, IDH2, MDH2, and PCK1 related to the TCA
 263 cycle, as well as the over-abundance of ACAT1, and HMGCS1 involved in ketogenesis, in the PP period relative to
 264 the AP period, strengthened the robustness of the proteome analysis and the ASCA analysis. Indeed most of them
 265 were previously identified by differential proteome during the transition period [11, 21, 23-25]. Some proteins may
 266 be highlighted such as the overabundance of both the ACO2 and IDH mitochondrial enzymes known to induce α -
 267 ketoglutarate (AKG) production (from citrate) that serves as an energy source and also as a precursor for glutamine,
 268 gluconeogenesis, and synthesis of acute-phase proteins [26]. We observed an overabundance of both cytosolic and
 269 mitochondrial IDH isozymes (IDH1 and IDH2, respectively) in the PP period, indicating activated IDH2/IDH1 shuttle
 270 transferring high energy electrons in the form of NADPH from mitochondria to cytosol [27]. Moreover, an over-
 271 abundance of ACAT1 and HMGCS1 was reported in feed-restricted ketotic cows in the PP period [28]. Part of the
 272 well-known DAP involved in FA oxidation and mevalonate pathway were highlighted in the schematic Figure 5.
 273



274
 275 Figure 5) Schematic of fatty acid oxidation in dairy cows' hepatocyte. In the pathway map, only the differentially abundant proteins in the
 276 postpartum period are highlighted; red colour indicates upregulation; green designated downregulation.

277 In dairy cows, propionate as a primary source but also lactate, AA (specifically L-alanine), and glycerol can be
278 oxidized indirectly through the TCA cycle to supply carbon for gluconeogenesis. The entry point of these substrates
279 differs and could be through either succinate, oxaloacetate (OAA), or Acetyl-CoA, which is under the control of
280 different isoforms of phosphoenolpyruvate carboxykinase (PEPCK). Here, we observed the PP overabundance of
281 PCK1 (cytosolic form) enzyme, which is a rate-limiting enzyme in gluconeogenesis [29], controlling the entry from
282 AA and propionate [30]. In line with our results, it has been reported that the expression of PCK1 is elevated with
283 increasing feed intake during early lactation [31, 32].

284 The oxidative capacity of the TCA cycle is dependent on the supply of OAA (carbon carrier) from pyruvate by the
285 action of pyruvate carboxylase (PC) to maintain a 1:1 relationship between OAA and acetyl-CoA [33]. The results
286 revealed an overexpression trend (fold change = 1.65) of the PC enzyme, although its expression was not modeled as
287 differentially abundant. It is critical to balance the synthesis of metabolic intermediates (anaplerosis) and the extraction
288 of metabolic intermediates for breakdown (cataplerosis), especially during the transition period to fuel
289 gluconeogenesis and maintaining carbon homeostasis [33]. Therefore, it can be concluded that the overabundance of
290 both PC and PCK1 probably concur to increase the gluconeogenesis capacity while keeping the balance between
291 anaplerosis and cataplerosis.

292 Moreover, we observed an enrichment of the peroxisome proliferator-activated receptors (PPAR) pathway, which is
293 known to have a pivotal role in cycling lipid and carbohydrate substrates into glycolytic/gluconeogenic pathways
294 favoring energy production [34]. Accordingly, an overabundance of Acyl-CoA dehydrogenase (ACADM) which is
295 involved in PPAR signaling and carbon and FA metabolism, combined with the overabundance of long FA transporter
296 (SLC25A1 and SLC25A13) in the PP period, suggest a higher transport activity of FA from the plasma into the
297 hepatocytes, thus supporting a higher level of FA α and β -oxidation for energy supply. In the PPAR pathway, the
298 relative abundance of Enoyl-CoA Hydratase and 3-Hydroxyacyl CoA Dehydrogenase (EHHADH) along with Enoyl-
299 CoA Hydratase, Short Chain 1 (ECHS1) was increased; both proteins have been previously reported to be involved in
300 milk FA metabolism in humans [35] and cow [36] studies, not only through the PPAR but also through AMPK (5'
301 AMP-activated protein kinase) signaling pathways. The significant effects of ECHS1 on long-chain unsaturated,
302 medium-chain saturated FA, and milk FA traits in dairy cattle were discussed elsewhere [36]. The enrichment of the
303 PPAR pathway is also in line with the repeatedly reported role of PPARs as a sensor of NEFA levels [37, 38].

304 Besides, PPAR are also involved in transcriptional regulatory mechanisms coordinating the abundance and enzyme
305 content of organelles [39]. In this regard, we observed the enrichment of pathways related to organelles, in particular,
306 peroxisomes and mitochondria in PP-cluster2, with more than 20 DAP in the peroxisome, including IDH, Acyl-CoA
307 dehydrogenases (ACADs), Sterol Carrier Protein 2 (SPC2), and ACADM. Both peroxisomes and mitochondria are
308 remarkably dynamic adapting their number and activity depending on the prevailing environmental conditions i.e.,
309 excessive NEFA can thus be used directly as substrate and indirectly through PPAR activation [39]. Along with
310 mitochondria, peroxisomes play a crucial role in cellular lipid hemostasis, in which the overabundance of SPC2
311 indicates activation of the peroxisomal cholesterol transport from the cytoplasm and an induced FA β -oxidation [40].
312 Moreover, AA metabolism, including glycine, serine, isoleucine, threonine, and tryptophan metabolism, was enriched
313 in synchronized with mobilizing skeletal muscle protein during the PP NEB period. The released AA were primarily

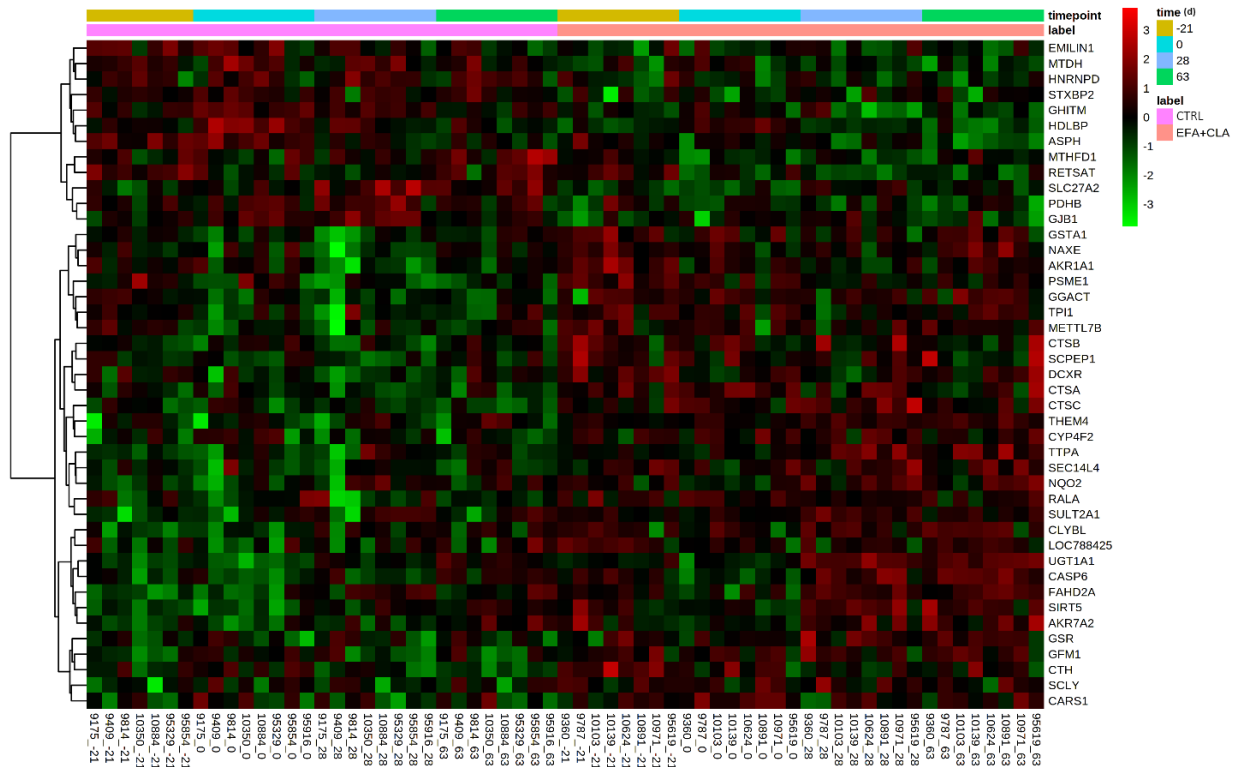
314 not metabolized in the liver to support mammary glands' milk protein synthesis [41, 42]. Considering the differences
315 between the AA profile of muscle and milk [43, 44], the enrichment of various AA metabolism was probably a
316 counter-regulation to maintain the AA ratio, precisely because AA are only available in limited quantities.
317 Interestingly, we observed the degradation of the branched-chain AA (BCAA, i.e., valine, leucine, and isoleucine)
318 among the most significantly enriched pathways in the PP-cluster2. In this regard, BCAA, in contrast to other AA, are
319 less degraded in the liver (first-pass hepatic catabolism) and are preferentially metabolized in extrahepatic tissues [45,
320 46]. Activated hepatic degradation of BCAA, in particular during the transition period may indicate that they primarily
321 converted to other AA or fed into TCA cycle/ketogenesis pathways. The present results suggest a strong relationship
322 between ketogenesis and BCAAs, accordingly to what was previously reported [11], in such a way that when citrate
323 synthesis (intensively driven by BCAA degradation but also FA oxidation) exceed the TCA capacity, its surplus is
324 directed to ketogenesis. In this pathway, 11 DAP were involved among which ECHS1, EHHADH, ACAA, and
325 ACADM were discussed previously. Here, the overabundance of the α and β subunits of the propionyl-CoA
326 carboxylase enzyme (PCCA and PCCB) that catalyzes the conversion of propionyl-CoA to methylmalonyl-CoA,
327 revealed an activated gluconeogenesis pathway using propionate as a substrate, and thus feeds the TCA cycle with
328 limiting intermediates.
329 Proteomic results provided an in-depth overview of metabolic adaptations during the NEB period. To summarize, FA
330 metabolism and degradation, PPAR signaling pathway, peroxisome, and TCA cycle were enriched to enhance lipid
331 and carbohydrate catabolic processes that fuel glycolytic/gluconeogenic pathways favoring energy production rather
332 than storage. Also, the enrichment of pathways related to FA biosynthesis, elongation, and biosynthesis of unsaturated
333 FA, along with α -linolenic acid metabolism, suggest that the identified proteins are involved in providing
334 intermediates/backbones to be used later by the mammary gland for milk fat synthesis. Furthermore, metabolic
335 adaptations were initiated in response to NEB by mobilizing energy substrate to fuel the TCA cycle with OAA,
336 succinate, and α -ketoglutarate, by activating a broad range of pathways related to carbohydrate, lipid, AA, and energy
337 metabolism.

338

339 **3.3. Gene ontology and functional enrichment analyses of differentially abundant proteins between** 340 **treatment groups**

341 We have previously reported in detail proteins and their associated pathways affected by FA supplementation at
342 several timepoints around parturition [60]. Here, we pooled all timepoints and reported the enriched pathways affected
343 by EFA+CLA treatment (regardless of time). Of the 43 DAP modeled within the treatment, 31 proteins had higher,
344 and 12 proteins had lower abundance in EFA+CLA with a fold change ranging from -3 to 3 (Figure 6).

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



351

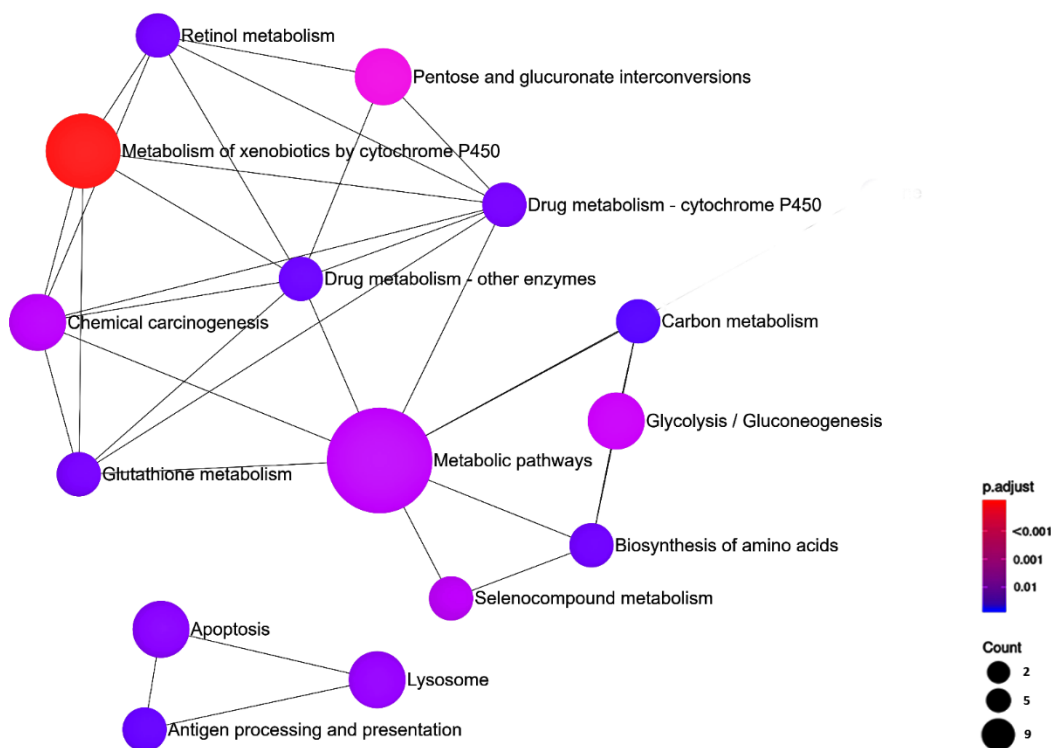
352 Figure 6) Hierarchical clustering and heatmap presentation of differentially abundant proteins between CTRL and EFA+CLA. Rows are
 353 respectively sorted by similarity as indicated by the left (proteins) dendrograms. Red and green represent increased and decreased proteins
 354 abundance, respectively.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

355

356 Moreover, 11 KEGG pathways were found to be enriched when the 43 DAP were considered: metabolism of
 357 xenobiotics by cytochrome P450, pentose and glucuronate interconversions, glycolysis/gluconeogenesis, lysosome,
 358 apoptosis, glutathione metabolism, retinol metabolism, chemical carcinogenesis, drug metabolism - cytochrome P450,
 359 and drug metabolism - other enzymes (Figure 7).

360 The most significantly enriched KEGG pathway was the metabolism of xenobiotics by cytochrome P450 with four
 361 DAP, including an overabundance of glutathione S-transferase A1 (GSTA1), aldo_ket_red domain-containing protein
 362 (AKR7A2), sulfotransferase family 2A member 1 (SULT2A1), UDP-glucuronosyltransferase family 1 member A1
 363 (UGT1A1). Cytochrome P450 (CYP) pathways constitute a superfamily of more than 1000 enzymes containing heme,
 364 capable of affecting various metabolic and biosynthetic processes by oxidizing different structural compounds,
 365 including steroids, prostaglandins, FA, derivatives of retinoic acid, and xenobiotics [47, 48]. For instance, the
 366 involvement of CYP enzymes in the hepatic biotransformation of cholesterol, its degradation to bile acids (BA),
 367 detoxification, and metabolic homeostasis has been the subject of many research studies [49, 50]. We have previously
 368 reported the involvement of specific CYP enzymes in different time points during the transition period that could be
 369 time-dependent or related to the fluctuating concentration of FA (NEFA and supplemented FA) serving as specific
 370 substrates [60]. Different CYP enzymes are capable of catalyzing the oxidative biotransformation of FA which is
 371 known as hepatic ω -oxidation of FA and functions primarily to facilitate their elimination when mitochondrial β -

372 oxidation is saturated. Compared to β -oxidation, ω -oxidation take place in the endoplasmic reticulum and involves
 373 the oxidation of the ω -carbon of FA to provide succinyl-CoA [60].
 374



375
 376 Figure 7) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of differentially abundant proteins (DAP) between
 377 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
 378 pathway.

379
 380 Another mechanism that regulates CYPs expression is through the activation of the PPAR pathway [51]. It has been
 381 shown that PUFA, especially ω -3 FA, compete with NEFA for ligand activation of PPAR [37], suggesting a potential
 382 role of these receptors in drug metabolism as well as metabolic homeostasis related to FA metabolism. All these pieces
 383 of evidence imply that the cytochrome P450 system may play a key role in regulating hepatic lipid homeostasis, as
 384 proposed earlier [49].

385 Another study indicated the involvement of CYP genes in steroidogenesis converting cholesterol to pregnenolone and
 386 consequently to dehydroepiandrosterone [52]. In this study, the steroid hormone biosynthesis pathway was enriched
 387 by the overabundance of the LOC100138004 protein. Steroid biosynthesis mainly occurs in the gonads and the adrenal
 388 glands, while the liver is considered a site for steroid hormone inactivation [53]. Recent observations in dairy cows
 389 have shown that providing a gluconeogenic feed (propylene glycol) or treatment with insulin infusion decreased the
 390 hepatic expression of CYP enzymes (CYP2C and CYP3A activity) responsible for hepatic progesterone catabolism,
 391 which could result in early fetal losses [54]. In the current study, neither the concentration of insulin [55] nor the
 392 hepatic abundance of CYP2C and CYP3A enzymes were affected by the treatment. Hence, identified CYP enzymes

393 were time-specific; they were not presented at all time points to be considered DAPs with the repeated measurement
394 ASCA model. Thus, the ASCA method has identified additional proteins with the CYP pathways that exemplify first
395 the benefit of combining ASCA and PLS-DA analysis, and second the centrality of CYP pathways in responses to
396 EFA+CLA supplementation in dairy cows.

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

404 Associated with the glutathione and cytochrome metabolism pathway, GSTM 3 and 4 both belonging to the
405 glutathione S-transferase (GST) superfamily, were downregulated. Members of the GST family are upregulated in
406 response to oxidative stress and are involved in catalyzing the xenobiotic-derived electrophilic metabolites, in steroid
407 hormone biosynthesis, in eicosanoid metabolism and, and in MAPK pathway (for review, see [56]). Moreover,
408 PRDX6, a member of the peroxiredoxin antioxidant enzymes family, is involved in the detoxification process against
409 oxidative stress through glutathione peroxidase. In this regard, Abuelo et al. [57] reported a gradual increase in
410 oxidative stress status after calving due to fat mobilization. Due to the higher ω -oxidation capacity in EFA+CLA
411 supplemented cows [60], it seems conceivable to activate GSH synthesis for avoiding oxidative stress. Collectively,
412 the results indicated that EFA+CLA supplementation enriched cytochrome P450 as a core affected pathway.

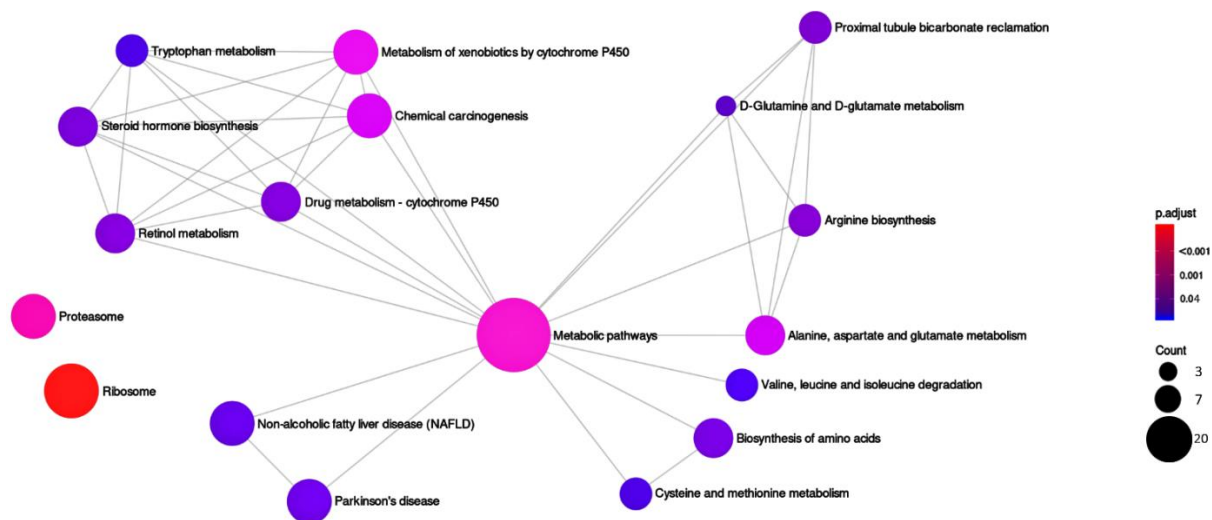
413 It is worth mentioning that identifying DAP between CTRL and EFA+CLA group in each timepoint [60] provided
414 partially different patterns (only a few proteins in common) in comparison to identifying DAP between pooled CTRL
415 and EFA+CLA group (without considering time). This is because we observed a time-specific pattern for DAP, which
416 would not be detectable by the ASCA model. The ASCA would only consider a protein as DAP if it had a constantly
417 lower/higher abundance in all timepoints. Interestingly, metabolism of xenobiotics by cytochrome P450, drug
418 metabolism - cytochrome P450, drug metabolism - other enzymes, and retinol metabolism were enriched as the main
419 affected pathways by both methods.

420

421 **3.4. Gene ontology and functional enrichment analyses of differentially abundant proteins within the** 422 **interaction of transition period and fatty acid supplementation**

423 Herein, 97 proteins were found to be affected by the interaction of time and FA supplementation (with a fold change
424 ranging from -6 to +6); proteins were fluctuating between two independent parameters ($\alpha*\beta$) and therefore reporting
425 the individual over- or under-abundancy for each protein is not feasible. The relative abundance of proteins modelled
426 in the interaction effect is graphically presented in a Heatmap (Figure 8). The GO enrichment analysis revealed that
427 these proteins were annotated by 65 enriched GO terms within the BP category such as cellular process (GO:0009987),
428 organonitrogen compound metabolic process (GO:1901564), protein metabolic process (GO:0019538), peptide

435 Also, the functional analyses of the DAP revealed the enrichment of nine KEGG pathways, including metabolic
 436 pathways, metabolism of xenobiotics by cytochrome P450, drug metabolism - cytochrome P450, drug metabolism -
 437 other enzymes, retinol metabolism, steroid hormone biosynthesis, ribosome, chemical carcinogenesis, proteasome
 438 (Figure 9) which were mainly the same pathways found enriched for the DAP in the FA-supplemented group (β).
 439 Among the KEGG enriched pathways, ribosome (with seven DAP including different ribosomal proteins (RP) S2,
 440 S3A, L34, L18A, L10A, L27A, and S4Y1), metabolism of xenobiotics by cytochrome P450 (with five DAP, CYP1A2,
 441 UGT2B4, alcohol dehydrogenase 4 (ADH4), hydroxysteroid 11-beta dehydrogenase 1 (HSD11B1), and GSTM4), and
 442 proteasome (with four hits including different proteasome subunits, PSMD1, PSMD4, PSMA1, and PSMD14) were
 443 the top enriched ones. In line with current results, a previous *in vitro* study on human liver microsomes indicated an
 444 inhibitory effect of PUFA containing linoleic acid, α -linolenic acid, arachidonic acid, eicosapentaenoic acid and,
 445 docosahexaenoic acid on CYP1A2 [58]. In contrast, the inhibition of CYP1A2 was not observed in an *in vivo* study
 446 when linseed oil was infused directly into the abomasum of dairy cows [59]. However, the authors concluded that the
 447 infusion might not have achieved sufficient concentrations to inhibit the key enzymes involved in steroid metabolism.
 448 The enrichment of ribosome and proteasome pathways is probably due to elevated protein biosynthesis and turnover
 449 in the PP-cluster2 and the EFA+CLA group.
 450



451
 452 Figure 9) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of proteins identified by the interaction effect. The
 453 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
 454 pathway.

455
 456 Metabolism of xenobiotics by cytochrome P450 was commonly enriched during the transition to lactation (α), between
 457 treatments (β), and interaction of them ($\alpha\beta$), and thus can likely be considered as central mechanisms responsible for
 458 maintaining the metabolic homeostasis in response to NEFA mobilization and FA supplementation. Given that CYP
 459 are involved in the metabolism of both endogenous and exogenous substrates, it could be speculated that supplemented
 460 FA and their intermediate metabolites had xenobiotic-like potential and induced a series of reactions initiated by the

461 ligand activation of PPAR. Consequently, CYP enzymes and their associated pathways such as retinol and glutathione
462 metabolism and steroid hormone biosynthesis were being activated to regulate lipid homeostasis. However, further
463 studies are required to verify this notion.

464

465 **3.5. Comparison of ASCA with PLS-DA method**

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

475

476

477 **4. Conclusion**

478 The present results revealed the molecular signature of metabolic shifts during the transition from gestation to lactation
479 in dairy cows and its interaction with supplemented EFA+CLA using the repeated measurement ASCA model. During
480 the transition from gestation to lactation, DAP enriched metabolic pathways were mainly related to FA metabolism
481 and degradation, AA metabolism, biosynthesis and degradation, and carbohydrate and energy metabolism in favor of
482 energy production. Herein, the NEFA ligand activation of the nuclear PPAR orchestrates lipid metabolism, involving
483 regulation of hepatic mitochondrial and peroxisome metabolism. Supplemented EFA+CLA amplified FA oxidation
484 mechanisms induced by NEFA. The enrichment of cytochrome P450 as an interaction effect was to maintain metabolic
485 homeostasis by oxidation/detoxifying endogenous and exogenous produced xenobiotics. Collectively, it could be
486 concluded that EFA+CLA supplementation in dairy cows having a low level of these two FA, had some marginal
487 beneficial effects on hepatic lipid metabolism and metabolic health.

488

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491 concentration for mass spectrometry analyses and R. Furioso Ferreira for drawing Figure 5.

492

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497

498 **Data availability**

499 The data and related analyses are available through the link <https://doi.org/10.15454/Z2K0OR>.

500 **Figure legends**

501 Figure 1) Schematic diagram of the (A) study design, (B) proteomics workflow and peptide identification, and (C) statistical analysis and
502 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
503 +63 d relative to parturition). Bold lines indicate liver biopsy sampling time points. (B) High-resolution LC-MS/MS analysis, peptide alignment
504 (progenesis), and protein identification (mascot) procedure were performed by Progenesis software coupled with the Mascot search engine,
505 statistical analysis was based on Multivariate Analysis of variance – simultaneous component analysis (ASCA), and (C) ASCA design and
506 bioinformatics analysis.

507
508 Figure 2) Major patterns associated with transition time (A), FA treatment (B) and their interaction (C) calculated by analysis of variance -
509 simultaneous component analysis (ASCA), in dairy cows supplemented with or without EFA+CLA in 4 time-points (-21, +1, +28, and +63 d
510 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
511 interaction of them (ab).

512
513 Figure 3) Hierarchical clustering and heatmap representation of differentially abundant proteins during the transition from late gestation to lactation
514 in dairy cows. Rows are respectively sorted by similarity as indicated by the left (proteins) dendrograms. Red and green represent increased and
515 decreased protein abundance, respectively. The colour code for different time points and treatments is provided on the right-hand side.

516
517 Figure 4) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of differentially abundant proteins (DAP) during the
518 transition from late gestation to lactation in dairy cows. The colour of the nodes represents the $-\log_{10}$ (adjusted P-value); Node size represents the
519 number of DAP contained in the node (smaller indicates lesser DAP, bigger indicates more DAP).

520
521 Figure 5) Schematic of fatty acid oxidation in dairy cows' hepatocyte. In the pathway map, only the differentially abundant proteins in the
522 postpartum period are highlighted; red colour indicates upregulation; green designated downregulation.

523
524 Figure 6) Hierarchical clustering and heatmap presentation of differentially abundant proteins between CTRL and EFA+CLA. Rows are
525 respectively sorted by similarity as indicated by the left (proteins) dendrograms. Red and green represent increased and decreased proteins
526 abundance, respectively. The colour code for different time points and treatments is provided on the right-hand side.

527
528 Figure 7) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of differentially abundant proteins (DAP) between
529 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
530 pathway.

531
532 Figure 8) Hierarchical clustering and heatmap representation of differentially abundant proteins by interaction effect. Rows are the average of
533 protein abundances in each group at each timepoint. Red and green represent increased and decreased protein abundance, respectively. The colour
534 code for different time points and treatments is provided on the right-hand side.

535
536 Figure 9) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of proteins identified by the interaction effect. The
537 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
538 pathway.

539

540 **Table heading**

541 Table 1) The differentially abundant proteins identified during the time, between treatment groups, and their interaction.

Num.	Protein	Gene name	Differentially abundant at (α , β , $\alpha\beta$)*
1	Acetyl-CoA acyltransferase 1	ACAA1	α
2	Acetyl-CoA acyltransferase 2	ACAA2	α
3	Acyl-CoA dehydrogenase, C-4 to C-12 straight chain	ACADM	α
4	Acetyl-CoA acetyltransferase 1	ACAT1	α
5	Aconitase 2	ACO2	α
6	Acyl-CoA thioesterase 8	ACOT8	α
7	Acyl-CoA oxidase 1	ACOX1	α
8	Acyl-CoA oxidase 2	ACOX2	α
9	Acyl-CoA synthetase long-chain family member 1	ACSL1	α
10	Acyl-CoA synthetase long-chain family member 5	ACSL5	α
11	Acyl-CoA synthetase short-chain family member 3	ACSS3	α
12	Aldo-keto reductase family 1 member D1	AKR1D1	α
13	Aldehyde dehydrogenase 3 family member A2	ALDH3A2	α
14	Alpha-methylacyl-CoA racemase	AMACR	α
15	Annexin A4	ANXA4	α
16	Annexin A9	ANXA9	α
17	Argininosuccinate synthase 1	ASS1	α
18	AU RNA binding methylglutaconyl-CoA hydratase	AUH	α
19	Branched chain keto acid dehydrogenase E1, alpha polypeptide	BCKDHA	α
20	Retinyl ester hydrolase type 1	BREH1	α
21	Basigin	BSG	α
22	Complement C1q binding protein	C1QBP	α
23	Carbonyl reductase 4	CBR4	α
24	Conglutinin	CGN1	α
25	Coenzyme Q6, monooxygenase	COQ6	α
26	Carnitine palmitoyltransferase 2	CPT2	α
27	Carnitine O-acetyltransferase	CRAT	α
28	Cysteine sulfinic acid decarboxylase	CSAD	α
29	Cytochrome P450, family 27, subfamily A, polypeptide 1	CYP27A1	α
30	Cytochrome P450, family 2, subfamily E, polypeptide 1	CYP2E1	α
31	Cytochrome P450, family 51, subfamily A, polypeptide 1	CYP51A1	α
32	Dehydrogenase/reductase 1	DHRS1	α
33	Dehydrogenase/reductase	DHRS4	α
34	Dihydrolipoamide S-succinyltransferase	DLST	α
35	Dimethylglycine dehydrogenase	DMGDH	α
36	Enoyl-CoA hydratase, short chain 1	ECHS1	α
37	Enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase	EHHADH	α
38	Esterase D	ESD	α
39	Electron transfer flavoprotein dehydrogenase	ETFDH	α
40	Farnesyl-diphosphate farnesyltransferase 1	FDFT1	α

41	Farnesyl diphosphate synthase	FDPS	α
42	Ferritin light chain	FTL	α
43	Glutamate dehydrogenase 1	GLUD1	α
44	Glycerate kinase	GLYCK	α
45	Glutamic-oxaloacetic transaminase 1	GOT1	α
46	Glycerol-3-phosphate dehydrogenase 1	GPD1	α
47	Glucose-6-phosphate isomerase	GPI	α
48	Glutathione S-transferase mu 3	GSTM3	α
49	2-hydroxyacyl-CoA lyase 1	HACL1	α
50	Hydroxyacid oxidase	HAO1	α
51	Hydroxyacid oxidase 2	HAO2	α
52	3-hydroxy-3-methylglutaryl-CoA synthase 1	HMGCS1	α
53	Hemopexin	HPX	α
54	Hydroxysteroid 17-beta dehydrogenase 4	HSD17B4	α
55	Hydroxysteroid 17-beta dehydrogenase 8	HSD17B8	α
56	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7	HSD3B7	α
57	Hydroxysteroid dehydrogenase like 2	HSDL2	α
58	Heat shock protein family D	HSPD1	α
59	Isocitrate dehydrogenase	IDH1	α
60	Isocitrate dehydrogenase	IDH2	α
61	Isopentenyl-diphosphate delta isomerase 1	IDI1	α
62	Immunoglobulin heavy constant mu	IGHM	α
63	L-2-hydroxyglutarate dehydrogenase	L2HGDH	α
64	lamin A/C	LMNA	α
65	Phylloquinone omega-hydroxylase CYP4F2	LOC100295883	α
66	Nicotinamide N-methyltransferase	LOC511161	α
67	Cytochrome P450 2C31	LOC785540	α
68	Lon peptidase 1, mitochondrial	LONP1	α
69	Lanosterol synthase	LSS	α
70	mannosidase alpha class 2B member 1	MAN2B1	α
71	Malate dehydrogenase 2	MDH2	α
72	Malectin	MLEC	α
73	Malonyl-CoA decarboxylase	MLYCD	α
74	Methylsterol monooxygenase 1	MSMO1	α
75	Mevalonate diphosphate decarboxylase	MVD	α
76	Nicotinamide phosphoribosyltransferase	NAMPT	α
77	NAD	NSDHL	α
78	Propionyl-CoA carboxylase alpha subunit	PCCA	α
79	Propionyl-CoA carboxylase beta subunit	PCCB	α
80	Phosphoenolpyruvate carboxykinase 1	PCK1	α
81	Peroxisomal trans-2-enoyl-CoA reductase	PECR	α
82	Peroxisomal biogenesis factor 14	PEX14	α
83	Phosphoglycerate dehydrogenase	PHGDH	α
84	Phytanoyl-CoA 2-hydroxylase	PHYH	α

85	Paraoxonase 1	PON1	α
86	Peroxioredoxin 6	PRDX6	α
87	Regucalcin	RGN	α
88	Ribosome binding protein 1	RRBP1	α
89	Sterol carrier protein 2	SCP2	α
90	Selenium binding protein 1	SELENBP1	α
91	Sideroflexin 1	SFXN1	α
92	Serine hydroxymethyltransferase 2	SHMT2	α
93	Solute carrier family 22	SLC22A9	α
94	Solute carrier family 25 member 1	SLC25A1	α
95	Solute carrier family 25 member 13	SLC25A13	α
96	Solute carrier family 25 member 4	SLC25A4	α
97	Squalene epoxidase	SQLE	α
98	Signal transducer and activator of transcription 3	STAT3	α
99	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	SULT1A1	α
100	Sulfotransferase family 1E member 1	SULT1E1	α
101	Thimet oligopeptidase 1	THOP1	α
102	Thymocyte nuclear protein 1	THYN1	α
103	TNF receptor associated protein 1	TRAP1	α
104	Thioredoxin like 1	TXNL1	α
105	Uridine phosphorylase 2	UPP2	α
106	Ubiquinol-cytochrome c reductase core protein I	UQCRC1	α
107	Ubiquinol-cytochrome c reductase core protein II	UQCRC2	α
108	ATPase Family AAA Domain Containing 3A	ATAD3	α
109	Dimethylarginine dimethylaminohydrolase 1	DDAH1	α, β
110	Hydroxysteroid 11-beta dehydrogenase 1	HSD11B1	α, β
111	UDP-glucuronosyltransferase 2B4	LOC615303	α, β
112	Mitochondrial trans-2-enoyl-CoA reductase	MECR	α, β
113	Glutathione S-Transferase Mu 4	GSTM4	α, β
114	Fumarylacetoacetate hydrolase domain containing 2A	FAHD2A	α, β
115	Solute carrier family 27 member 2	SLC27A2	α, β
116	UDP glucuronosyltransferase 1 family, polypeptide A1	UGT1A1	α, β
117	Acyl-CoA dehydrogenase family member 11	ACAD11	$\alpha\beta$
118	Aminocarboxymuconate semialdehyde decarboxylase	ACMSD	$\alpha\beta$
119	Actin, beta like 2	ACTBL2	$\alpha\beta$
120	Alcohol dehydrogenase 4	ADH4	$\alpha\beta$
121	Adenylate kinase 4	AK4	$\alpha\beta$
122	Activated leukocyte cell adhesion molecule	ALCAM	$\alpha\beta$
123	Amidohydrolase domain containing 1	AMDHD1	$\alpha\beta$
124	Arginase 1	ARG1	$\alpha\beta$
125	Actin related protein 2/3 complex subunit 2	ARPC2	$\alpha\beta$
126	Asparaginase like 1	ASRGL1	$\alpha\beta$
127	ATPase Na ⁺ /K ⁺ transporting subunit beta 3	ATP1B3	$\alpha\beta$
128	Bleomycin hydrolase	BLMH	$\alpha\beta$

129	Calnexin	CANX	$\alpha\beta$
130	Cystathionine-beta-synthase	CBS	$\alpha\beta$
131	Cell cycle and apoptosis regulator 2	CCAR2	$\alpha\beta$
132	Chaperonin containing TCP1 subunit 4	CCT4	$\alpha\beta$
133	Ceroid-lipofuscinosis, neuronal 5	CLN5	$\alpha\beta$
134	Collagen type XVIII alpha 1 chain	COL18A1	$\alpha\beta$
135	Coatomer protein complex subunit gamma 1	COPG1	$\alpha\beta$
136	COP9 signalosome subunit 6	COPS6	$\alpha\beta$
137	Catenin alpha 1	CTNNA1	$\alpha\beta$
138	Cullin 4A	CUL4A	$\alpha\beta$
139	Cytoplasmic FMR1 interacting protein 1	CYFIP1	$\alpha\beta$
140	Cytochrome P450, family 1, subfamily A, polypeptide 2	CYP1A2	$\alpha\beta$
141	DEXH-box helicase 9	DHX9	$\alpha\beta$
142	DnaJ heat shock protein family	DNAJA2	$\alpha\beta$
143	Eukaryotic elongation factor, selenocysteine-tRNA specific	EEFSEC	$\alpha\beta$
144	EF-hand domain family member D2	EFHD2	$\alpha\beta$
145	Eukaryotic translation initiation factor 2 subunit alpha	EIF2S1	$\alpha\beta$
146	Eukaryotic translation initiation factor 2 subunit beta	EIF2S2	$\alpha\beta$
147	ELAV like RNA binding protein 1	ELAVL1	$\alpha\beta$
148	FGGY carbohydrate kinase domain containing	FGGY	$\alpha\beta$
149	FK506 binding protein 11	FKBP11	$\alpha\beta$
150	Glutamine--fructose-6-phosphate transaminase 1	GFPT1	$\alpha\beta$
151	Glutaminase 2	GLS2	$\alpha\beta$
152	Glutathione synthetase	GSS	$\alpha\beta$
153	3-hydroxyisobutyrate dehydrogenase	HIBADH	$\alpha\beta$
154	Histone cluster 2, H2bf	HIST2H2BF	$\alpha\beta$
155	High mobility group box 2	HMGB2	$\alpha\beta$
156	Heterogeneous nuclear ribonucleoprotein F	HNRNPF	$\alpha\beta$
157	Heterogeneous nuclear ribonucleoprotein U	HNRNPU	$\alpha\beta$
158	Heterochromatin protein 1 binding protein 3	HP1BP3	$\alpha\beta$
159	Heparan sulfate proteoglycan 2	HSPG2	$\alpha\beta$
160	Cytochrome P450, family 2, subfamily J	LOC521656	$\alpha\beta$
161	Mesencephalic astrocyte derived neurotrophic factor	MANF	$\alpha\beta$
162	Methylcrotonoyl-CoA carboxylase 1	MCCC1	$\alpha\beta$
163	MX dynamin like GTPase 1	MX1	$\alpha\beta$
164	Myeloid derived growth factor	MYDGF	$\alpha\beta$
165	Asparaginyl-tRNA synthetase	NARS	$\alpha\beta$
166	NADH:ubiquinone oxidoreductase subunit A10	NDUFA10	$\alpha\beta$
167	NADH:ubiquinone oxidoreductase core subunit S2	NDUFS2	$\alpha\beta$
168	Nidogen 1	NID1	$\alpha\beta$
169	Non-metastatic cells 2, protein	NME2	$\alpha\beta$
170	Phosphate cytidylyltransferase 1, choline, alpha	PCYT1A	$\alpha\beta$
171	Programmed cell death 6	PDCD6	$\alpha\beta$
172	Pyruvate kinase, muscle	PKM	$\alpha\beta$

173	Protein O-fucosyltransferase 1	POFUT1	$\alpha\beta$
174	DNA polymerase delta interacting protein 2	POLDIP2	$\alpha\beta$
175	Peptidylprolyl isomerase A	PPIA	$\alpha\beta$
176	Peptidylprolyl isomerase D	PPID	$\alpha\beta$
177	Proline rich coiled-coil 1	PRRC1	$\alpha\beta$
178	Proteasome subunit alpha 1	PSMA1	$\alpha\beta$
179	Proteasome 26S subunit, non-ATPase 1	PSMD1	$\alpha\beta$
180	Proteasome 26S subunit, non-ATPase 14	PSMD14	$\alpha\beta$
181	Proteasome 26S subunit, non-ATPase 4	PSMD4	$\alpha\beta$
182	Ribosomal protein L10a	RPL10A	$\alpha\beta$
183	Ribosomal protein L18a	RPL18A	$\alpha\beta$
184	Ribosomal protein L27a	RPL27A	$\alpha\beta$
185	Ribosomal protein L34	RPL34	$\alpha\beta$
186	Ribosomal protein S2	RPS2	$\alpha\beta$
187	Ribosomal protein S3A	RPS3A	$\alpha\beta$
188	Ribosomal protein S4, Y-linked 1	RPS4Y1	$\alpha\beta$
189	Reticulon 4 interacting protein 1	RTN4IP1	$\alpha\beta$
190	RuvB like AAA ATPase 2	RUVBL2	$\alpha\beta$
191	SAMM50 sorting and assembly machinery component	SAMM50	$\alpha\beta$
192	Selenoprotein O	SELENOO	$\alpha\beta$
193	Solute carrier family 39 member 7	SLC39A7	$\alpha\beta$
194	Sorting nexin 12	SNX12	$\alpha\beta$
195	Sorting nexin 3	SNX3	$\alpha\beta$
196	Transcription elongation factor B subunit 2	TCEB2	$\alpha\beta$
197	Transmembrane p24 trafficking protein 7	TMED7	$\alpha\beta$
198	Transmembrane emp24 protein transport domain containing 9	TMED9	$\alpha\beta$
199	Tubulin alpha 4a	TUBA4A	$\alpha\beta$
200	Thioredoxin reductase 1	TXNRD1	$\alpha\beta$
201	Ubiquitin like modifier activating enzyme 1	UBA1	$\alpha\beta$
202	Ubiquitin conjugating enzyme E2 V1	UBE2V1	$\alpha\beta$
203	UDP glycosyltransferase family 3 member A2	UGT3A2	$\alpha\beta$
204	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	UQCRFS1	$\alpha\beta$
205	Ubiquitin specific peptidase 5	USP5	$\alpha\beta$
206	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein beta	YWHAB	$\alpha\beta$
207	NME/NM23 Nucleoside Diphosphate Kinase 1	NME1-1	$\alpha\beta$
208	Septin 7	SEPTIN7	$\alpha\beta$
209	Aldo-keto reductase family 1 member A1	AKR1A1	β
210	Aldo-keto reductase family 7 member A2	AKR7A2	β
211	Aspartate beta-hydroxylase	ASPH	β
212	Caspase 6	CASP6	β
213	Citrate lyase beta like	CLYBL	β
214	Cystathionine gamma-lyase	CTH	β
215	Cathepsin A	CTSA	β
216	Cathepsin B	CTSB	β

217	Cathepsin C	CTSC	β
218	Cytochrome P450, family 4, subfamily F, polypeptide 2	CYP4F2	β
219	L-xylulose reductase-like	DCXR	β
220	Elastin microfibril interfacer 1	EMILIN1	β
221	G elongation factor mitochondrial 1	GFM1	β
222	Gamma-glutamylamine cyclotransferase	GGACT	β
223	Growth hormone inducible transmembrane protein	GHITM	β
224	Gap junction protein beta 1	GJB1	β
225	Glutathione-disulfide reductase	GSR	β
226	Vigilin	HDLBP	β
227	Heterogeneous nuclear ribonucleoprotein D	HNRNPD	β
228	Aflatoxin B1 aldehyde reductase member 3	LOC788425	β
229	Methyltransferase like 7B	METTL7B	β
230	Metadherin	MTDH	β
231	Methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1	MTHFD1	β
232	NAD(P)H-hydrate epimerase	NAXE	β
233	N-ribosylidihyronicotinamide:quinone reductase	NQO2	β
234	Pyruvate dehydrogenase	PDHB	β
235	Proteasome activator subunit 1	PSME1	β
236	RAS like proto-oncogene A	RALA	β
237	Retinol saturase	RETSAT	β
238	Selenocysteine lyase	SCLY	β
239	Serine carboxypeptidase 1	SCPEP1	β
240	SEC14 like lipid binding 4	SEC14L4	β
241	Sirtuin 5	SIRT5	β
242	Syntaxin binding protein 2	STXBP2	β
243	Sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone	SULT2A1	β
244	Thioesterase superfamily member 4	THEM4	β
245	Triosephosphate isomerase 1	TPI1	β
246	Alpha tocopherol transfer protein	TTPA	β
247	Glutathione S-Transferase Alpha 1	GSTA1	β
248	Cysteinyl-TRNA Synthetase 1	CARS1	β

542 *identified as differentially abundant α = during transition period, β = between treatment groups (CTRL and EFA+CLA), $\alpha\beta$ = interaction of time
543 and treatment.
544

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