

1 **Proteomics and metabolomics characterizing the pathophysiology of adaptive reactions to the**
2 **metabolic challenges during the transition from late pregnancy to early lactation in dairy**
3 **cows**

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8 **List of abbreviations**

9 AGP, α_1 -acid glycoprotein; APP, acute phase protein; ATP, adenosine triphosphate; BHB, β -
10 hydroxybutyrate; CoA, Coenzyme A; DIM, days in milk; GPC, glycerophosphocholine; NADP,
11 nicotinamide adenine dinucleotide phosphate; NAFLD, non-alcoholic fatty liver disease; NEFA,
12 non-esterified fatty acids; NNB, negative nutrient balance; PC, phosphocholine; PI, physiological
13 imbalance; SAA, serum amyloid A; SARA, subacute ruminal acidosis; TCA cycle, tricarboxylic
14 acid cycle; TG, triglyceride; VLDL, very low density lipoprotein.

15 **Abstract**

16 The transition from late pregnancy to early lactation is a critical period in a dairy cow`s life due to
17 the rapidly increasing drain of nutrients from the maternal organism towards the foetus and into
18 colostrum and milk. In order to cope with the challenges of parturition and lactation, comprehensive
19 adaptive reactions comprising the endocrine and the immune system need to be accomplished.

20 There is high variation in this coping ability and both metabolic and infectious diseases,
21 summarized as “production diseases”, such as hypocalcaemia (milk fever), fatty liver syndrome,
22 laminitis and ketosis, may occur and impact welfare, productive lifespan and economic outcomes.

23 Proteomics and metabolomics have emerged as valuable techniques to characterize proteins and

24 metabolite assets from tissue and biological fluids, such as milk, blood and urine. In this review we
25 provide an overview on metabolic status and physiological changes during the transition period and
26 the related production diseases in dairy cows, and summarize the state of art on proteomics and
27 metabolomics of biological fluids and tissues involved in metabolic stress during the peripartum
28 period. We also provide a current and prospective view of the application of the recent
29 achievements generated by omics for biomarker discovery and their potential in diagnosis.

30

31 **Significance**

32 For high-yielding dairy cows there are several “occupational diseases” that occur mainly during the
33 metabolic challenges related to the transition from pregnancy to lactation. Such diseases and their
34 sequelae form a major concern for dairy production, and often lead to early culling of animals.

35 Beside the economical perspective, metabolic stress may severely influence animal welfare. There
36 is a multitude of studies about the metabolic backgrounds of such so called production diseases like
37 ketosis, fatty liver, or hypocalcaemia, although the investigations aiming to assess the complexity of
38 the pathophysiological reactions are largely focused on gene expression, i.e. transcriptomics. For
39 extending the knowledge towards the proteome and the metabolome, the respective technologies are
40 of increasing importance and can provide an overall view of how dairy cows react to metabolic
41 stress, which is needed for an in-depth understanding of the molecular mechanisms of the related
42 diseases. We herein review the current findings from studies applying proteomics and
43 metabolomics to transition-related diseases, including fatty liver, ketosis, endometritis,
44 hypocalcaemia and laminitis. For each disease, a brief overview of the up to date knowledge about
45 its pathogenesis is provided, followed by an insight into the most recent achievements on the
46 proteome and metabolome of tissues and biological fluids, such as blood serum and urine,
47 highlighting potential biomarkers. We believe that this review would help readers to become
48 more familiar with the recent progresses of molecular background of transition-related diseases thus
49 encouraging research in this field.

50

51 **Introduction**

52 The transition period from late pregnancy to early lactation is a critical period in a dairy cow`s life
53 due to the rapidly increasing drain of nutrients from the maternal organism towards the foetus and
54 into colostrum and milk. During this transition period, fetal growth reaches its exponential course
55 during the last weeks of pregnancy and concomitantly the mammary gland parenchyma mass
56 markedly grows [1]. After calving, the output of nutrients with milk exceeds the input by voluntary
57 feed intake. The negative nutrient balance (NNB) resulting therefrom requires a massive
58 mobilization of body reserves, mainly body fat but also protein. Albeit NNB is a common
59 phenomenon in mammals, both the duration and the extent observed in modern high yielding dairy
60 cows represent a biological extreme. To be able to cope with the challenges of parturition and
61 lactation, comprehensive adaptive mechanisms comprising the endocrine and the immune system
62 need to be accomplished. There is high variation in this coping ability and both metabolic and
63 infectious diseases, summarized as “production diseases”, may occur and have an impact on
64 welfare, productive lifespan and economic outcomes. The incidence of such diseases is greatest
65 during early lactation with hypocalcaemia (milk fever), fatty liver syndrome and ketosis (or
66 acetoanaemia) being the most common metabolic diseases. In case of infectious diseases, metritis
67 and mastitis, attributable to the immune-compromised situation during the metabolic challenge, are
68 most frequent. Fig.1 presents the relationship between metabolic stress and disease development.
69 Several studies have attempted to identify the causes and risk factors associated with the high
70 incidence of health problems observed during the periparturient period [2–5], and systems biology
71 approaches addressing the issue of the regulatory mechanisms of nutrient metabolism in lactation
72 are published [6–8] . Beside environmental factors, production diseases also have a genetic
73 component; e.g. for subclinical macromineral disorders and major clinical diseases the heritability
74 reported were low to moderate [9].

75 To further investigate the complexity of these diseases, omics approaches which include multi-
76 variate, and large-scale analyses, may be applied. Such studies gather information either at the level
77 of the DNA, RNA, miRNA, protein or the metabolites, and provide a snapshot of the current
78 condition in cells, tissues or body fluids (Fig 2). However, variation in sampling times between
79 different studies can yield different results and it is important to take this into account when
80 interpreting omics results or planning further studies. The multivariate results from omics
81 approaches require extensive bioinformatics resources that are mostly available online. Both
82 proteomics and metabolomics have evolved as the functional continuation of transcriptomics in less
83 than two decades, and have developed rapidly, due to improvements in technology and
84 bioinformatics tools. General reviews about the application of proteomics and metabolomics to
85 livestock science were published earlier [10–12]. Both proteomics and metabolomics involve the
86 resolution of a complex mixture of compounds into components that can then be identified and
87 characterized. For what concerns proteins, their identification always involves matching each the
88 amino acid sequence to the respective encoding gene and thus depends of the sequence information
89 available for the target species, but can also include the description of posttranslational protein
90 modifications.

91 Two major mass spectrometry (MS) platforms are available for proteomics, following the
92 mechanism through which ions are generated: these ion sources are termed matrix-assisted laser
93 desorption/ionization (MALDI) and electrospray ionization (ESI). Before analysis, proteins are
94 fractionated either by electrophoretic, for intact proteins, or chromatographic, for peptides generated
95 after protein cleavage, techniques. Protein fractions are then digested, to generate peptides that can
96 be further fractionate by chromatographic techniques and then characterized by MS, which can
97 record the mass of analytes to generate information about their structure. The resulting data are
98 further analysed with search engines, such as Mascot (Matrix Science Ltd), to generate in silico MS
99 data for the specified genome sequence database.

100 Absolute protein quantification is difficult to achieve with proteomics techniques. On the contrary,
101 relative quantitation can be achieved by gel-based methods, such as 2DE using semiquantitative
102 protein stains, or protein labeling strategies, such as difference gel electrophoresis (DIGE) [13]. As
103 an example, DIGE was applied to blood serum samples to identify potential biomarkers related to
104 hypocalcaemia [14].

105 At the peptide level, relative quantification can be achieved by stable isotope-labeling approaches
106 (iTRAQ) or by label-free comparison. Isolated proteins or tryptic peptides can be chemically
107 labeled before separation (iTRAQ) [15]. Quantification of proteins by means of iTRAQ was used,
108 among the others, to identify liver proteins related to physiological imbalance [16].

109 For metabolites, species-specificity is not an issue albeit relative quantities may differ. The major
110 analytical approaches used in metabolomics rely on two techniques: MS and NMR techniques [17].

111 For MS-based techniques, samples are fractionated through chromatographic techniques such as
112 Gas Chromatography (GC) or High Pressure Liquid Chromatography (HPLC), or capillary
113 electrophoresis. The GC is used to fractionate volatile metabolites, such as for example BHB or
114 other organic acids, or fatty acids, whereas HPLC is used for lipophilic metabolites, such as acyl
115 carnitines for example. Fractionated metabolites are then ionized and identified following mass
116 spectrometry analysis. NMR techniques can identify simultaneously all the analytes, and do not
117 require any prefractionation of the sample. Successful identification of individual metabolites
118 depends of high quality mass spectra, powerful spectral *matching* algorithms and comprehensive
119 and reliable spectral libraries [18].

120 Metabolomics strategies are commonly divided into targeted and non-targeted metabolomics.

121 Targeted metabolomics aims to quantify defined groups of metabolites, such as for example, in case
122 of transition period-related diseases, acylcarnitines, carbohydrates, amino acids or organic acids.

123 Internal standards, such as stable isotope (^1H and ^{13}C) labelled metabolites are used to measure
124 analytes in a quantitative or semi-quantitative ways [19]. Non-targeted metabolomics allows for the
125 detection of all the metabolites in a sample, in theory. Indeed, non-targeted metabolomics is able to

126 detect up to 10,000 independent spectral features in biological samples [20], but only a fraction of
127 them can be actually identified. Targeted metabolomics was already applied in several dairy cow
128 studies e.g. to characterize the respective changes in blood throughout the transition phase [21–24]
129 and non-targeted metabolomics for testing a supplement aiming to ameliorate metabolic stress [25],
130 respectively. The aim of this review is to provide an overview of the state of the art applications of
131 proteomics and metabolomics to address production diseases of dairy cows, whose prevention
132 represents a priority for intensive milk production. We reconcile and summarize the information
133 currently available on metabolic status and physiological changes during the transition period, and
134 discuss the most recent achievements in proteomics and metabolomics applications for biomarker
135 discovery, their potential as diagnostic tools, but also for comprehension of complex (patho)
136 physiological contexts. However, the review will be largely limited to metabolic diseases and to
137 metritis. For mastitis, the reader is referred to the specific “mastitomics” literature [26–28] and also
138 to the latest considerations of meta-proteomics and meta-metabolomics which include the milk
139 microbiome as recently reviewed by Addis and coworkers [29].

140 Metabolomics by means of near infrared spectroscopy is used since many years in routine milk
141 recordings to assess gross milk composition (mainly the content of fat, protein and lactose), but is
142 also increasingly considered for minor milk constituents to provide information about the
143 physiological status of the animals [30]. Such information could be used for herd management
144 decisions and for phenotyping in animal breeding as well. However, to be able to relate the spectra
145 obtained to certain metabolites and, in consequence, to identify health disturbances, circumspect
146 validations and algorithms are necessary [31–33]. Those applications are beyond the scope of this
147 review that will be limited to the identification of metabolites related to production diseases in
148 tissues, mainly liver and adipose tissue, and biological fluids, such as blood, but also urine and milk
149 where appropriate.

150

151 **Metritis and endometritis**

152 The uterine mucosal environment is protected, in principle, from invading pathogens by physical
153 anatomical barriers and molecular mechanisms. However, dairy cows may develop endometritis or
154 metritis (an inflammation of the inner mucosal layer of the uterus, or of the entire uterine wall,
155 respectively), when these mechanisms are compromised. The opening of the cervix for giving birth
156 as well as tissue lesions related to labour and expulsion of the calf and placental membranes violate
157 the anatomical barriers, concomitantly the innate immune defense is suppressed thus facilitating
158 bacterial infections that cause metritis and endometritis [34]. These diseases have a high incidence
159 postpartum [35–37] and often result in decreased fertility as shown by reduced conception rates and
160 increased calving-to-conception intervals [38].

161 A pioneering proteomic study provided first clues about the alterations in blood serum in
162 pathological (endometritis) versus physiological states [39]. Patterns indicative for an inflammatory
163 reaction were also found in healthy animals around calving as demonstrated by increasing
164 concentrations of positive acute phase proteins (APP), such as α_1 -acid glycoprotein (AGP) and
165 haptoglobin, and a correspondent decrease of negative APP, such as α_2 HS glycoprotein (fetuin-A)
166 suggesting that the approaching calving resembles an acute-phase reaction. This is in line with the
167 common observation of an inflammatory reaction peripartum that is considered as physiological and
168 even necessary for the successful adaptation to the metabolic challenge as recently reviewed [40].

169 In the proteome study [39], the concentrations of AGP two weeks before calving were lower in
170 animals that developed endometritis postpartum, but were higher two weeks after calving,
171 suggesting AGP not only as a potential prepartum bioindicator for an early detection of an increased
172 risk for endometritis but also indicating a complex functional role for AGP in the context of
173 endometritis.

174 In a 2-DGE analysis followed by MALDI-TOF, endometritis-associated proteins were identified in
175 endometrial samples [41]. Several proteins, including desmin, α -actin-2, heat-shock protein (HSP)
176 27, peroxiredoxin-6, luteinizing hormone receptor isoform 1, collectin-43 precursor,
177 deoxyribonuclease-I (DNase-I), and MHC class I heavy chain (MHC-Ih) were up-regulated in

178 endometritis, whereas, transferrin, interleukin-2 precursor, hemoglobin β subunit, and potassium
179 channel tetramerisation domain containing 11 (KCTD11) were down-regulated as compared to
180 normal endometrium. Desmin and α -actin-2 identified in this proteomic study to be related with
181 endometritis are common in mammalian cells, but being also up-regulated during other diseases,
182 such as cancer [42], they would not qualify as specific biomarkers for endometritis. However, truly
183 specific associations between the proteins found to be divergently regulated are unlikely anyway, in
184 view of the pleiotropic defense reactions. Fig. 3 summarizes the differences in proteomes of blood
185 and endometrial samples associated with the development of metritis. When comparing the uterine
186 proteome of cows infected with a specific bacterium (*Trueperella pyogenes* - formerly
187 *Arcanobacterium*), with that of uninfected cows, using 2-DGE , annexins A1 and A2 (ANXA1 and
188 ANXA2), apolipoprotein A-1, calprotectin (S100A9), cathelicidin, enolase 1 (ENO1),
189 peptidoglycan recognition protein 1 (PGLYRP1), phosphoglycerate mutase 1 (PGAM1), serine
190 dehydratase (SDS) and serine protease inhibitors (SERPIN) B1, B3 and B4 proteins were found to
191 be differentially regulated [43]. In the second part of the study ten of these proteins were monitored
192 in uterine samples from dairy cows at 15 and 42 days post-partum, and strong positive correlations
193 between the cytology scores (percentage of polymorphonuclear neutrophils) and cathelicidin,
194 PGLYRP1, SERPINB1 and S100A9 levels at day 15 were found.

195 Retention of the placenta e.g. the failure to expel the placenta within 12 - 24 h after calving, is
196 known as a major predisposing factor for the development of endometritis or metritis and thus
197 impairs fertility but also animal health in general [44]. Beside infectious diseases, non-infectious
198 risk factors like dystocia, but also nutritional deficiencies, are listed as causes for the placental
199 retention albeit the aetiology is not completely understood [44]. Few investigations on proteomic
200 differences between retained and normal placenta were carried out so far. A review describing the
201 involvement of extracellular matrix proteins in placenta release was recently published [45]. The 2-
202 DGE reference map for bovine placenta during late pregnancy identified 273 proteins, providing the

203 background for studies on molecular mechanism of placenta modification and diseases during late
204 pregnancy [46].

205 Protein differences between retained and normally delivered placentae were studied starting from
206 tissue obtained from both the fetal and the maternal side of the placenta (i.e. cotyledon villi and
207 caruncle crypts). Using 1-D and 2-DE, differences between the protein profiles in the two groups
208 were assessed by means of computer-aided analysis but the identification of specific proteins
209 remained undone [47]. In a follow-up study, the protein patterns in normal and in retained placentae
210 were investigated by 2-DIGE [48]. In this study, differentially regulated proteins were identified by
211 means of MALDI-TOF analysis. Comparisons between fetal healthy/retained and maternal
212 healthy/retained placentae yielded only five differentially regulated proteins. Albeit preliminary, the
213 results point to an involvement of RabGTPases, which are known as master regulators of
214 intracellular trafficking: Ras-related protein Rab-7b was up-regulated only in healthy maternal
215 placenta, whereas Rab GDP dissociation inhibitor beta was up-regulated in the cotyledons of both
216 retained and healthy placentae. In addition, short transient receptor potential channel 5 was
217 identified in caruncles of both retained and healthy placentae, and transforming growth factor β
218 was highly abundant in both maternal and fetal parts of retained placenta. The proteins identified in
219 placental tissues indeed aggrandize the spectrum of relevant pathways to consider in the context of
220 placental maturation. However, these results were limited to tissue analyses and thus realistic
221 predictive approaches for retained placentae using body fluids are not coming into reach so far.
222 The application of metabolomics techniques to metritis and endometritis is still lacking, in both
223 human and veterinary medicine.

224 Further studies have to be conducted to distinguish between physiological and pathological protein
225 and metabolite patterns due to the challenges of parturition and thus to unravel the complexity of
226 the underlying processes; identifying early indicators for the cow`s ability to cope with the situation
227 for eventually providing metaphylactic measures is a further goal.

228

229 **Hypocalcaemia (milk fever)**

230 The requirements for calcium (Ca) increase dramatically towards the end of pregnancy and the
231 onset of lactation. The Ca content of milk is about 1.2 g/L and a modern dairy cow may produce up
232 to 60 L per day during peak lactation, resulting in a daily Ca loss of more than 70 g/d. Thus,
233 metabolic adaptations need to be activated, otherwise the blood concentration of Ca falls below a
234 critical threshold and clinical and subclinical hypocalcaemia can result [49]. Hypocalcaemia
235 impacts health, future milk production, and reproductive performance and has been demonstrated to
236 be linked with compromised immune function; cows with lower blood Ca concentrations within the
237 first day after calving were more likely to have retained placenta and resulting metritis, and mastitis
238 [50]. In addition, hypocalcaemia is also associated with metabolic diseases such as left displaced
239 abomasum, ketosis and fatty liver [51,52].

240 Both proteomics and metabolomics provided some molecular insight into pathogenesis of
241 hypocalcaemia. Proteomic comparisons of plasma samples from dairy cows with or without milk
242 fever were performed by 2-DIGE, followed by in-gel digestion and MALDI-TOF-MS analysis for
243 peptide mass fingerprinting of selected protein spots [53]. Out of 23 protein spots found to be
244 different between the groups, eight were isolated and identified representing five unique proteins:
245 serpin peptidase inhibitor (angiotensin) and endopin 2B were increased in hypocalcaemic animals,
246 whereas albumin, fibrinogen beta chain, and IgG heavy-chain C-region (IgG-C(H)) were down-
247 regulated. Interestingly, the study demonstrated also a shift in the electrophoretic mobility of
248 albumin and angiotensin, suggesting that milk fever not only changes their concentration, but
249 possibly also their post-translational modification. In another study using weak cationic exchange
250 protein chips for plasma protein profiling by SELDI-TOF-MS, six proteins were identified in
251 animals with subclinical hypocalcaemia (average milk yield 30 kg/day) differing from the healthy
252 controls (average milk yield 28 kg/day) [54]: albumin, fibrinogen alpha chain, amyloid beta A4
253 proteins and VGF were increased, and apolipoprotein A-II and serum amyloid A proteins were
254 decreased. In a very recent study [14], serum samples from cows were collected on days -3, 0 and

255 +3 relative to calving. According to the Ca serum concentrations the animals were classified as
256 either healthy or having clinical or subclinical hypocalcaemia. Using samples from day -3, DIGE
257 and MALDI-TOF MS were used to search for proteins suitable as predictors for postpartum
258 hypocalcaemia. Five proteins were differentially regulated when comparing cows that developed
259 clinical milk fever or stayed healthy: Vitamin-D binding protein precursor, paraoxonase,
260 apolipoprotein A-IV precursor and alpha-1-antitrypsin were decreased, and A2M protein was more
261 abundant in cows with clinical hypocalcaemia post partum. There was no overlap in these proteins
262 when comparing healthy cows with those that developed subclinical hypocalcaemia later: compared
263 to healthy animals, complement C4 precursor, A2M protein, endopin 1 and haptoglobin were
264 decreased in cows with subclinical hypocalcaemia, and no protein was found to be increased [14].
265 The issue of hypocalcaemia was also addressed by assessing the metabolome in serum samples
266 from hypocalcaemic versus normocalcaemic cows yielding around 30kg milk/day: using a 500-
267 MHz digital (1)H-NMR spectrometer, nine metabolites with differing concentrations between the
268 groups were found [55]. Glucose, alanine, glycerol, phosphocreatine, and γ -aminobutyrate (GABA)
269 were decreased, and β -hydroxybutyrate (BHB), acetone, pyruvate, and lysine were increased in
270 cows with milk fever. The increase of pyruvate is probably also related to the decrease of
271 phosphocreatinine observed in cows with milk fever, since elevated pyruvate decreases the
272 production of phosphocreatinine, by inhibiting creatinine-pyruvate kinase at least in humans [56].
273 The decrease of phosphocreatine also reduces ATP (adenosine triphosphate) production in muscles,
274 may partially explain the paresis, ataxia and paralysis that are associated with milk fever. In
275 addition, the decrease of the inhibitory neurotransmitter GABA may also account for the
276 neurological symptoms of the disease, e.g. depression and coma. However, the importance of
277 GABA in the circulation is unknown.

278 Results provided by proteomics applied to hypocalcaemia are somehow contradictory: for example,
279 in one study albumin abundance is increased [53], whereas in another the albumin abundance is
280 decreased [54]. This apparent inconsistencies might be related to the fact that results were obtained

281 following two different proteomics techniques (2D-DIGE separation followed by identification with
282 MALDI-TOF-MS and SELDI-TOF, respectively), rather than to different sampling times (in both
283 studies sample collection was close to delivery (6 and 24 h, respectively). In view of positive APP,
284 such as SAA, haptoglobin, complement C4 precursor and alpha-1-antitrypsin being decreased in
285 hypocalcaemic cows, hypocalcemia might also be related to systemic inflammation. Metabolomics
286 results are probably more interesting: taken together, the findings reported by metabolomic analyses
287 indicate a relationship between hypocalcaemia and energy metabolism, rather than a specific
288 association with Ca metabolism. Although limited to one study [57], metabolomics confirmed in
289 cows what has been already reported in humans, i.e. that calcium plays a pivotal role in regulating
290 energy homeostasis. One important finding in this context is that by increasing the Ca^{2+}
291 concentration in adipocytes, adipogenesis and a coordinated inhibition of lipolysis were stimulated,
292 thus demonstrating that Ca is capable of regulating adiposity [58,59]. In dairy cows in which
293 hypocalcaemia was experimentally induced by intravenous infusion of ethylene glycol tetraacetic
294 acid (EGTA), a selective Ca-chelator, reduced blood concentrations of insulin and increased levels
295 of glucose and NEFA were reported, together with reduced phagocytotic and oxidative burst
296 activity of neutrophils [60]. Even though some of the observed effects in this study might have
297 partly been caused by the reduced feed intake during EGTA infusion, the findings are in line with a
298 role of Ca in energy metabolism. Fig. 4 summarizes the differences in metabolome and proteome
299 between healthy and hypocalcaemic cows. Future omics studies could allow to find possible
300 explanations for the contradictory results stated above and deepen the insight into the relationships
301 between hypocalcaemia and energy metabolism.

302

303 **Metabolic diseases related to energy metabolism:**

304 During the transition period, nutrients need to be directed towards the growing foetus and the
305 mammary gland even though feed intake is often depressed around calving and does not increase as
306 does milk yield. To accomplish an adequate supply of nutrients to foetus and mother, several

307 adaptive mechanisms are activated. The main ones are: increasing gluconeogenesis, reducing
308 peripheral insulin sensitivity and increasing lipolysis. As outlined below, these reactions may also
309 overshoot and result in the most common metabolic production diseases, i.e. in ketosis and fatty
310 liver. Ruminants almost entirely depend on gluconeogenesis since glucose from plant carbohydrates
311 hardly reaches the small intestine due to fermentation in the forestomachs which yields propionate
312 as the main gluconeogenetic substrate. In particular the mammary gland has a high demand for
313 glucose to produce lactose, the major osmole in milk. In contrast to other organs, glucose uptake of
314 the mammary gland is insulin-independent and by decreasing the insulin-sensitivity in skeletal
315 muscle and adipose tissue, glucose can be drained towards the mammary gland [61]. The increase
316 in lipolysis provides fatty acids as energy substrates but also for milk fat synthesis; albeit the
317 contribution of fatty acids from the mobilization of body fat is normally less than 10% of the milk
318 fatty acids, this share increases proportionally in early lactation with the extent of the energy deficit
319 [62]. When the rate of lipolysis exceeds the capacity of the liver, fatty liver and ketosis (or
320 acetoanaemia) can occur. With the importance of lipolysis, the central role of adipose tissue comes
321 into play and indeed many studies including proteomics and metabolomics investigated adipose
322 tissue in context with peripartum diseases. Oxidation of fatty acids provides acetyl-CoA which is
323 than condensed with oxaloacetate to form citrate for entering the TCA cycle. However, when
324 glucose requirements are high, oxaloacetate is increasingly used for gluconeogenesis and thus
325 acetyl-CoA cannot be completely oxidized but is converted into ketone bodies, mainly acetone,
326 acetoacetate and BHB. Ketosis, in particular subclinical ketosis, is a common disease in dairy cows
327 and often concurs with other peripartum diseases such as retained placenta and metritis [63]. Excess
328 fatty acids can also be re-esterified in the liver and deposited as triglycerides; however, their export
329 into the circulation is limited based on the low intrinsic capacity for mainly VLDL (very low
330 density lipoprotein) in ruminants [57,64]. Fatty liver is thus another production disease affecting
331 many animals at least in mild forms. Taken together the main adaptations to accomplish partitioning

332 of nutrients towards foetus and milk are basically known, including the temporal patterns of some
333 proteins and metabolites during the transition period [65].

334 However, what makes these physiological adaptive mechanisms shift towards pathological
335 conditions is largely unknown. Using proteomics and metabolomics provides new explanatory
336 approaches and also potentially also predictors for unfavourable conditions which might be
337 mitigated if diagnosed early enough. For the latter applied aspects, the use of body fluids, in
338 particular those that can be collected non-invasively like milk, is certainly preferable; however,
339 including those tissues that are the major players in energy metabolism, i.e. liver and adipose tissue,
340 is necessary for clarifying the pathways included and their complex interrelationships.

341 ***Fatty liver:*** The fatty liver observed in dairy cows has many similarities with non-alcoholic
342 fatty liver disease (NAFLD) in humans. Several proteomic and metabolomics studies were
343 published about NAFLD [66–68]. Yet the respective literature on fatty liver in dairy animals is
344 surprisingly limited. To the best of our knowledge there is only one review about fatty liver
345 proteomics in farm animals, but it mainly focused on poultry [69]. In dairy cows a proteomic
346 analysis was carried out in liver obtained from animals (1st lactation, 16-201 DIM) fed ad libitum as
347 compared with feed-deprived cows [70]. Proteins were separated by 2-DE, and those that were
348 differently regulated were identified by MALDI-TOF. Several pathways related to lipid and to
349 carbohydrate metabolism were found to be dysregulated. Acyl-CoA dehydrogenase and Acyl-CoA
350 acetyltransferase 2 were both down-regulated in feed-deprived animals, suggesting decreased fatty
351 acid degradation and contributing to explain the insurgency of liver disease. The fatty acid binding
352 protein 1 was also found to be decreased. Other enzymes involved in fatty acid degradation that
353 were decreased in feed-deprived cows include aldehyde dehydrogenase, which converts fatty acids to
354 their corresponding aldehydes. On the contrary, sterol carrier protein 2, which catalyzes the
355 transfer/exchange of cholesterol and phospholipids between membranes, was more abundant in
356 feed-restricted cows suggesting an increase in lipid trafficking. The decrease of peroxiredoxin-6,
357 whose main role is to protect against oxidative damages, suggests a possible increase in oxidative

358 stress in the ruminant liver during feed restriction. With regard to carbohydrate metabolism, several
359 enzymes, including 6-phosphofructokinase, enolase1, and triosephosphate isomerase, fructose-
360 bisphosphate aldolase B, sorbitol dehydrogenase and aldehyde dehydrogenase 2 were less abundant
361 in feed-deprived versus ad libitum fed cows. The corresponding up-regulation of parathymosin, an
362 inhibitor for glycolytic enzymes, confirms a reduction of glycolysis [71]. Besides, the results
363 indicated that protein metabolism was also affected by feed deprivation: proteins involved in
364 protein degradation, such as ubiquitin carboxyl-terminal esterase L3, proteasome 26S subunit, and
365 protein disulfide-isomerase-related protein 5 were decreased. This result was unexpected, since feed
366 restriction is believed to result in increased protein degradation. The authors suggested that
367 downregulating proteins involved in protein degradation might help to protect the liver from
368 excessive autophagy. In addition, skeletal muscle rather than liver is the greatest labile source of
369 amino acids for energy needs [72]. However, other “protecting” proteins, such as heat-shock 70
370 kDa protein 5 (HSPA5), a chaperon, were less abundant in liver of feed-restricted cows. The urea
371 cycle in particular was dysregulated: proteins, such as arginase-1 and argininosuccinate synthetase,
372 were up-regulated in feed restriction. Conversely, L-arginine:glycine amidinotransferase and
373 glutamate dehydrogenase 1, were decreased. Finally, proteins involved in calcium metabolism, such
374 as regucalcin, annexin IV and calcium binding protein SPEC 2D were increased in the liver of feed-
375 restricted cows. In 2012, a comparison of the liver proteome of cows with either low or high liver
376 triglyceride (TG) content in early lactation was published [57]. A high liver TG content was found
377 to be associated with increased oxidation of saturated fatty acids, oxidative stress, and urea
378 synthesis and decreased oxidation of unsaturated fatty acids, but not with impaired gluconeogenesis.
379 Aiming to identify hepatic biomarkers for physiological imbalances (PI), the liver proteome of dairy
380 cows at early and mid lactation (49 ± 22 DIM, average milk yield 42 ± 7 kg/day versus 159 ± 39 DIM,
381 29 ± 7 kg/day) was determined by means of ITRAQ-based profiling; PI was calculated based on
382 plasma free fatty acids, BHB, and glucose concentrations and was compared between 6 cows with
383 greatest and least PI in early and mid lactation, respectively. PI was increased by a 4 day feed

384 restriction period and liver biopsies were collected one day before and on day 3 of the feed
385 restriction [16]. In early lactation, enzymes involved in gluconeogenesis and β -oxidation, such as
386 pyruvate carboxylase and very long chain specific acyl-CoA dehydrogenase, respectively, were
387 increased in cows with a higher PI indicating increased gluconeogenesis and fatty acid oxidation. In
388 addition, three enzymes involved in energy metabolism, such as mitochondrial isocitrate NADP+
389 (nicotinamide adenine dinucleotide phosphate)-dependent dehydrogenase, glycine N-
390 acyltransferase and UDP-glucose 6-dehydrogenase were decreased, partially explaining the
391 molecular background of PI. By increasing nutrient restriction, thus aggravating the status of PI, the
392 increasing demand of energy coupled with a decrease of anti-oxidant defense is confirmed by an
393 upregulation of mitochondrial trifunctional protein, subunit α , enoyl-CoA hydratase and pyruvate
394 carboxylase and downregulation of glutathione S-transferase Mu 1, manganese superoxide
395 dismutase, aldehyde oxidase, and glycine N-acyltransferase. Proteins related to apoptosis (14-3-3
396 protein β/α), and mobilization and targeting of fatty acid (liver fatty acid binding protein) were also
397 decreased.

398 During mid lactation, before feed restriction, PI increased the amount of proteins involved in ketone
399 biosynthesis, such as alcohol dehydrogenase 4 and alcohol dehydrogenase NADP+, and in TCA
400 cycle, including dihydrolipoamide dehydrogenase 2 and methylmalonate-semialdehyde
401 dehydrogenase. Again, proteins involved in antioxidant defense and CO₂ transport, such as carbonic
402 anhydrase 3 were decreased. After increasing PI by means of nutrient restriction, the upregulation
403 of proteins involved in fatty acid oxidation, such as acetyl-CoA oxidase 2, acyl-CoA-binding
404 protein and carnitine O-palmitoyltransferase 2 was even more pronounced. Proteins involved in
405 ketone biosynthesis, such as acetyl-CoA acyltransferase were increased as well. Consistent with
406 changes related to PI during early lactation, the experiment amplification of PI decreased proteins
407 involved in antioxidant defenses, such as peptide methionine sulfoxide reductase, Glutathione S-
408 transferase A1, and carbonic anhydrase 3 . Proteins involved in amino acid metabolism, such as
409 peptide methionine sulphoxide reductase and ornithine carbamoyltransferase, and fatty acid

410 oxidation, such as short-chain specific acyl-CoA dehydrogenase and hydroxyacyl-CoA
411 dehydrogenase, were also downregulated. Beside the identification of potential biomarkers for
412 different degree of PI, the study of Moyes and coworkers [16] provided a better understanding of
413 the molecular background of liver diseases during PI, as shown by the downregulation of proteins
414 related to anti-oxidant defense, which might be responsible for the increasing cellular damage. For
415 aldehyde dehydrogenase and HSP70, relationships with NAFLD in rats and in humans,
416 respectively, were identified earlier [73,74].

417 Metabolomics approaches were also applied to study the pathogenesis of and the search for
418 biomarkers in fatty liver disease. A recent review highlighted the state of the art in human and
419 laboratory animal metabolomics investigations on fatty liver diseases [75]. Several studies
420 addressed the modifications induced by the development of fatty liver disease in dairy ruminants by
421 means of metabolomic techniques. A serum metabolomic profile using a triple quadrupole
422 spectrometry identified a total of 29 metabolites which allowed to discriminate healthy cows from
423 animals with hepatic lipidosis [23]. The experimental design included animals with different ranges
424 of hepatic lipidosis, ranging from low, medium to severe grade. Some animals also displayed, in
425 addition, other diseases, such as displaced abomasum, bronchopneumonia, retained placenta, and
426 mastitis. Six phosphatidylcholines were identified as promising predictive biomarkers of hepatic
427 lipidosis. The other discriminating metabolites included amino acids, such as glycine and
428 glutamine, sphingomyelins and hydroxy-sphingomyelins and other phosphatidylcholines, but could
429 only discriminate the three unhealthy groups from the healthy animals. Beside their possible use as
430 biomarkers, the finding that the phosphatidylcholine asset is modified during lipidosis is interesting:
431 phosphatidylcholines are precursors of hepatic triacylglycerols [76], and can decrease peripartum
432 due to an increased triacylglycerol production [77–79]. Assembly and secretion of lipoproteins
433 require the contribution of phosphatidylcholines. By limiting the hepatic synthesis of lipoproteins,
434 which export fatty acids from hepatocytes, the decrease of phosphatidylcholine content may
435 aggravate the accumulation of lipids in the liver [80].

436 A parallel study focusing on the plasma lipidome also drove to similar conclusions [81]. The
437 investigation was carried out on animals with different grades of fatty liver, displaying weak,
438 medium and severe disease. The lipid extracts were profiled by means of separation with ultra
439 performance LC and identification with LC-MS. The study confirmed that phosphatidylcholines are
440 reduced in animals with medium and severe fatty liver disease. Five bile acids were decreased as
441 related to increased severity of fatty liver. Remarkably, the insurgency of fatty liver diseases was
442 positively correlated to the presence of Resolvin E1 and palmytoil-ethanolamine. Resolvin E1 is
443 synthesized from ω -3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and
444 docosahexaenoic acid (DHA), and is an anti-inflammatory lipid mediator[82,83][82] [83]. Also
445 palmytoil-ethanolamine is an endogenous amide with anti-inflammatory activity [84]. It is known
446 that metabolic diseases such as fatty liver also induce an inflammation [85]. The study of Gerspach
447 and coworkers [81], therefore, confirmed that anti-inflammatory pathways are activated during fatty
448 liver disease. One estrogen metabolite, not further specified, was found to discriminate between
449 mild or strong and weak fatty liver in the same study.

450 A novel approach relying on (1)H-NMR investigated the metabolome in fatty livers of 171 Holstein
451 cows [86]. The main advantage of this technique in the metabolomic field is that it can provide a
452 profile of proton-containing, low-molecular-weight metabolites, starting from a limited amount of
453 sample [87]. Plasma and liver tissue samples from animals with fatty liver disease (16.31 ± 4.30
454 DIM, average milk yield 28.32 ± 4.73 kg/day) and healthy animals (15.5 ± 6.02 DIM, average milk
455 yield 30.75 ± 3.7 kg/day) were included in the study. As expected, plasma from animals with fatty
456 liver disease had increased BHB, isobutyrate and acetone concentrations. The amino acids glycine,
457 valine, trimethylamine-N-oxide, and citrulline were also increased. Conversely, other amino acids
458 such as alanine and asparagine were decreased. Glucose, GABA, glycerol, and creatinine were
459 decreased as well. The decrease of alanine and asparagine is consistent with their role as
460 gluconeogenic amino acids, since both can enter the TCA cycle to generate glucose during an
461 energy-deficient status. The finding that trimethylamine-N-oxide and citrulline are increased what is

462 usually accompanied by oxidative stress and liver damage, corresponds to what has been
463 demonstrated in other species, such as mice [88] and humans [89].

464 ***Ketosis:*** to extend the understanding of the pathogenic effects of ketosis, protein
465 modifications were determined by using 2-DE coupled with MALDI-TOF for comparing the
466 proteomic profiles in liver from healthy and ketotic cows [90]. Several metabolic pathways were
467 found to be altered in ketosis. Structural proteins, such as myosin related proteins (myosin light
468 chain for example, and tropomyosin) and MGC128326 were significantly up-regulated in liver from
469 cows with ketosis. As shown by the increased number of isoforms, post translational modifications
470 related to ketosis were assumed. Myoglobin was also more abundant, suggesting an activation of
471 oxidative stress defense pathways, which is confirmed by the upregulation of proteins belonging to
472 the peroxiredoxin families, such as peroxiredoxin – 5 and -6, glutathione S-transferase alpha-1,
473 flavin reductase and sulfotransferases, all of them fulfilling anti-oxidant activities. As expected,
474 proteins related to gluconeogenesis, such as α -enolase, were also increased during ketosis. The list
475 of proteins that were decreased in ketotic liver included also proteins involved in fatty acid
476 oxidation, such as acetyl-CoA acetyltransferase 2 and 3-hydroxyacyl-CoA dehydrogenase,
477 suggesting a further possible relationship between ketosis and lipidosi: as a consequence of the
478 decreased ability to utilize them, fatty acids accumulate in liver cells, contributing to the
479 development of hepatic lipidosi.

480 Beside liver and plasma, the difference of proteomic profiles in animals with ketosis were also
481 explored in urine using SELDI-TOF techniques [91]. Samples were collected 7 – 28 DIM from two
482 groups of animals, one affected by clinical ketosis and the other from healthy cows, with a milk
483 production of 9625 kg/year. The urinary profile of animals with ketosis showed a decrease for 11
484 proteins, most of them involved in the inflammatory response, such as fibrinogen, C1 inhibitor,
485 osteopontin, also hepcidin and human neutrophil peptides 1-3. Interestingly, also proteins associated
486 with the neuron function, such as VGF (non-acronymic) protein and amyloid precursor protein,
487 were decreased in the animals with ketosis as compared with the control group. Proteins related to

488 lipid metabolism, as well as inflammation, were also decreased during ketosis, namely SAA and
489 apolipoprotein C-III, indicating a change in lipid metabolism during ketosis. Two other proteins, i.e.
490 transthyretin, a transport protein, and cystatin C, a protease inhibitor, were also decreased.
491 Fibrinogen, hepcidin and SAA are acute phase proteins [92] and their concentration is supposed to
492 increase during an inflammatory status. Targeted assessments of acute phase proteins in blood have
493 shown increased concentrations of inflammatory markers in ketotic cows. Thus the finding of
494 decreased concentrations of SAA in urine of ketotic cows is opposing the situation in blood and
495 deserves further investigation. The proteins differentially regulated in liver and urine of cows with
496 ketosis as compared to healthy animals are presented in Fig. 5.

497 More than proteomics, it is metabolomics that contributed to address the changes in biological
498 fluids during ketosis. A NMR-based metabolomic analysis on milk from animals with ketosis was
499 carried out during a time course study on 264 high yielding dairy cows with an average milk yield
500 of 32.8 ± 4.7 kg energy corrected milk/day [93]. Milk samples were collected weekly for 5 weeks
501 and once again 6 months post partum. NMR spectroscopy was carried out, and presented evidence
502 that high milk glycerophosphocholine (GPC) levels and high ratios of GPC to phosphocholine (PC)
503 during the first four weeks of lactation, and GPC at mid lactation, could provide reliable biomarkers
504 for the development of ketosis. Although milk provides an ideal substrate for metabolomic analysis,
505 being collected routinely and non-invasively, more studies were carried out on plasma metabolome,
506 which, conversely, yields a higher amount of potential biomarker metabolites. A metabolomic
507 approach using GC/MS techniques analysed the differential plasma metabolomes of dairy cows
508 with clinical (12 ± 5 DIM, 32.1 ± 7.8 kg milk/day) and subclinical ketosis (14 ± 6 DIM, 35.0 ± 7.2
509 kg milk/day), as compared with healthy animals (16 ± 6 DIM, 37.0 ± 6.2 kg milk/day) [94]. The
510 study revealed that the metabolomes of animals with subclinical and clinical ketosis were mostly
511 identical, whereas 25 potential biomarkers were found between animals with ketosis, both clinical
512 and subclinical, when compared with healthy animals indicating that several biochemical pathways
513 were modified. The nine metabolites decreased during ketosis suggested a decrease in

514 gluconeogenesis in affected animals, and a parallel activation of the pentose-phosphate pathway. A
515 decrease of ribitol levels, related to riboflavin deficiency, and vitamin C, may increase the oxidative
516 stress. The decrease of lactic acid and L-alanine, which are both gluconeogenetic precursors,
517 suggested a close relationship between ketosis and carbohydrate metabolism, as a consequence of
518 hypoglycaemia and lack of precursors of gluconeogenesis. Sixteen metabolites were increased in
519 animals with ketosis. As expected, this list included ketone bodies, and fatty acids, such as BHB,
520 palmitic acid, heptadecanoic acid, stearic acid, trans-9-octadecenoic acid, myristic acid, cis-9-
521 hexadecenoic acid, confirming also the mobilization of adipose tissue. Amino acids were also found
522 to be increased, namely L-isoleucine and a catabolic product of lysine, 2-piperidinecarboxylic acid,
523 two amino acids involved in ketogenesis, and glycine, suggesting an increase of proteolysis needed
524 to fuel gluconeogenesis. The study confirmed BHB acid as gold biomarker for ketosis, and
525 suggested cis-9-hexadecenoic acid as novel biomarker for clinical ketosis, and an indicator of fat
526 mobilization. Plasma metabolic profiling from cows affected by clinical ketosis was determined
527 with two other different techniques, namely LC/MS and (1)-NMR. In a study whose experimental
528 design was very similar to that of Zhang and coworkers [94] plasma metabolomics of cow with
529 clinical and subclinical ketosis was determined by means of 1H-NMR [95]. A total of 25
530 metabolites were found to be dysregulated as a consequence of various stages of the disease, and as
531 compared with healthy animals. Amino acids including histidine, glutamic acid, glutamine, lysine
532 and phenylalanine, together with lactate and glucose, were decreased during ketosis. Amino acids
533 such as alanine, proline and tyrosine decreased only in clinical ketosis, as well as LDL and VLDL.
534 As expected, metabolites related to biosynthesis of ketone bodies, such as BHB, N-
535 Acetylglucosamine, acetate, acetoacetate and acetone were increased in ketotic cows. Glycine,
536 leucine, isoleucine and valine were increased in clinical ketosis only. A last study, this time focused
537 on the differences in metabolomes between Holstein Friesian cows, 12 - 16 DIM and 18 - 21 kg
538 milk /day, affected with clinical ketosis or being healthy, was carried out by means of LC/MS [96]:
539 aminoacids such as valine, glycine, and lipids, such as glycocholic, tetradecenoic and palmitoleic

540 acid, were increased in clinical ketosis, whereas other amino acids, such as arginine, leucine,
541 isoleucine, tryptophan and lysine decreased. Aminobutyric acid, creatinine, undecanoic acid and
542 norcotinine were decreased as well. Consistent with previous reports, obtained with different
543 metabolomic techniques, the study from Li and coworkers [96] confirmed that amino acids are
544 affected during ketosis: e.g. glycine was increased whereas lysine was decreased.

545

546 **Pathophysiological alterations in the proteome and metabolome of adipose tissue related to** 547 **the transition period**

548 Adipose tissue fulfills a dual role: it regulates energy storage by storing and releasing fatty acids,
549 and is a major endocrine gland, capable of modulating metabolism by secreting hormones and
550 adipokines [97]. As mentioned earlier, the transition from late pregnancy to early lactation is
551 accompanied by an increased rate of lipolysis. Furthermore, adipose tissue can regulate, by means
552 of modifying its adipokine asset, the development of major metabolic changes such as insulin
553 resistance or sensitivity [7,61,98].

554 The proteome of subcutaneous adipose tissue from cows classified as either insulin-resistant or
555 insulin-sensitive according to phosphorylation of protein kinase B (AKT) in this tissue was
556 characterized by quantitative shotgun proteomics (nanoLC-MS/MS) [99]. Adipose tissue biopsies
557 were collected 17 days before and 3 – 5 days after calving. From 586 proteins detected, 143 were
558 differentially regulated in prepartum versus postpartum tissue. Several functions, such as those
559 related to lipid metabolism, including fatty acid metabolism, the esterification of lipids and
560 oxidation of fatty acids, appeared as changed. The proteins whose abundance was decreased after
561 calving included fatty acid synthase, complement C3, annexin-A1 and acyl-CoA desaturase.

562 Comparing insulin-resistant and insulin-sensitive subcutaneous adipose tissue yielded 111 proteins
563 that were differentially regulated. Most of them (a total number of 106) were more abundant in the
564 insulin-resistant state, whereas only five were decreased. Insulin resistance was associated with a
565 dysregulation of pathways related to energy and lipid metabolism, including gluconeogenesis and

566 glycolysis, signaling mediated by 14-3-3, TCA cycle, ERK/MAPK signaling, lipid accumulation,
567 release and lipolysis. Inflammatory responses, such as leukocyte migration and proliferation of T
568 lymphocytes, were also activated in insulin-resistant adipose tissue.

569 During the transition period, adipose tissue may also react to stress related to environmental factors,
570 such as heat. A label-free, quantitative shotgun proteomics (nano-LC-MS/MS) approach
571 investigated the effects of seasonal heat stress on the adipose proteome, aiming to highlight
572 biomarkers of heat stress on late pregnant cows during summer heat stress (average milk yield 33.8
573 kg/day) as compared to the winter season (38.2 kg/day) [100,101]. The proteome in subcutaneous
574 adipose tissue biopsies obtained 14 day before calving yielded a total number of 107 out of 1495
575 proteins identified that were differentially abundant between summer and winter. The pathways that
576 were found to be modified included the Keap1-Nrf2 pathway which is the major regulator of
577 cytoprotective responses to oxidative and electrophilic stress [102], such as STIP1 and ubiquitin-
578 conjugating enzyme E2 K, which were increased in summer, and GSTM1, microsomal GST 1,
579 (MGST1), GST Mu 3 (GSTM3), ferritin heavy chain and MAP2K1 which were decreased. The
580 acute phase response was also modified. In particular, albumin, hemopexin, serotransferrin, AGP,
581 apolipoprotein A-II, α -2-HS-glycoprotein, C-reactive protein and MAP2K1 were decreased in
582 summer, whereas the abundance of the von Willebrand factor and fibrinogen α chain was increased.

583 Protein related to the farnesoid X receptor (FXR)[103], which is a member of the nuclear family of
584 receptors in control of numerous metabolic pathways, and, jointly with retinoid X receptor (RXR),
585 plays a crucial role in linking bile acid productions with lipoprotein, lipid and glucose metabolism,
586 were also modified, as well as proteins belonging to Liver X receptor/RXR pathways [104], whose
587 function is to regulate cholesterol, fatty acid, and glucose homeostasis. The finding of this study
588 provided the evidence that heat stress has a local impact on adipose tissue in late pregnant cows,
589 highlighting meanwhile a list of possible biomarkers for heat stress related to transition period.

590

591 **Pathophysiological alterations in the proteome and metabolome of skeletal muscle related to**
592 **the transition period**

593 Several studies point to the importance of amino acid metabolism in context with the
594 pathophysiological changes related to the metabolic challenges of the transition period. An
595 increased need for amino acids results from fetal growth and milk protein synthesis but is also
596 related to the use of amino acids for generating energy by direct oxidation or as precursors for
597 gluconeogenesis. The dogma that amino acids are significant contributors to hepatic
598 gluconeogenesis has recently been revised based on quantitative data on the uptake of amino acids
599 by the liver; only alanine remains in the list of quantitatively important gluconeogenic amino
600 acids [105]. However, there is also an increased need for amino acids for positive acute phase
601 proteins [106–108] which show a distinct and typical peak around calving [39]. The biggest labile
602 source for amino acids in the body is skeletal muscle, but the number of proteomics or
603 metabolomics studies actually addressing this tissue in context with transition cow is very limited.
604 Kuhla and coworkers [105] used 2-DE and MALDI-TOF-MS on muscle biopsies collected in week
605 -3, 0, +2 and +4 relative to calving. In total 43 differentially regulated muscle protein spots were
606 identified throughout the periparturient period. In early lactation, abundance of cytoskeletal proteins
607 and enzymes involved in glycogen synthesis and in the TCA cycle was decreased, whereas proteins
608 related to glycolysis, fatty acid degradation, lactate, and ATP production were increased.
609 Metabolomic investigations of skeletal muscle in transition cows are only at the verge of being
610 published: in view of several abstracts presented at the International Animal Science and Dairy
611 Science meeting in 2016 and 2017, several publications on this topic can be expected [109–111].

612

613 **Associations of proteomes and metabolomes with productive life span, energy balance and**
614 **feed regulation**

615 Several studies focusing on the metabolic situation during the transition period in general rather
616 than on specific production diseases also contributed to our understanding on the pathophysiology

617 of the underlying adaptive responses. Aiming to find biomarkers for any kind of production
618 diseases relevant for transition dairy cows, a quantitative targeted metabolomics approach was used
619 [21]. Plasma collected at 4 time points from 4 weeks prepartum to 4 weeks post partum from 12
620 cows of which 6 developed multiple peripartum diseases, including laminitis, mastitis, metritis and
621 retained placenta whereas the other 6 cows remained healthy was compared. The study identified
622 five plasma metabolites that could be related to periparturient diseases: carnitine, valerylcarnitine,
623 propionyl carnitine, lysophosphatidylcholine acyl C18:2 and lysophosphatidylcholine acyl C14:0
624 were increased in animals that developed peripartum related diseases as compared with healthy
625 controls as early as 4 weeks before parturition. Two phosphatidylcholines, namely
626 phosphatidylcholine acyl-alkyl C42:4 and phosphatidylcholine diacyl C42:6, were increased 1 week
627 before delivery. Carnitine, lysophosphatidylcholine acyl C18:2 and lysophosphatidylcholine acyl
628 C14:0 were increased 1 week postpartum as well, whereas carnitine was decreased after 4 weeks.
629 These results are remarkable, since they highlighted the possible use of three metabolites, namely
630 carnitine, propionyl carnitine, and lysophosphatidylcholine acyl C14:0 as potentially predictive of
631 peripartum-related diseases up to 4 weeks before delivery [21,96].

632 The occurrence of production diseases exerts profound effects on productive life span since
633 continued disorders like decreased fertility may result which in turn give reason to premature
634 culling. Even in case of non-ouvert disease, a metabolic predisposition for the risk of leaving the
635 herd prematurely might exist. Huber and colleagues [22] applied targeted metabolomics but also
636 “classical” variables (e.g. insulin, free fatty acids, BHB) to search for factors predisposing for
637 shorter or longer productive lifespan in 19 cows that remained apparently healthy during the first
638 100 days of lactation. Eight of these cows left productive life within the current lactation due to
639 various health and fertility problems and 11 cows finished the current lactation without any signs of
640 clinical illness. Long-chain acylcarnitines and biogenic amines were found to be associated with
641 extended productive life span. These metabolites are mainly secreted by the liver and depend on the
642 functionality of hepatic mitochondria. The concentrations of biogenic amines and some

643 acylcarnitines differed already before the onset of lactation thus indicating their predictive potential
644 for continuation or early ending of productive life.

645 Using milk samples from cows with great differences in energy balance, achieved by varying the
646 length of the dry period, Lu and coworkers [112] applied untargeted metabolomics and proteomics
647 techniques, i.e. as NMR in milk serum and milk lipids as well as FASP Dimethyl Labeling-
648 NanoLC-Orbitrap-MS/MS on milk fat globule membrane proteins. They found that a severely
649 negative energy balance was related to greater concentrations of acute phase response proteins,
650 unsaturated fatty acids, and galactose-1-phosphate. In contrast, the concentrations of cholesterol,
651 cholesterol synthesis-related proteins, and stomatin were increased in improved energy balance. The
652 appropriateness of using milk proteomic and metabolomic data to draw conclusions not only about
653 milk quality but about the individual cow's health situation is of outstanding importance. Using
654 NMR techniques in routine milk recordings is aimed at applying such information in herd health
655 management programs or for breeding purposes. Maher and co-workers [113] compared the
656 metabolic profiles from blood and from milk samples obtained from Holstein cows via ¹H NMR
657 methods and statistical heterospectroscopy. The authors summarized their results as being
658 confirmative for milk being a distinct metabolic compartment with a metabolite composition largely
659 not influenced by plasma composition under normal circumstances. Similarly, Ilves and coworkers
660 [114] reported that there is only little correlation between the composition of the metabolomes in
661 milk and in blood. However, the group of Maher [113] found trimethylamine and dimethylsulfone,
662 both originating from rumen fermentation, being correlated across both body fluids, indicating that
663 measuring these substances in either body fluid might allow to evaluate rumen function. Taken
664 together, omics techniques provide a detailed view on a great number of metabolites or proteins and
665 thus enable to also consider additional factors previously not considered. The knowledge of the
666 factors influencing or indicating the metabolic situation and productive lifespan of dairy cows can
667 thus be deepened. Nevertheless, extrapolating results from a given experimental design, body fluid

668 or tissue, and performance level of cows likely has its limitations when conditions e.g. timing of
669 samples relative to physiological status, are different.

670

671 **Proteomics during laminitis.**

672 Laminitis (*Pododermatitis aseptica diffusa*), also known as sole-haemorrhage, is an inflammation of
673 the laminar corium of the hoof. Cow laminitis accounts for 41% of cases of lameness [115] and is
674 most prevalent around day 50 to day 100 of lactation [116]. It is thus not a typical disease for early
675 lactation as the other diseases included in this review, but the course of the preceding reactions
676 during the transition period might form predisposing elements for developing laminitis. Related to
677 laminitis are several claw horn lesions, such as white line disease and ulcers of the sole. Metabolic
678 diseases, in particular acidosis, both clinical and subacute ruminal acidosis (SARA) are regarded as
679 underlying cause of laminitis [117], although the pathogenesis is not fully understood. The digestive
680 disorder SARA is found in up to 19% of early lactation dairy cows as well as 26% of mid-lactation
681 cows, and is related to diets with high portions of concentrate. Increasing the portion of concentrate
682 is a common strategy to improve the energy supply for cows, but there is a risk for shifting the
683 rumen microbiota toward increased lactate production and thus acidification of rumen content
684 which may result in increased histamine secretion and thereby also affect the claw capillaries [118].
685 However, the causes of laminitis and associated claw horn lesions are multi-factorial in nature
686 [119,120].

687 In equine medicine, laminitis can be induced by feeding and using such models several omics
688 studies have been carried out to unravel the pathogenesis of the disease, or to look for predictive
689 biomarkers [121,122]. As a preliminary step to unravel the changes in plasma of dairy cows
690 affected from laminitis, a proteome analysis carried out by 2-DE coupled with MALDI-TOF
691 identification of differentially regulated proteins between animals with spontaneously occurring
692 clinical laminitis and healthy animals used as controls was carried out [123]. A semi-quantitative
693 analysis of the 2-DE gels revealed that 16 proteins were differentially regulated between the two

694 groups of animals, of which 12 were more abundant in laminitis, and 4 were decreased, as
695 compared to healthy animals. Proteins involved in inflammation and in defensive mechanisms were
696 increased during laminitis, namely complement component C9, haptoglobin and conglutinin, as
697 well as apolipoprotein A-IV, and apolipoprotein A-I, which are also involved in inflammatory
698 reaction, but also in lipid metabolism, together with 3-hydroxy-3-methylglutaryl-CoA reductase.
699 The group of more abundant proteins includes also zinc finger protein 300-like, transmembrane
700 protein TMP10, isocitrate dehydrogenase, and serum albumin. The upregulation of serum albumin
701 is remarkable, and apparently in contradiction with the behaviour of albumin during acute
702 inflammation. Serum albumin is a negative acute phase protein [92,124] and, therefore, its serum
703 concentration decreases during acute inflammation, as it has been demonstrated also in laminitis
704 [125]. It must be said that the upregulation of albumin is hardly demonstrable in the context of the
705 experimental design of the study, on the background that the samples were analyzed after depletion
706 of abundant proteins, such as albumin and immunoglobulins, to decrease the dynamic range of the
707 samples. Besides, it has been demonstrated that albumin may be regarded as a local positive acute
708 phase protein during mastitis, being up-regulated by epithelial cells of the mammary gland [126].
709 The proteins that were less abundant in plasma from animals affected by clinical laminitis include
710 two members of complement pathways, namely C4BP, which is an inhibitor of complement, and
711 Complement C9 precursor, and Glycerol-3-phosphate dehydrogenase 1-like protein, and Ectoderm-
712 neural cortex protein 1, which is an actin-binding protein playing a role in the oxidative stress
713 response. The number of proteomics studies on bovine laminitis is yet too limited to draw final
714 conclusions about the pathogenesis. Nevertheless, proteomics results confirmed the development of
715 a pro-inflammatory loop, as demonstrated by the upregulation of inflammation-related pathways,
716 and a converse down-regulation of anti-inflammatory factors, as demonstrated by the decrease of
717 C4BP. The increase of complement C9 and the parallel decrease of complement C9 precursor
718 suggests that the complement pathway is activated, since the C9 precursor is less abundant, likely to

719 produce the active C9 protein. To the best of the knowledge of the authors, no metabolomics studies
720 on cow laminitis were carried out to date.

721

722 **Proteomics and metabolomics in transition period related diseases: gaps and perspectives**

723 The main features of the metabolic derangements occurring in the typical production diseases of
724 dairy cows that are related to the transition from pregnancy to lactation are basically known since
725 several years. In particular for the metabolic diseases, overshooting lipolysis and ketogenesis were
726 identified as aetiologic key elements. However, the knowledge of the molecular basis of successful
727 versus compromised adaptation to the metabolic challenge of early lactation is still incomplete.

728 Omics technologies such as proteomics and metabolomics provide important tools to close this gap
729 in understanding the pathophysiology and also hold some promise for developing biomarkers.

730 *Understanding the pathophysiology:* beside transcriptomics, proteomics applied to adipose
731 tissue, for example, has evidently contributed to a huge advancement in the knowledge of the
732 involvement of this tissue in the development of transition-related diseases [127]. Proteome maps
733 have been established for several biological fluids and tissues in cattle [128], and the amount of the
734 literature available on cow proteomics and metabolomics is steadily growing, as shown in Fig. 6.
735 Nonetheless, although increasing exponentially during the last few years, the application of
736 proteomics and metabolomics in veterinary and animal science is lagging behind those in human
737 medicine [11], and several gaps have yet to be covered. A wider application of proteomics
738 techniques in bovine peripartum-related diseases is hampered by the lack of tools to validate the MS
739 findings. The availability of the bovine genome [129] will allow for a very precise identification of
740 selected proteins and is poised for closing this gap. Moreover, the complete annotation of the
741 genome provided a full application of bioinformatics tools to characterize the pathways where these
742 proteins are involved in. Yet, for a biological validation of proteins identified at different abundance
743 in proteomics approaches, the options are limited to the use of antibodies in immunoassays (e.g.
744 ELISA) or in Western blotting. For both approaches, the specificity of the antibodies is limiting,

745 and species determined differences in the amino acid sequence might hamper the applicability of
746 antibodies developed against the target protein in different species. Besides, the performance of
747 antibodies in different methodological set-ups can differ. The quantitative power of immunoassays
748 like ELISA is usually good, but developing a valid ELISA system is laborious and time consuming.
749 In Western blotting, the effort of setting up a system with a working antibody is often considered as
750 faster, but when aiming to work quantitatively, comprehensive validation is also required and
751 determining differences as low as 2 to 4-fold may remain impossible [130]. In view of these
752 limitations, assessing the matching mRNA concentration, preferably from identical sample for
753 validating results from proteomics is often used as “biological validation”. However, the
754 abundance of mRNA may not correspond to the abundance of the respective protein [131] and
755 therefore relying on the direct, *absolute* correlation between protein and mRNA levels is hardly an
756 adequate validation measurement [132]. Nevertheless, albeit being far from perfect, in many cases
757 quantifying the mRNA abundance remains the only approach available [131], but is further limited
758 to cells and tissues, where both mRNA and proteins are accessible. When proteomics is carried out
759 in biological fluids, such as saliva, urine and blood serum, only protein but not mRNA material is
760 available. Nonetheless, the growing economic interests in producing antibodies and also assays
761 specific for various animal species, including cattle, might cover this gap in the near future. The
762 validation of metabolomics results is easier, there is no species issue and expectedly the comparison
763 between results obtained via MS-based targeted metabolomics with classical assays yielded good
764 correlations. For example, in a study in which 17 free amino acids were measured in 54 dairy cows’
765 sera both via classical methods or via a target metabolomics approach [133] the concentrations
766 obtained by the two methods were all correlated ($P < 0.0001$), with an average r-value of $0.82 \pm$
767 0.14 (mean \pm SD; Dr Hassan Sadri, personal communication). In non-targeted metabolomics, the
768 ongoing improvement of the livestock metabolome database (LMBDB, available at
769 <http://www.lmdb.ca>), will facilitate future untargeted metabolomics studies by increasing the
770 number of identified metabolites. The main gap to be covered includes metabolite coverage and

771 their quantification, however improving the data set and information included in the livestock
772 metabolome database (LMBDB, available at <http://www.lmdb.ca>), will facilitate future untargeted
773 metabolomics studies. For NMR-based approaches, there is a large variability of reference methods
774 used for calibration, and thus standardizing the methods used within and across countries is still a
775 major challenge [134]. Moreover, albeit the application in milk is attractive in terms of being non-
776 invasive and combined with well-established routine assessments of macro nutrients in milk, it
777 should be kept in mind that there is only little correlation between the composition of the
778 metabolomes in milk and in blood [114].

779 Standardization and comprehensive reporting of experimental conditions and animal characteristics
780 is a basic requirement but is often incomplete, albeit respective guidelines are available, at least in
781 laboratory animals [135]. This is a general shortcoming that, albeit not specific for proteomics and
782 metabolomics studies, is of particular importance for omics techniques due to the snapshot character
783 of the results generated. In large animals as dairy cows, standardization of experimental conditions
784 and animals is close to impossible but important information such as age, lactation number and
785 stage, body condition, diet composition and feeding regimen as well as detailed description of
786 sampling procedures and timing relative to physiological state are sometimes missing. Given the
787 background that the development of metabolic diseases in ruminants is strongly related to the
788 nutrient requirements which in turn depend mainly on the level of milk production, the need for
789 reporting milk yield and composition for an appropriate interpretation of data is obvious.

790 Applying proteomics and metabolomics to the complex of transition period-related diseases for
791 elucidating the underlying pathophysiological processes is beneficial and allows for developing a
792 holistic imagination but also a faster progress in research. Diseases apparently not related, such as
793 fatty liver, hypocalcaemia, ruminal acidosis and laminitis, could be identified as being associated
794 not only from epidemiology data but also for what concerns their molecular backgrounds. Examples
795 for newly emerging disease associated pathways from proteomics and metabolomics comprise the
796 relationship with energy status in hypocalcaemia [57], the involvement of RabGTPases in retained

797 placenta [48] or the observation of decreased cystatin concentrations in urine from ketosis-affected
798 cows [43]. In addition, the concept of NEFA oxidation rather than lipolysis alone influences the
799 adaptive capability to the metabolic challenge of early lactation, was substantially supported by the
800 acyl-carnitine data from metabolomics [22,96] but also from proteomics [16]. Applying omics
801 techniques distal from transcriptomics is also particularly promising in terms of quantitative aspects
802 since the relative importance of pathways can be evaluated by the flux changes and thus may allow
803 for identifying molecular targets most promising for prophylactic, metaphylactic or therapeutic
804 interventions. For making more efficient use of the analytical techniques available for
805 understanding the adaptive responses in dairy cows, a systemic approach is required. Integration of
806 different omics techniques, namely metabolomics, proteomics and transcriptomics in the same
807 study, will help to produce a holistic and comprehensive interpretation of multi-omics data. Hereby
808 production data and, if available, genotype information, should be combined for providing an
809 integrated network of the single elements, the knowledge of which could yield a level of
810 information higher than the sum of individual parts. It must be said that this gap may be difficult to
811 bridge, given the shortage of funding available to livestock research as compared to medical studies.
812 Nevertheless when balancing the amount of information provided from metabolomics and
813 proteomics against the one from classical assays for the different targets, the omics approaches, in
814 particular those where no further biological validation is required as in case of metabolomics, might
815 nevertheless work out superior in terms of costs.

816 ***Development of Biomarkers:*** Selecting biomarker candidates from proteomes and
817 metabolomes that show great differences when comparing diseases cows against healthy controls
818 and that can be assessed in body fluids may yield predictive and diagnostic tools. However, such
819 biomarkers will be based on quantitative results since none of the candidates will be found in
820 healthy or in diseased state only. Proteomics studies do mostly not provide truly quantitative data,
821 but refer to trends, e.g. an increase or a decrease as compared to internal standards. Applying
822 proteomic techniques to metabolic diseases has not provided any robust and consistent list of

823 biomarkers so far, although it has shed some light into the pathogenesis of many transition-period
824 related diseases. More than proteomics, metabolomics contributed more to discovering potential
825 biomarkers. As mentioned above, carnitines emerged as potential biomarkers for metabolic diseases
826 related to transition period [21,22,96,109]. When considering the application of biomarker
827 candidates it is probably more realistic to assess patterns of different metabolites rather than single
828 components. Moreover, due to the common basis of many transition period-related diseases,
829 specific markers for individual diseases are improbable to emerge. In addition, comprehensive
830 biological validation is needed before such measurements can indeed be considered as assessments
831 of biomarkers for predictive and diagnostic purposes. However, the integration of several
832 pathophysiological aspects e.g. lipolysis, ketogenesis and oxidative capacity in such patterns, by
833 combining fatty acids, ketone bodies AND acylcarnitines, will likely yield more information than
834 the classical measurement of NEFA and BHB. Moreover, the expansion of the dynamic range of
835 detection of low-abundance proteins and metabolites is likely poised to pave the way for the
836 detection of peripartum-specific biomarker patterns. Such (complex) biomarkers, will enable the
837 identification of phenotypes less sensitive to metabolic stress and thus the development and
838 implementation of strategies for early diagnosis, prognosis and prevention of transition-related
839 diseases.

840

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844

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1314 **Figure legends**

1315 **Fig. 1: The relationship between metabolic stress and disease development.**

1316 The figure presents a schematic flow of the events during the pathogenesis of peripartum related
1317 diseases

1318 **Fig. 2: The application of OMICS technologies to production diseases in dairy cows**

1319 **Fig. 3: Changes in protein abundance during endometritis.**

1320 The figure presents the differences in proteomes of blood and endometrial samples associated with
1321 the development of metritis. The figure was drawn following the papers of Cairoli et al. [39] and
1322 Choe et al. [41]. As indicated by the arrows, the abundance of proteins listed on the left was
1323 increased, whereas the ones on the right were decreased when compared to healthy animals.
1324 Abbreviations: α 1-acid glycoprotein (AGP), Heat-shock protein (HSP), deoxyribonuclease-I
1325 (DNase-I), luteinizing hormone receptor isoform 1 (LH receptor isoform 1); MHC class I heavy
1326 chain (MHC-Ih), Interleukin (IL), Hemoglobin (hb), potassium channel tetramerisation domain
1327 containing (KCTD).

1328 **Fig. 4: Changes in protein abundance and metabolites between healthy and hypocalcaemic** 1329 **cows.**

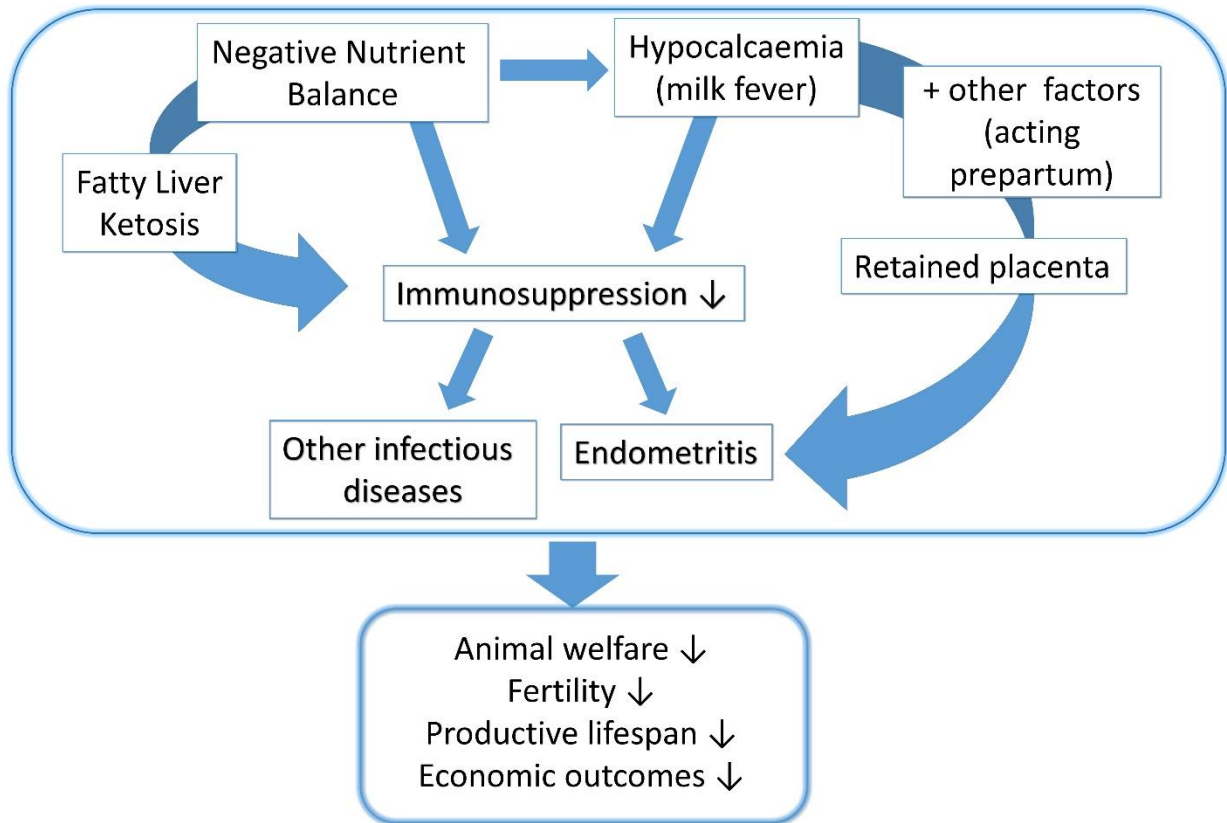
1330 The figure highlights the differences in metabolome and proteome between healthy and
1331 hypocalcaemic cows. The figure was drawn following the papers of Xia et al. [53], Wang et al. [54]
1332 and Shu et al. [14], for what concerns proteomics, and Sun et al. [55] for what concerns
1333 metabolomics. The two arrows represent an increase (arrow up, left side) and a decrease (arrow
1334 down, right side) of the abundance of the respective proteins.

1335 **Fig. 5: Hepatic proteins differentially expressed during ketosis.**

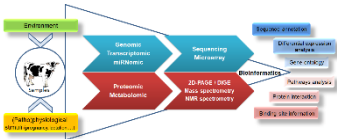
1336 The metabolic processes involved in increased formation of ketone bodies are schematically shown
1337 together with the list of proteins found to be differentially expressed in liver [90] of cows with
1338 ketosis as compared to healthy animals. The two arrows represent an increase (arrow up, left side)
1339 and a decrease (arrow down, right side) of the abundance of the respective proteins.

1340 **Fig 6: Proteomics and metabolomics literature as related to bovine species.**

1341 Farm animal proteomics literature: number of manuscripts within the keyword cow proteomics and
1342 cow metabolomics from Medline up to July 2017.

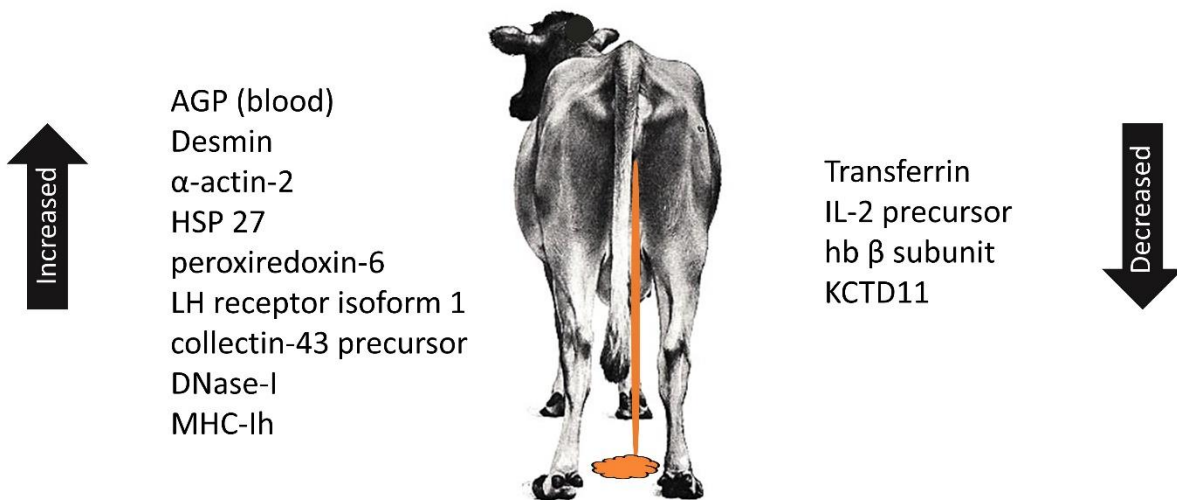


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1344

Blood and endometrial proteins associated with the development of endometritis



1345

Altered blood proteins in hypocalcaemia

- Serpin peptidase inhibitor
- Endopin 2B
- Albumin
- Fibrinogen Alpha Chain
- Amyloid beta A4 proteins
- VGF
- A2M protein



- Fibrinogen Beta Chain
- IgG -C(H)
- Albumin
- Apolipoprotein A-II
- Serum amyloid A
- Vit-D binding protein precursor
- Paraoxonase,

- *β*-hydroxybutyrate
- acetone
- pyruvate
- lysine

- Glucose
- alanine
- glycerol
- Phosphocreatine
- γ -aminobutyrate

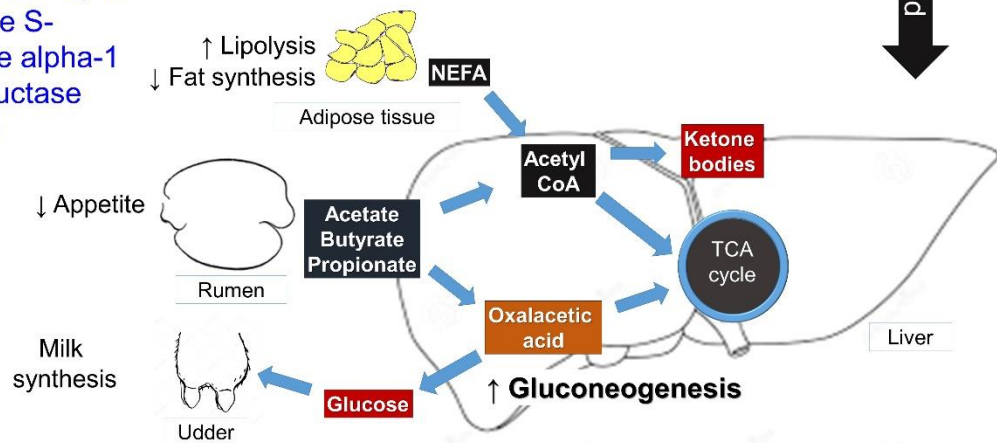
Altered blood metabolites in hypocalcaemia

1346

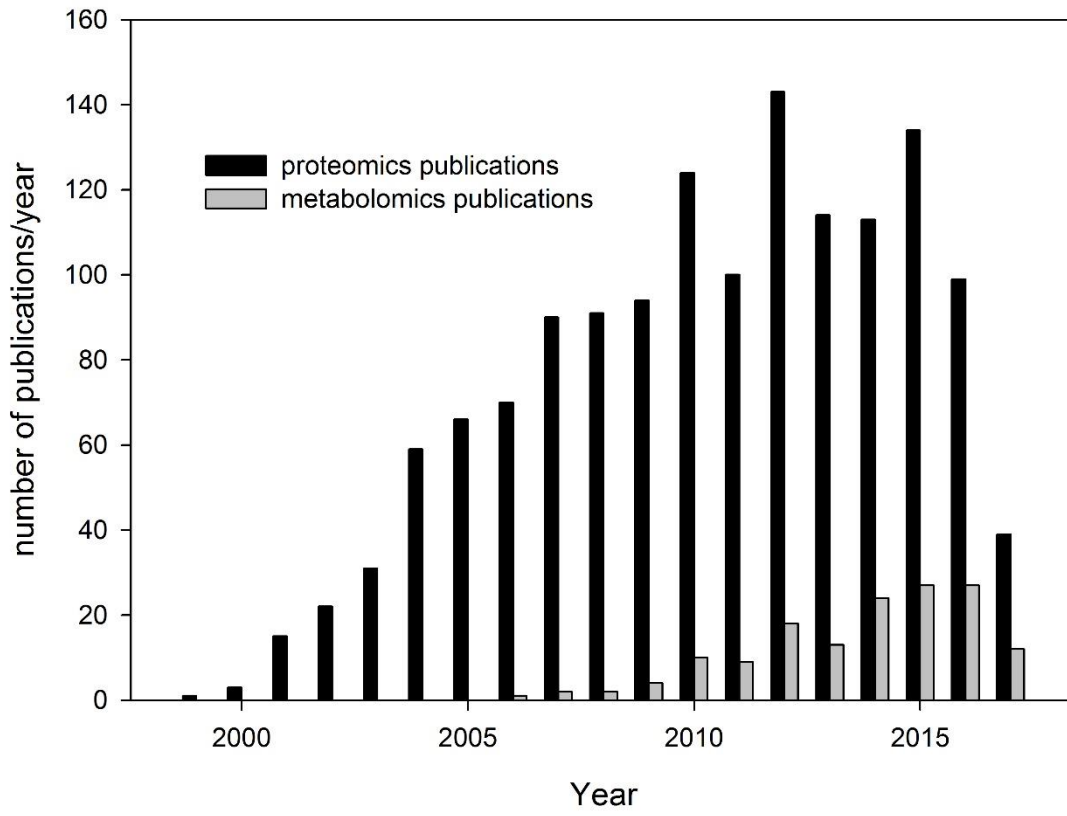
Hepatic proteins differentially expressed during ketosis

- Myosin light chain
- Tropomyosin
- MGC128326
- Myoglobin
- Peroxiredoxin -5, -6
- Glutathione S-transferase alpha-1
- Flavin reductase
- α -enolase

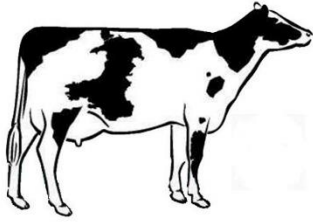
- Acetyl-CoA acetyltransferase 2
- 3-hydroxyacyl-CoA dehydrogenase



1347



1348



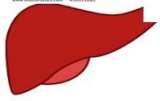
Understanding and assessing metabolic health in dairy cows



Blood



Tissues



Endometrial brush



Urine



Milk

**Proteomics
&
Metabolomics**