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

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List of abbreviations

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

Abstract

The transition from late pregnancy to early lactation is a critical period in a dairy cow`s life due to the rapidly increasing drain of nutrients from the maternal organism towards the foetus and into colostrum and milk. In order to cope with the challenges of parturition and lactation, comprehensive adaptive reactions comprising the endocrine and the immune system need to be accomplished. There is high variation in this coping ability and both metabolic and infectious diseases, summarized as “production diseases”, such as hypocalcaemia (milk fever), fatty liver syndrome, laminitis and ketosis, may occur and impact welfare, productive lifespan and economic outcomes. Proteomics and metabolomics have emerged as valuable techniques to characterize proteins and metabolite assets from tissue and biological fluids, such as milk, blood and urine. In this review we provide an overview on metabolic status and physiological changes during the transition period and the related production diseases in dairy cows, and summarize the state of art on proteomics and metabolomics of biological fluids and tissues involved in metabolic stress during the peripartum period. We also provide a current and prospective view of the application of the recent achievements generated by omics for biomarker discovery and their potential in diagnosis.

Significance

For high-yielding dairy cows there are several “occupational diseases” that occur mainly during the metabolic challenges related to the transition from pregnancy to lactation. Such diseases and their sequelae form a major concern for dairy production, and often lead to early culling of animals. Beside the economical perspective, metabolic stress may severely influence animal welfare. There is a multitude of studies about the metabolic backgrounds of such so called production diseases like ketosis, fatty liver, or hypocalcaemia, although the investigations aiming to assess the complexity of the pathophysiological reactions are largely focused on gene expression, i.e. transcriptomics. For extending the knowledge towards the proteome and the metabolome, the respective technologies are of increasing importance and can provide an overall view of how dairy cows react to metabolic stress, which is needed for an in-depth understanding of the molecular mechanisms of the related diseases. We herein review the current findings from studies applying proteomics and metabolomics to transition-related diseases, including fatty liver, ketosis, endometritis, hypocalcaemia and laminitis. For each disease, a brief overview of the up to date knowledge about its pathogenesis is provided, followed by an insight into the most recent achievements on the proteome and metabolome of tissues and biological fluids, such as blood serum and urine, highlighting potential biomarkers. We believe that this review would help readers to be become more familiar with the recent progresses of molecular background of transition-related diseases thus encouraging research in this field.

Introduction

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

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

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

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

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

For metabolites, species-specificity is not an issue albeit relative quantities may differ. The major analytical approaches used in metabolomics rely on two techniques: MS and NMR techniques [17]. For MS-based techniques, samples are fractionated through chromatographic techniques such as Gas Chromatography (GC) of High Pressure Liquid Chromatography (HPLC), or capillary electrophoresis. The GC is used to fractionate volatile metabolites, such as for example BHB of other organic acids, or fatty acids, whereas HPLC is used for lipophilic metabolites, such as acyl carnitines for example. Fractionated metabolites are then ionized and identified following mass spectrometry analysis. NMR techniques can identify simultaneously all the analytes, and do not require any prefractionation of the sample. Successful identification of individual metabolites depends of high quality mass spectra, powerful spectral *matching* algorithms and comprehensive and reliable spectral libraries [18].

Metabolomics strategies are commonly divided into targeted and non-targeted metabolomics. Targeted metabolomics aims to quantify defined groups of metabolites, such as for example, in case of transition period-related diseases, acylcarnitines, carbohydrates, amino acids or organic acids. Internal standards, such as stabile isotope (^{1}H and ^{13}C) labelled metabolites are used to measure analytes in a quantitative or semi-quantitative ways [19]. Non-targeted metabolomics allows for the detection of all the metabolites in a sample, in theory. Indeed, non-targeted metabolomics is able to detect up to 10,000 independent spectral features in biological samples [20], but only a fraction of them can be actually identified. Targeted metabolomics was already applied in several dairy cow studies e.g. to characterize the respective changes in blood throughout the transition phase [21–24] and non-targeted metabolomics for testing a supplement aiming to ameliorate metabolic stress [25], respectively. The aim of this review is to provide an overview of the state of the art applications of proteomics and metabolomics to address production diseases of dairy cows, whose prevention represents a priority for intensive milk production. We reconcile and summarize the information currently available on metabolic status and physiological changes during the transition period, and discuss the most recent achievements in proteomics and metabolomics applications for biomarker discovery, their potential as diagnostic tools, but also for comprehension of complex (patho) physiological contexts. However, the review will be largely limited to metabolic diseases and to metritis. For mastitis, the reader is referred to the specific "mastitomics" literature [26–28] and also to the latest considerations of meta-proteomics and meta-metabolomics which include the milk microbiome as recently reviewed by Addis and coworkers [29].

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

Metritis and endometritis

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

A pioneering proteomic study provided first clues about the alterations in blood serum in pathological (endometritis) versus physiological states [39]. Patterns indicative for an inflammatory reaction were also found in healthy animals around calving as demonstrated by increasing concentrations of positive acute phase proteins (APP), such as $\square_1$-acid glycoprotein (AGP) and haptoglobin, and a correspondent decrease of negative APP, such as $\square_2$HS glycoprotein (fetuin-A) suggesting that the approaching calving resembles an acute-phase reaction. This is in line with the common observation of an inflammatory reaction peripartum that is considered as physiological and even necessary for the successful adaptation to the metabolic challenge as recently reviewed [40]. In the proteome study [39], the concentrations of AGP two weeks before calving were lower in animals that developed endometritis postpartum, but were higher two weeks after calving, suggesting AGP not only as a potential prepartum bioindicator for an early detection of an increased risk for endometritis but also indicating a complex functional role for AGP in the context of endometritis.

In a 2-DGE analysis followed by MALDI-TOF, endometritis-associated proteins were identified in endometrial samples [41]. Several proteins, including desmin, α-actin-2, heat-shock protein (HSP) 27, peroxiredoxin-6, luteinizing hormone receptor isoform 1, collectin-43 precursor, deoxyribonuclease-I (DNase-I), and MHC class I heavy chain (MHC-Ih) were up-regulated in endometritis, whereas, transferrin, interleukin-2 precursor, hemoglobin β subunit, and potassium channel tetramerisation domain containing 11 (KCTD11) were down-regulated as compared to normal endometrium. Desmin and α-actin-2 identified in this proteomic study to be related with endometritis are common in mammalian cells, but being also up-regulated during other diseases, such as cancer [42], they would not qualify as specific biomarkers for endometritis. However, truly specific associations between the proteins found to be divergently regulated are unlikely anyway, in view of the pleiotropic defense reactions. Fig. 3 summarizes the differences in proteomes of blood and endometrial samples associated with the development of metritis. When comparing the uterine proteome of cows infected with a specific bacterium (*Trueperella pyogenes* - formerly *Arcanobacterium*), with that of uninfected cows, using 2-DGE , annexins A1 and A2 (ANXA1 and ANXA2), apolipoprotein A-1, calprotectin (S100A9), cathelicidin, enolase 1 (ENO1), peptidoglycan recognition protein 1 (PGLYRP1), phosphoglycerate mutase 1 (PGAM1), serine dehydratase (SDS) and serine protease inhibitors (SERPIN) B1, B3 and B4 proteins were found to be differentially regulated [43]. In the second part of the study ten of these proteins were monitored in uterine samples from dairy cows at 15 and 42 days post-partum, and strong positive correlations between the cytology scores (percentage of polymorphonuclear neutrophils) and cathelicidin, PGLYRP1, SERPINB1 and S100A9 levels at day 15 were found.

Retention of the placenta e.g. the failure to expel the placenta within 12 - 24 h after calving, is known as a major predisposing factor for the development of endometritis or metritis and thus impairs fertility but also animal health in general [44]. Beside infectious diseases, non-infectious risk factors like dystocia, but also nutritional deficiencies, are listed as causes for the placental retention albeit the aetiology is not completely understood [44]. Few investigations on proteomic differences between retained and normal placenta were carried out so far. A review describing the involvement of extracellular matrix proteins in placenta release was recently published [45]. The 2-DGE reference map for bovine placenta during late pregnancy identified 273 proteins, providing the background for studies on molecular mechanism of placenta modification and diseases during late pregnancy [46].

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

The application of metabolomics techniques to metritis and endometritis is still lacking, in both human and veterinary medicine.

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

Hypocalcaemia (milk fever)

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

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

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

Results provided by proteomics applied to hypocalcaemia are somehow contradictory: for example, in one study albumin abundance is increased [53], whereas in another the albumin abundance is decreased [54]. This apparent inconsistencies might be related to the fact that results were obtained following two different proteomics techniques (2D-DIGE separation followed by identification with MALDI-TOF-MS and SELDI-TOF, respectively), rather than to different sampling times (in both studies sample collection was close to delivery (6 and 24 h, respectively). In view of positive APP, such as SAA, haptoglobin, complement C4 precursor and alpha-1-antitrypsin being decreased in hypocalcaemic cows, hypocalcemia might also be related to systemic inflammation. Metabolomics results are probably more interesting: taken together, the findings reported by metabolomic analyses indicate a relationship between hypocalcaemia and energy metabolism, rather than a specific association with Ca metabolism. Although limited to one study [57], metabolomics confirmed in cows what has been already reported in humans, i.e. that calcium plays a pivotal role in regulating energy homeostasis. One important finding in this context is that by increasing the Ca^{2+} concentration in adipocytes, adipogenesis and a coordinated inhibition of lipolysis were stimulated, thus demonstrating that Ca is capable of regulating adiposity [58,59]. In dairy cows in which hypocalcaemia was experimentally induced by intravenous infusion of ethylene glycol tetraacetic acid (EGTA), a selective Ca-chelator, reduced blood concentrations of insulin and increased levels of glucose and NEFA were reported, together with reduced phagocytotic and oxidative burst activity of neutrophils [60]. Even though some of the observed effects in this study might have partly been caused by the reduced feed intake during EGTA infusion, the findings are in line with a role of Ca in energy metabolism. Fig. 4 summarizes the differences in metabolome and proteome between healthy and hypocalcaemic cows. Future omics studies could allow to find possible explanations for the contradictory results stated above and deepen the insight into the relationships between hypocalcaemia and energy metabolism.

Metabolic diseases related to energy metabolism:

During the transition period, nutrients need to be directed towards the growing foetus and the mammary gland even though feed intake is often depressed around calving and does not increase as does milk yield. To accomplish an adequate supply of nutrients to foetus and mother, several adaptive mechanisms are activated. The main ones are: increasing gluconeogenesis, reducing peripheral insulin sensitivity and increasing lipolysis. As outlined below, these reactions may also overshoot and result in the most common metabolic production diseases, i.e. in ketosis and fatty liver. Ruminants almost entirely depend on gluconeogenesis since glucose from plant carbohydrates hardly reaches the small intestine due to fermentation in the forestomaches which yields propionate as the main gluconeogenetic substrate. In particular the mammary gland has a high demand for glucose to produce lactose, the major osmole in milk. In contrast to other organs, glucose uptake of the mammary gland is insulin-independent and by decreasing the insulin-sensitivity in skeletal muscle and adipose tissue, glucose can be drained towards the mammary gland [61]. The increase in lipolysis provides fatty acids as energy substrates but also for milk fat synthesis; albeit the contribution of fatty acids from the mobilization of body fat is normally less than 10% of the milk fatty acids, this share increases proportionally in early lactation with the extent of the energy deficit [62]. When the rate of lipolysis exceeds the capacity of the liver, fatty liver and ketosis (or acetonaemia) can occur. With the importance of lipolysis, the central role of adipose tissue comes into play and indeed many studies including proteomics and metabolomics investigated adipose tissue in context with peripartum diseases. Oxidation of fatty acids provides acetyl-CoA which is than condensed with oxaloacetate to form citrate for entering the TCA cycle. However, when glucose requirements are high, oxaloacetate is increasingly used for gluconeogenesis and thus acetyl-CoA cannot be completely oxidized but is converted into ketone bodies, mainly acetone, acetoacetate and BHB. Ketosis, in particular subclinical ketosis, is a common disease in dairy cows and often concurs with other peripartum diseases such as retained placenta and metritis [63]. Excess fatty acids can also be re-esterified in the liver and deposited as triglycerides; however, their export into the circulation is limited based on the low intrinsic capacity for mainly VLDL (very low density lipoprotein) in ruminants [57,64]. Fatty liver is thus another production disease affecting many animals at least in mild forms. Taken together the main adaptations to accomplish partitioning of nutrients towards foetus and milk are basically known, including the temporal patterns of some proteins and metabolites during the transition period [65].

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

***Fatty liver*:** The fatty liver observed in dairy cows has many similarities with non-alcoholic fatty liver disease (NAFLD) in humans. Several proteomic and metabolomics studies were published about NAFLD [66–68]. Yet the respective literature on fatty liver in dairy animals is surprisingly limited. To the best of our knowledge there is only one review about fatty liver proteomics in farm animals, but it mainly focused on poultry [69]. In dairy cows a proteomic analysis was carried out in liver obtained from animals (1st lactation,16-201 DIM) fed ad libitum as compared with feed-deprived cows [70]. Proteins were separated by 2-DE, and those that were differently regulated were identified by MALDI-TOF. Several pathways related to lipid and to carbohydrate metabolism were found to be dysregulated. Acyl-CoA dehydrogenase and Acyl-CoA acetyltransferase 2 were both down-regulated in feed-deprived animals, suggesting decreased fatty acid degradation and contributing to explain the insurgency of liver disease. The fatty acid binding protein 1 was also found to be decreased. Other enzymes involved in fatty acid degradation that were decreased in fed-deprived cows include aldehyde dehydrogenase, which converts fatty acids to their corresponding aldehydes. On the contrary, sterol carrier protein 2, which catalyzes the transfer/exchange of cholesterol and phospholipids between membranes, was more abundant in feed-restricted cows suggesting an increase in lipid trafficking. The decrease of peroxiredoxin-6, whose main role is to protect against oxidative damages, suggests a possible increase in oxidative stress in the ruminant liver during feed restriction. With regard to carbohydrate metabolism, several enzymes, including 6-phosphofructokinase, enolase1, and triosephosphate isomerase, fructose-bisphosphate aldolase B, sorbitol dehydrogenase and aldehyde dehydrogenase 2 were less abundant in feed-deprived versus ad libitum fed cows. The corresponding up-regulation of parathymosin, an inhibitor for glycolytic enzymes, confirms a reduction of glycolysis [71]. Besides, the results indicated that protein metabolism was also affected by feed deprivation: proteins involved in protein degradation, such as ubiquitin carboxyl-terminal esterase L3, proteasome 26S subunit, and protein disulfide-isomerase-related protein 5 were decreased. This result was unexpected, since feed restriction is believed to result in increased protein degradation. The authors suggested that downregulating proteins involved in protein degradation might help to protect the liver form excessive autophagy. In addition, skeletal muscle rather than liver is the greatest labile source of amino acids for energy needs [72]. However, other “protecting” proteins, such as heat-shock 70 kDa protein 5 (HSPA5), a chaperon, were less abundant in liver of feed-restricted cows. The urea cycle in particular was dysregulated: proteins, such as arginase-1 and argininosuccinate synthetase, were up-regulated in feed restriction. Conversely, L-arginine:glycine amidinotransferase and glutamate dehydrogenase 1, were decreased. Finally, proteins involved in calcium metabolism, such as regucalcin, annexin IV and calcium binding protein SPEC 2D were increased in the liver of feed-restricted cows. In 2012, a comparison of the liver proteome of cows with either low or high liver triglyceride (TG) content in early lactation was published [57]. A high liver TG content was found to be associated with increased oxidation of saturated fatty acids, oxidative stress, and urea synthesis and decreased oxidation of unsaturated fatty acids, but not with impaired gluconeogenesis. Aiming to identify hepatic biomarkers for physiological imbalances (PI), the liver proteome of dairy cows at early and mid lactation (49±22 DIM, average milk yield 42±7 kg/day versus 159±39 DIM, 29±7 kg/day) was determined by means of ITRAQ-based profiling; PI was calculated based on plasma free fatty acids, BHB, and glucose concentrations and was compared between 6 cows with greatest and least PI in early and mid lactation, respectively. PI was increased by a 4 day feed restriction period and liver biopsies were collected one day before and on day 3 of the feed restriction [16]. In early lactation, enzymes involved in gluconeogenesis and -oxidation, such as pyruvate carboxylase and very long chain specific acyl-CoA dehydrogenase, respectively, were increased in cows with a higher PI indicating increased gluconeogenesis and fatty acid oxidation. In addition, three enzymes involved in energy metabolism, such as mitochondrial isocitrate NADP+ (nicotinamide adenine dinucleotide phosphate)-dependent dehydrogenase, glycine N-acyltransferase and UDP-glucose 6-dehydrogenase were decreased, partially explaining the molecular background of PI. By increasing nutrient restriction, thus aggravating the status of PI, the increasing demand of energy coupled with a decrease of anti-oxidant defense is confirmed by an upregulation of mitochondrial trifunctional protein, subunit α, enoyl-CoA hydratase and pyruvate carboxylase and downregulation of glutathione S-transferase Mu 1, manganese superoxide dismutase, aldehyde oxidase, and glycine N-acyltransferase. Proteins related to apoptosis (14-3-3 protein β/α), and mobilization and targeting of fatty acid (liver fatty acid binding protein) were also decreased.

During mid lactation, before feed restriction, PI increased the amount of proteins involved in ketone biosynthesis, such as alcohol dehydrogenase 4 and alcohol dehydrogenase NADP+, and in TCA cycle, including dihydrolipoamide dehydrogenase 2 and methylmalonate-semialdehyde dehydrogenase. Again, proteins involved in antioxidant defense and CO_2 transport, such as carbonic anhydrase 3 were decreased. After increasing PI by means of nutrient restriction, the upregulation of proteins involved in fatty acid oxidation, such as acetyl-CoA oxidase 2, acyl-CoA-binding protein and carnitine O-palmitoyltransferase 2 was even more pronounced. Proteins involved in ketone biosynthesis, such as acetyl-CoA acyltransferase were increased as well. Consistent with changes related to PI during early lactation, the experiment amplification of PI decreased proteins involved in antioxidant defenses, such as peptide methionine sulfoxide reductase, Glutathione S-transferase A1, and carbonic anhydrase 3 . Proteins involved in amino acid metabolism, such as peptide methionine sulphoxide reductase and ornithine carbamoyltransferase, and fatty acid oxidation, such as short-chain specific acyl-CoA dehydrogenase and hydroxyacyl-CoA dehydrogenase, were also downregulated. Beside the identification of potential biomarkers for different degree of PI, the study of Moyes and coworkers [16] provided a better understanding of the molecular background of liver diseases during PI, as shown by the downregulation of proteins related to anti-oxidant defense, which might be responsible for the increasing cellular damage. For aldehyde dehydrogenase and HSP70, relationships with NAFLD in rats and in humans, respectively, were identified earlier [73,74].

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

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

A novel approach relying on (1)H-NMR investigated the metabolome in fatty livers of 171 Holstein cows [86]. The main advantage of this technique in the metabolomic field is that it can provide a profile of proton-containing, low-molecular-weight metabolites, starting from a limited amount of sample [87]. Plasma and liver tissue samples from animals with fatty liver disease (16.31±4.30 DIM, average milk yield 28.32±4.73 kg/day) and healthy animals (15.5±6.02 DIM, average milk yield 30.75±3.7 kg/day) were included in the study. As expected, plasma from animals with fatty liver disease had increased BHB, isobutyrate and acetone concentrations. The amino acids glycine, valine, trimethylamine-N-oxide, and citrulline were also increased. Conversely, other amino acids such as alanine and asparagine were decreased. Glucose, GABA, glycerol, and creatinine were decreased as well. The decrease of alanine and asparagine is consistent with their role as gluconeogenic amino acids, since both can enter the TCA cycle to generate glucose during an energy-deficient status. The finding that trimethylamine-N-oxide and citrulline are increased what is usually accompanied by oxidative stress and liver damage, corresponds to what has been demonstrated in other species, such as mice [88] and humans [89].

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

Beside liver and plasma, the difference of proteomic profiles in animals with ketosis were also explored in urine using SELDI-TOF techniques [91]. Samples were collected 7 – 28 DIM from two groups of animals, one affected by clinical ketosis and the other from healthy cows, with a milk production of 9625 kg/year. The urinary profile of animals with ketosis showed a decrease for 11 proteins, most of them involved in the inflammatory response, such as fibrinogen, C1 inhibitor, osteopontin, also hepcidin and human neutrophil peptides 1-3. Interestingly, also proteins associated with the neuron function, such as VGF (non-acronymic) protein and amyloid precursor protein, were decreased in the animals with ketosis as compared with the control group. Proteins related to lipid metabolism, as well as inflammation, were also decreased during ketosis, namely SAA and apolipoprotein C-III, indicating a change in lipid metabolism during ketosis. Two other proteins, i.e. transthyretin, a transport protein, and cystatin C, a protease inhibitor, were also decreased. Fibrinogen, hepcidin and SAA are acute phase proteins [92] and their concentration is supposed to increase during an inflammatory status. Targeted assessments of acute phase proteins in blood have shown increased concentrations of inflammatory markers in ketotic cows. Thus the finding of decreased concentrations of SAA in urine of ketotic cows is opposing the situation in blood and deserves further investigation. The proteins differentially regulated in liver and urine of cows with ketosis as compared to healthy animals are presented in Fig. 5.

More than proteomics, it is metabolomics that contributed to address the changes in biological fluids during ketosis. A NMR-based metabolomic analysis on milk from animals with ketosis was carried out during a time course study on 264 high yielding dairy cows with an average milk yield of 32.8 ± 4.7 kg energy corrected milk/day [93]. Milk samples were collected weekly for 5 weeks and once again 6 months post partum. NMR spectroscopy was carried out, and presented evidence that high milk glycerophosphocholine (GPC) levels and high ratios of GPC to phosphocholine (PC) during the first four weeks of lactation, and GPC at mid lactation, could provide reliable biomarkers for the development of ketosis. Although milk provides an ideal substrate for metabolomic analysis, being collected routinely and non-invasively, more studies were carried out on plasma metabolome, which, conversely, yields a higher amount of potential biomarker metabolites. A metabolomic approach using GC/MS techniques analysed the differential plasma metabolomes of dairy cows with clinical (12 ± 5 DIM, 32.1 ± 7.8 kg milk/day) and subclinical ketosis (14 ± 6 DIM, 35.0 ± 7.2 kg milk/day), as compared with healthy animals (16 ± 6 DIM, 37.0 ± 6.2 kg milk/day) [94]. The study revealed that the metabolomes of animals with subclinical and clinical ketosis were mostly identical, whereas 25 potential biomarkers were found between animals with ketosis, both clinical and subclinical, when compared with healthy animals indicating that several biochemical pathways were modified. The nine metabolites decreased during ketosis suggested a decrease in gluconeogenesis in affected animals, and a parallel activation of the pentose-phosphate pathway. A decrease of ribitol levels, related to riboflavin deficiency, and vitamin C, may increase the oxidative stress. The decrease of lactic acid and L-alanine, which are both gluconeogenetic precursors, suggested a close relationship between ketosis and carbohydrate metabolism, as a consequence of hypoglycaemia and lack of precursors of gluconeogenesis. Sixteen metabolites were increased in animals with ketosis. As expected, this list included ketone bodies, and fatty acids, such as BHB, palmitic acid, heptadecanoic acid, stearic acid, trans-9-octadecenoic acid, myristic acid, cis-9-hexadecenoic acid, confirming also the mobilization of adipose tissue. Amino acids were also found to be increased, namely L-isoleucine and a catabolic product of lysine, 2-piperidinecarboxylic acid, two amino acids involved in ketogenesis, and glycine, suggesting an increase of proteolysis needed to fuel gluconeogenesis. The study confirmed BHB acid as gold biomarker for ketosis, and suggested cis-9-hexadecenoic acid as novel biomarker for clinical ketosis, and an indicator of fat mobilization. Plasma metabolic profiling from cows affected by clinical ketosis was determined with two other different techniques, namely LC/MS and (1)-NMR. In a study whose experimental design was very similar to that of Zhang and coworkers [94] plasma metabolomics of cow with clinical and subclinical ketosis was determined by means of 1H-NMR [95]. A total of 25 metabolites were found to be dysregulated as a consequence of various stages of the disease, and as compared with healthy animals. Amino acids including histidine, glutamic acid, glutamine, lysine and phenylalanine, together with lactate and glucose, were decreased during ketosis. Amino acids such as alanine, proline and tyrosine decreased only in clinical ketosis, as well as LDL and VLDL. As expected, metabolites related to biosynthesis of ketone bodies, such as BHB, N-Acetylglucosamine, acetate, acetoacetate and acetone were increased in ketotic cows. Glycine, leucine, isoleucine and valine were increased in clinical ketosis only. A last study, this time focused on the differences in metabolomes between Holstein Friesian cows, 12 - 16 DIM and 18 - 21 kg milk /day, affected with clinical ketosis or being healthy, was carried out by means of LC/MS [96]: aminoacyls such as valine, glycine, and lipids, such as glycocholic, tetradecenoic and palmitoleic acid, were increased in clinical ketosis, whereas other amino acids, such as arginine, leucine, isoleucine, tryptophan and lysine decreased. Aminobutyric acid, creatinine, undecanoic acid and norcotinine were decreased as well. Consistent with previous reports, obtained with different metabolomic techniques, the study from Li and coworkers [96] confirmed that amino acids are affected during ketosis: e.g. glycine was increased whereas lysine was decreased.

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

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

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

Comparing insulin-resistant and insulin-sensitive subcutaneous adipose tissue yielded 111 proteins that were differentially regulated. Most of them (a total number of 106) were more abundant in the insulin-resistant state, whereas only five were decreased. Insulin resistance was associated with a dysregulation of pathways related to energy and lipid metabolism, including gluconeogenesis and glycolysis, signaling mediated by 14−3−3, TCA cycle, ERK/MAPK signaling, lipid accumulation, release and lipolysis. Inflammatory responses, such as leukocyte migration and proliferation of T lymphocytes, were also activated in insulin-resistant adipose tissue.

During the transition period, adipose tissue may also react to stress related to environmental factors, such as heat. A label-free, quantitative shotgun proteomics (nano-LC-MS/MS) approach investigated the effects of seasonal heat stress on the adipose proteome, aiming to highlight biomarkers of heat stress on late pregnant cows during summer heat stress (average milk yield 33.8 kg/day) as compared to the winter season (38.2 kg/day) [100,101]. The proteome in subcutaneous adipose tissue biopsies obtained 14 day before calving yielded a total number of 107 out of 1495 proteins identified that were differentially abundant between summer and winter. The pathways that were found to be modified included the Keap1-Nrf2 pathway which is the major regulator of cytoprotective responses to oxidative and electrophilic stress [102], such as STIP1 and ubiquitin-conjugating enzyme E2 K, which were increased in summer, and GSTM1, microsomal GST 1, (MGST1), GST Mu 3 (GSTM3), ferritin heavy chain and MAP2K1 which were decreased. The acute phase response was also modified. In particular, albumin, hemopexin, serotransferrin, AGP, apolipoprotein A-II, α-2-HS-glycoprotein, C-reactive protein and MAP2K1 were decreased in summer, whereas the abundance of the von Willebrand factor and fibrinogen α chain was increased. Protein related to the farnesoid X receptor (FXR)[103], which is a member of the nuclear family of receptors in control of numerous metabolic pathways, and, jointly with retinoid X receptor (RXR), plays a crucial role in linking bile acid productions with lipoprotein, lipid and glucose metabolism, were also modified, as well as proteins belonging to Liver X receptor/RXR pathways [104], whose function is to regulate cholesterol, fatty acid, and glucose homeostasis. The finding of this study provided the evidence that heat stress has a local impact on adipose tissue in late pregnant cows, highlighting meanwhile a list of possible biomarkers for heat stress related to transition period.

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

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

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

Several studies focusing on the metabolic situation during the transition period in general rather than on specific production diseases also contributed to our understanding on the pathophysiology of the underlying adaptive responses. Aiming to find biomarkers for any kind of production diseases relevant for transition dairy cows, a quantitative targeted metabolomics approach was used [21]. Plasma collected at 4 time points from 4 weeks prepartum to 4 weeks post partum from 12 cows of which 6 developed multiple peripartum diseases, including laminitis, mastitis, metritis and retained placenta whereas the other 6 cows remained healthy was compared. The study identified five plasma metabolites that could be related to periparturient diseases: carnitine, valerylcarnitine, propionyl carnitine, lysophosphatidylcholine acyl C18:2 and lysophosphatidylcholine acyl C14:0 were increased in animals that developed peripartum related diseases as compared with healthy controls as early as 4 weeks before parturition. Two phosphatidylcholines, namely phosphatidylcholine acyl-alkyl C42:4 and phosphatidylcholine diacyl C42:6, were increased 1 week before delivery. Carnitine, lysophosphatidylcholine acyl C18:2 and lysophosphatidylcholine acyl C14:0 were increased 1 week postpartum as well, whereas carnitine was decreased after 4 weeks. These results are remarkable, since they highlighted the possible use of three metabolites, namely carnitine, propionyl carnitine, and lysophosphatidylcholine acyl C14:0 as potentially predictive of peripartum-related diseases up to 4 weeks before delivery [21,96].

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

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

Proteomics during laminitis.

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

In equine medicine, laminitis can be induced by feeding and using such models several omics studies have been carried out to unravel the pathogenesis of the disease, or to look for predictive biomarkers [121,122]. As a preliminary step to unravel the changes in plasma of dairy cows affected from laminitis, a proteome analysis carried out by 2-DE coupled with MALDI-TOF identification of differentially regulated proteins between animals with spontaneously occurring clinical laminitis and healthy animals used as controls was carried out [123]. A semi-quantitative analysis of the 2-DE gels revealed that 16 proteins were differentially regulated between the two groups of animals, of which 12 were more abundant in laminitis, and 4 were decreased, as compared to healthy animals. Proteins involved in inflammation and in defensive mechanisms were increased during laminitis, namely complement component C9, haptoglobin and conglutinin, as well as apolipoprotein A-IV, and apolipoprotein A-I, which are also involved in inflammatory reaction, but also in lipid metabolism, together with 3-hydroxy-3-methylglutaryl-CoA reductase. The group of more abundant proteins includes also zinc finger protein 300-like, transmembrane protein TMP10, isocitrate dehydrogenase, and serum albumin. The upregulation of serum albumin is remarkable, and apparently in contradiction with the behaviour of albumin during acute inflammation. Serum albumin is a negative acute phase protein [92,124] and, therefore, its serum concentration decreases during acute inflammation, as it has been demonstrated also in laminitis [125]. It must be said that the upregulation of albumin is hardly demonstrable in the context of the experimental design of the study, on the background that the samples were analyzed after depletion of abundant proteins, such as albumin and immunoglobulins, to decrease the dynamic range of the samples. Besides, it has been demonstrated that albumin may be regarded as a local positive acute phase protein during mastitis, being up-regulated by epithelial cells of the mammary gland [126]. The proteins that were less abundant in plasma from animals affected by clinical laminitis include two members of complement pathways, namely C4BP, which is an inhibitor of complement, and Complement C9 precursor, and Glycerol-3-phosphate dehydrogenase 1-like protein, and Ectoderm-neural cortex protein 1, which is an actin-binding protein playing a role in the oxidative stress response. The number of proteomics studies on bovine laminitis is yet too limited to draw final conclusions about the pathogenesis. Nevertheless, proteomics results confirmed the development of a pro-inflammatory loop, as demonstrated by the upregulation of inflammation-related pathways, and a converse down-regulation of anti-inflammatory factors, as demonstrated by the decrease of C4BP. The increase of complement C9 and the parallel decrease of complement C9 precursor suggests that the complement pathway is activated, since the C9 precursor is less abundant, likely to produce the active C9 protein. To the best of the knowledge of the authors, no metabolomics studies on cow laminitis were carried out to date.

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

The main features of the metabolic derangements occurring in the typical production diseases of dairy cows that are related to the transition from pregnancy to lactation are basically known since several years. In particular for the metabolic diseases, overshooting lipolysis and ketogenesis were identified as aetiologic key elements. However, the knowledge of the molecular basis of successful versus compromised adaptation to the metabolic challenge of early lactation is still incomplete. Omics technologies such as proteomics and metabolomics provide important tools to close this gap in understanding the pathophysiology and also hold some promise for developing biomarkers.

Understanding the pathophysiology: beside transcriptomics, proteomics applied to adipose tissue, for example, has evidently contributed to a huge advancement in the knowledge of the involvement of this tissue in the development of transition-related diseases [127]. Proteome maps have been established for several biological fluids and tissues in cattle [128], and the amount of the literature available on cow proteomics and metabolomics is steadily growing, as shown in Fig. 6. Nonetheless, although increasing exponentially during the last few years, the application of proteomics and metabolomics in veterinary and animal science is lagging behind those in human medicine [11], and several gaps have yet to be covered. A wider application of proteomics techniques in bovine peripartum-related diseases is hampered by the lack of tools to validate the MS findings. The availability of the bovine genome [129] will allow for a very precise identification of selected proteins and is poised for closing this gap. Moreover, the complete annotation of the genome provided a full application of bioinformatics tools to characterize the pathways where these proteins are involved in. Yet, for a biological validation of proteins identified at different abundance in proteomics approaches, the options are limited to the use of antibodies in immunoassays (e.g. ELISA) or in Western blotting. For both approaches, the specificity of the antibodies is limiting, and species determined differences in the amino acid sequence might hamper the applicability of antibodies developed against the target protein in different species. Besides, the performance of antibodies in different methodological set-ups can differ. The quantitative power of immunoassays like ELISA is usually good, but developing a valid ELISA system is laborious and time consuming. In Western blotting, the effort of setting up a system with a working antibody is often considered as faster, but when aiming to work quantitatively, comprehensive validation is also required and determining differences as low as 2 to 4-fold may remain impossible [130]. In view of these limitations, assessing the matching mRNA concentration, preferably from identical sample for validating results from proteomics is often used as "biological validation". However, the abundance of mRNA may not correspond to the abundance of the respective protein [131] and therefore relying on the direct, *absolute* correlation between protein and mRNA levels is hardly an adequate validation measurement [132]. Nevertheless, albeit being far from perfect, in many cases quantifying the mRNA abundance remains the only approach available [131], but is further limited to cells and tissues, where both mRNA and proteins are accessible. When proteomics is carried out in biological fluids, such as saliva, urine and blood serum, only protein but not mRNA material is available. Nonetheless, the growing economic interests in producing antibodies and also assays specific for various animal species, including cattle, might cover this gap in the near future. The validation of metabolomics results is easier, there is no species issue and expectedly the comparison between results obtained via MS-based targeted metabolomics with classical assays yielded good correlations. For example, in a study in which 17 free amino acids were measured in 54 dairy cows' sera both via classical methods or via a target metabolomics approach [133] the concentrations obtained by the two methods were all correlated ($P < 0.0001$), with an average r-value of 0.82 ± 0.14 (mean ± SD; Dr Hassan Sadri, personal communication). In non-targeted metabolomics, the ongoing improvement of the livestock metabolome database (LMBDB, available at http://www.lmdb.ca), will facilitate future untargeted metabolomics studies by increasing the number of identified metabolites. The main gap to be covered includes metabolite coverage and their quantification, however improving the data set and information included in the livestock metabolome database (LMBDB, available at http://www.lmdb.ca), will facilitate future untargeted metabolomics studies. For NMR-based approaches, there is a large variability of reference methods used for calibration, and thus standardizing the methods used within and across countries is still a major challenge [134]. Moreover, albeit the application in milk is attractive in terms of being non-invasive and combined with well-established routine assessments of macro nutrients in milk, it should be kept in mind that there is only little correlation between the composition of the metabolomes in milk and in blood [114].

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

Applying proteomics and metabolomics to the complex of transition period-related diseases for elucidating the underlying pathophysiological processes is beneficial and allows for developing a holistic imagination but also a faster progress in research. Diseases apparently not related, such as fatty liver, hypocalcaemia, ruminal acidosis and laminitis, could be identified as being associated not only from epidemiology data but also for what concerns their molecular backgrounds. Examples for newly emerging disease associated pathways from proteomics and metabolomics comprise the relationship with energy status in hypocalcaemia [57], the involvement of RabGTPases in retained placenta [48] or the observation of decreased cystatin concentrations in urine from ketosis-affected cows [43]. In addition, the concept of NEFA oxidation rather than lipolysis alone influences the adaptive capability to the metabolic challenge of early lactation, was substantially supported by the acyl-carnitine data from metabolomics [22,96] but also from proteomics [16]. Applying omics techniques distal from transcriptomics is also particularly promising in terms of quantitative aspects since the relative importance of pathways can be evaluated by the flux changes and thus may allow for identifying molecular targets most promising for prophylactic, metaphylactic or therapeutic interventions. For making more efficient use of the analytical techniques available for understanding the adaptive responses in dairy cows, a systemic approach is required. Integration of different omics techniques, namely metabolomics, proteomics and transcriptomics in the same study, will help to produce a holistic and comprehensive interpretation of multi-omics data. Hereby production data and, if available, genotype information, should be combined for providing an integrated network of the single elements, the knowledge of which could yield a level of information higher than the sum of individual parts. It must be said that this gap may be difficult to bridge, given the shortage of funding available to livestock research as compared to medical studies. Nevertheless when balancing the amount of information provided from metabolomics and proteomics against the one from classical assays for the different targets, the omics approaches, in particular those where no further biological validation is required as in case of metabolomics, might nevertheless work out superior in terms of costs.

Development of Biomarkers: Selecting biomarker candidates from proteomes and metabolomes that show great differences when comparing diseases cows against healthy controls and that can be assessed in body fluids may yield predictive and diagnostic tools. However, such biomarkers will be based on quantitative results since none of the candidates will be found in healthy or in diseased state only. Proteomics studies do mostly not provide truly quantitative data, but refer to trends, e.g. an increase or a decrease as compared to internal standards. Applying proteomic techniques to metabolic diseases has not provided any robust and consistent list of biomarkers so far, although it has shed some light into the pathogenesis of many transition-period related diseases. More than proteomics, metabolomics contributed more to discovering potential biomarkers. As mentioned above, carnitines emerged as potential biomarkers for metabolic diseases related to transition period [21,22,96,109]. When considering the application of biomarker candidates it is probably more realistic to assess patterns of different metabolites rather than single components. Moreover, due to the common basis of many transition period-related diseases, specific markers for individual diseases are improbable to emerge. In addition, comprehensive biological validation is needed before such measurements can indeed be considered as assessments of biomarkers for predictive and diagnostic purposes. However, the integration of several pathophysiological aspects e.g. lipolysis, ketogenesis and oxidative capacity in such patterns, by combining fatty acids, ketone bodies AND acylcarnitines, will likely yield more information than the classical measurement of NEFA and BHB. Moreover, the expansion of the dynamic range of detection of low-abundance proteins and metabolites is likely poised to pave the way for the detection of peripartum-specific biomarker patterns. Such (complex) biomarkers, will enable the identification of phenotypes less sensitive to metabolic stress and thus the development and implementation of strategies for early diagnosis, prognosis and prevention of transition-related diseases.

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Figure legends

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

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

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

Fig. 3: Changes in protein abundance during endometritis.

The figure presents the differences in proteomes of blood and endometrial samples associated with the development of metritis. The figure was drawn following the papers of Cairoli et al. [39] and Choe et al. [41]. As indicated by the arrows, the abundance of proteins listed on the left was increased, whereas the ones on the right were decreased when compared to healthy animals. Abbreviations: α1-acid glycoprotein (AGP), Heat-shock protein (HSP), deoxyribonuclease-I (DNase-I), luteinizing hormone receptor isoform 1 (LH receptor isoform 1); MHC class I heavy chain (MHC-Ih), Interleukin (IL), Hemoglobin (hb), potassium channel tetramerisation domain containing (KCTD).

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

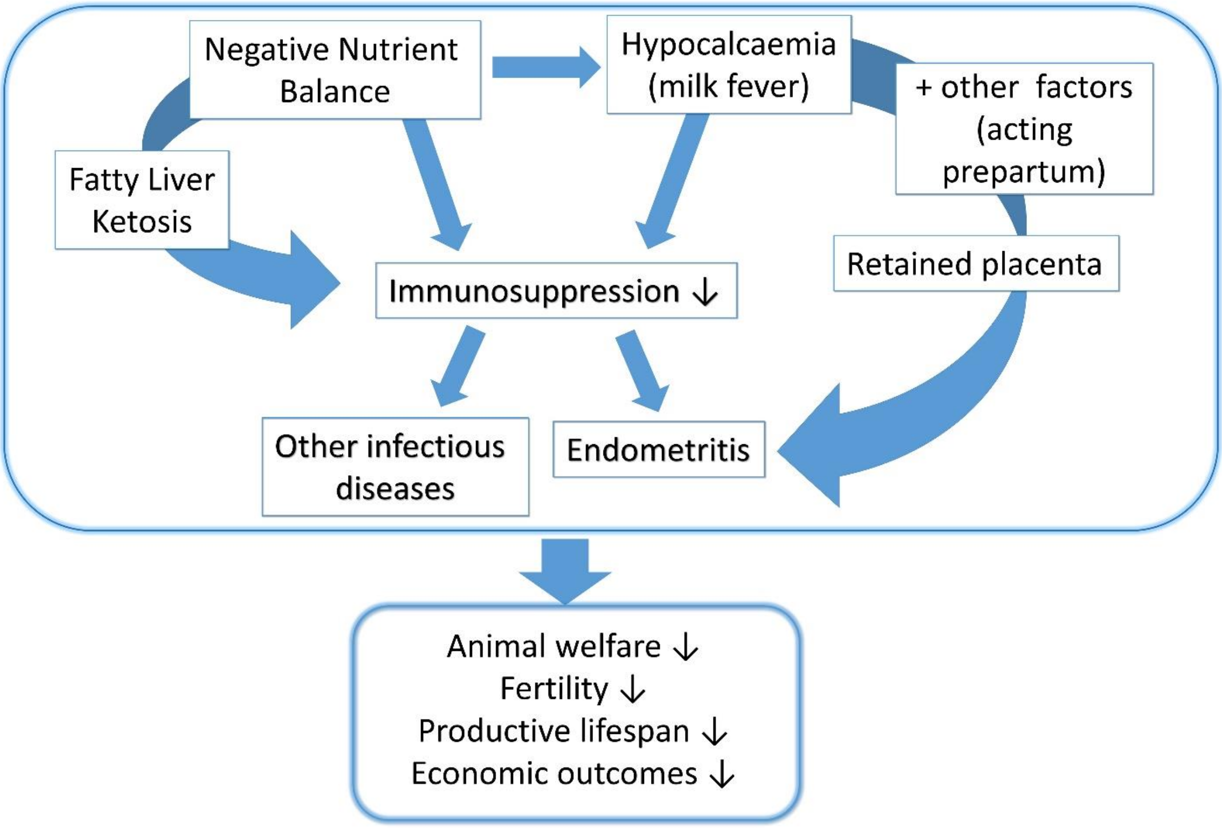
The figure highlights the differences in metabolome and proteome between healthy and hypocalcaemic cows. The figure was drawn following the papers of Xia et al. [53], Wang et al. [54] and Shu et al. [14], for what concerns proteomics, and Sun et al. [55] for what concerns metabolomics. The two arrows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

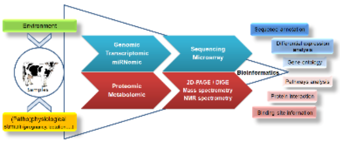
Fig. 5: Hepatic proteins differentially expressed during ketosis.

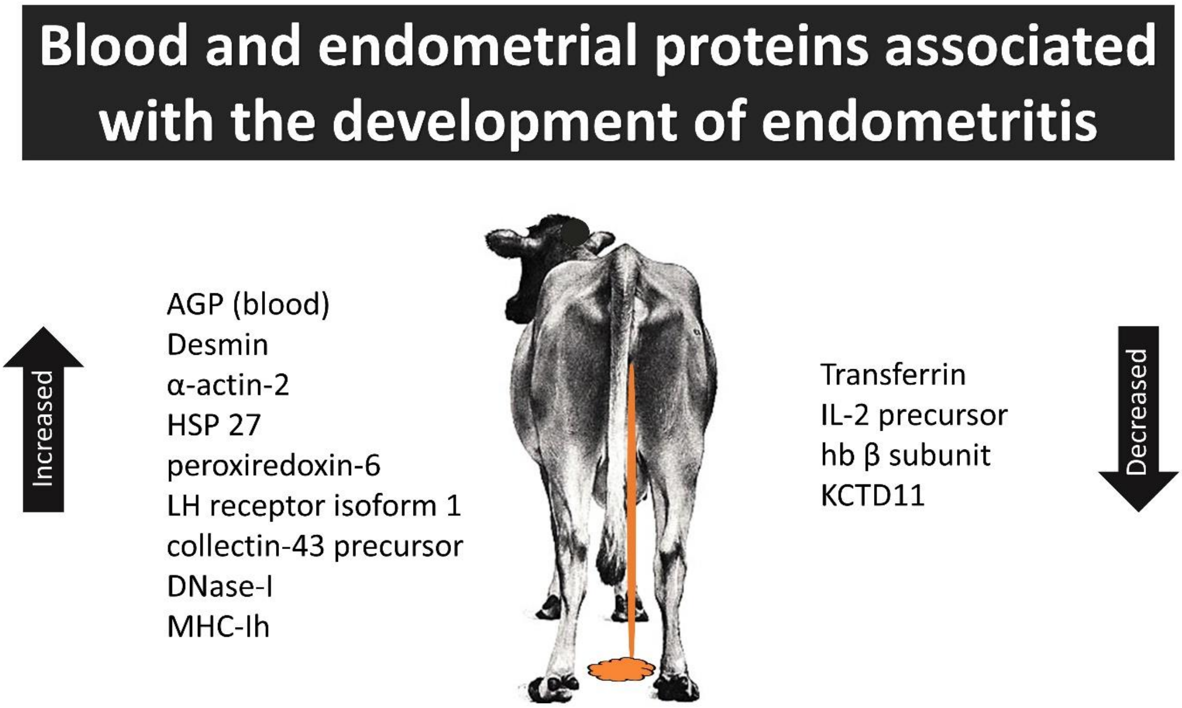
The metabolic processes involved in increased formation of ketone bodies are schematically shown together with the list of proteins found to be differentially expressed in liver [90] of cows with ketosis as compared to healthy animals. The two arrows represent an increase (arrow up, left side) and a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

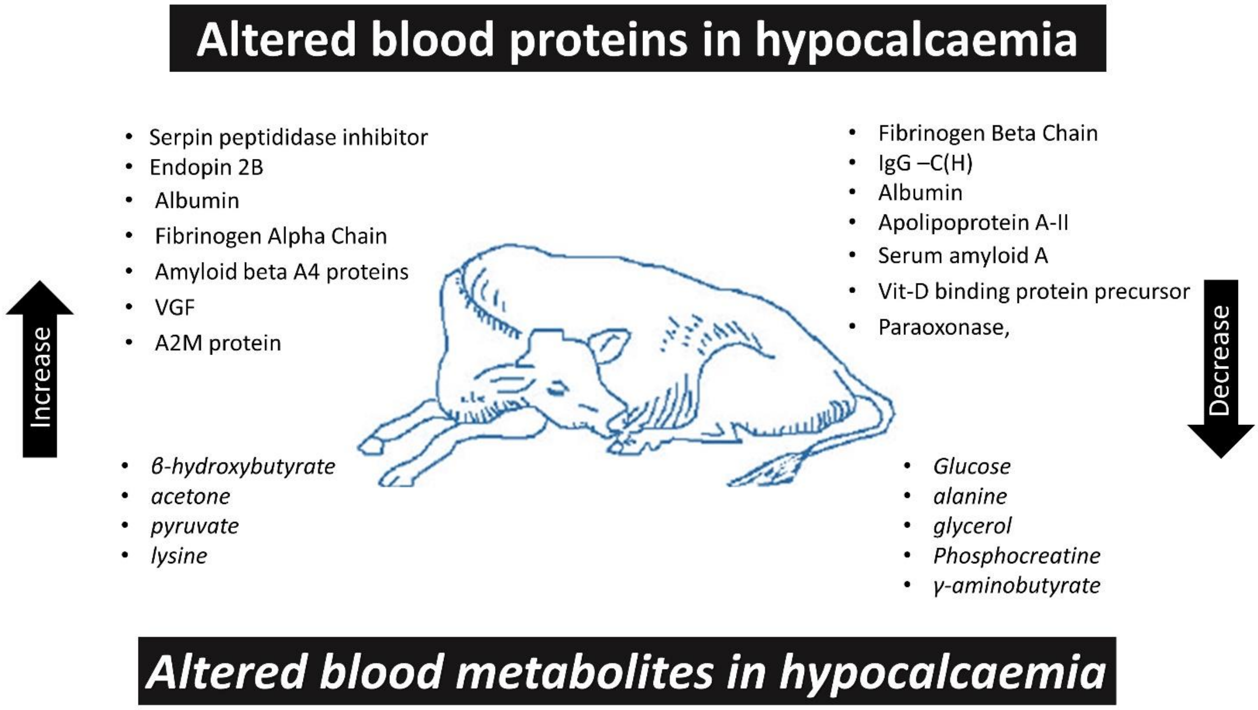
Fig 6: Proteomics and metabolomics literature as related to bovine species.

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

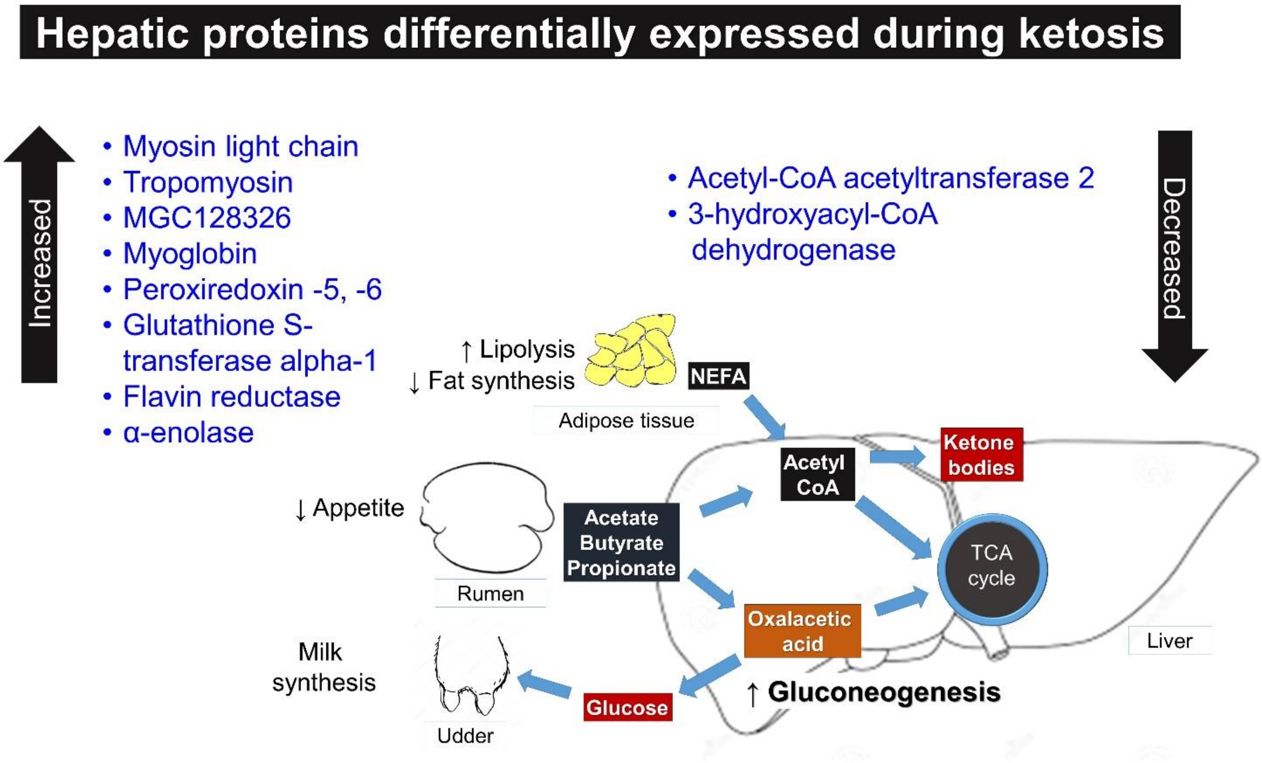

Negative Nutrient Balance
Hypocalcaemia (milk fever)
+ other factors (acting prepartum)
Fatty Liver Ketosis
Immunosuppression ↓
Retained placenta
Other infectious diseases
Endometritis
Animal welfare ↓
Fertility ↓
Productive lifespan ↓
Economic outcomes ↓



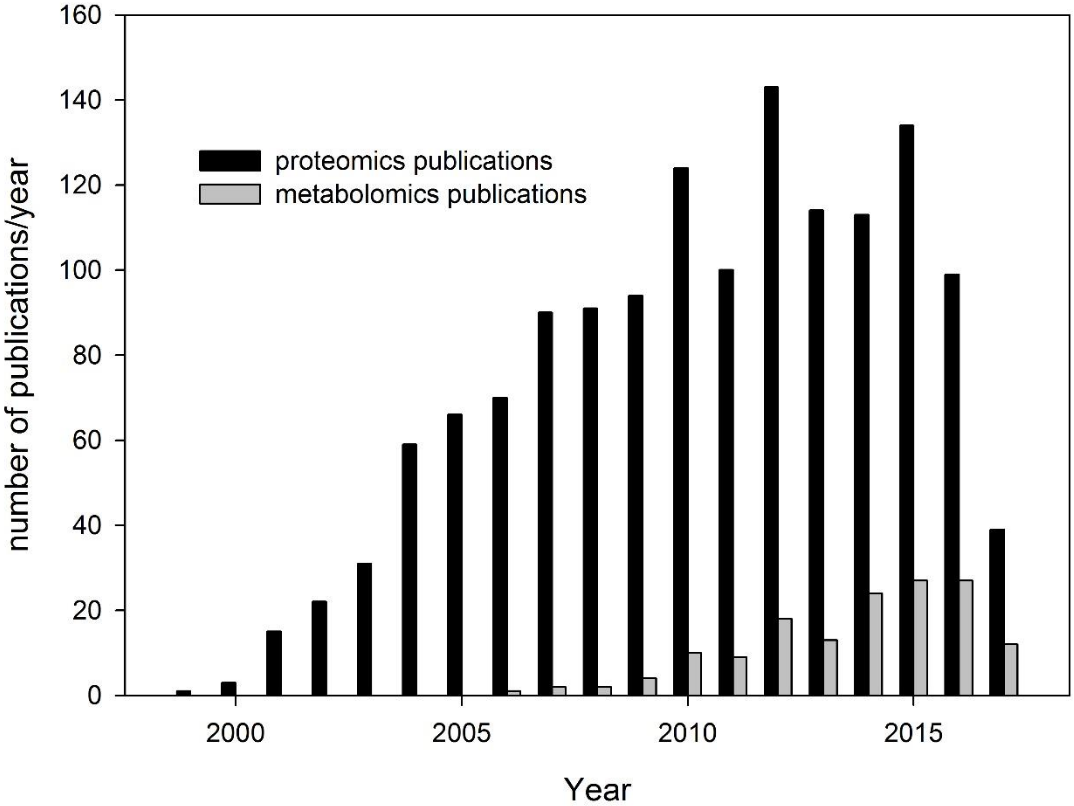

Blood and endometrial proteins associated with the development of endometritis
Increased
AGP (blood)
Desmin
α-actin-2
HSP 27
peroxiredoxin-6
LH receptor isoform 1
collectin-43 precursor
DNase-I
MHC-Ih
Transferrin
IL-2 precursor
hb β subunit
KCTD11
Decreased



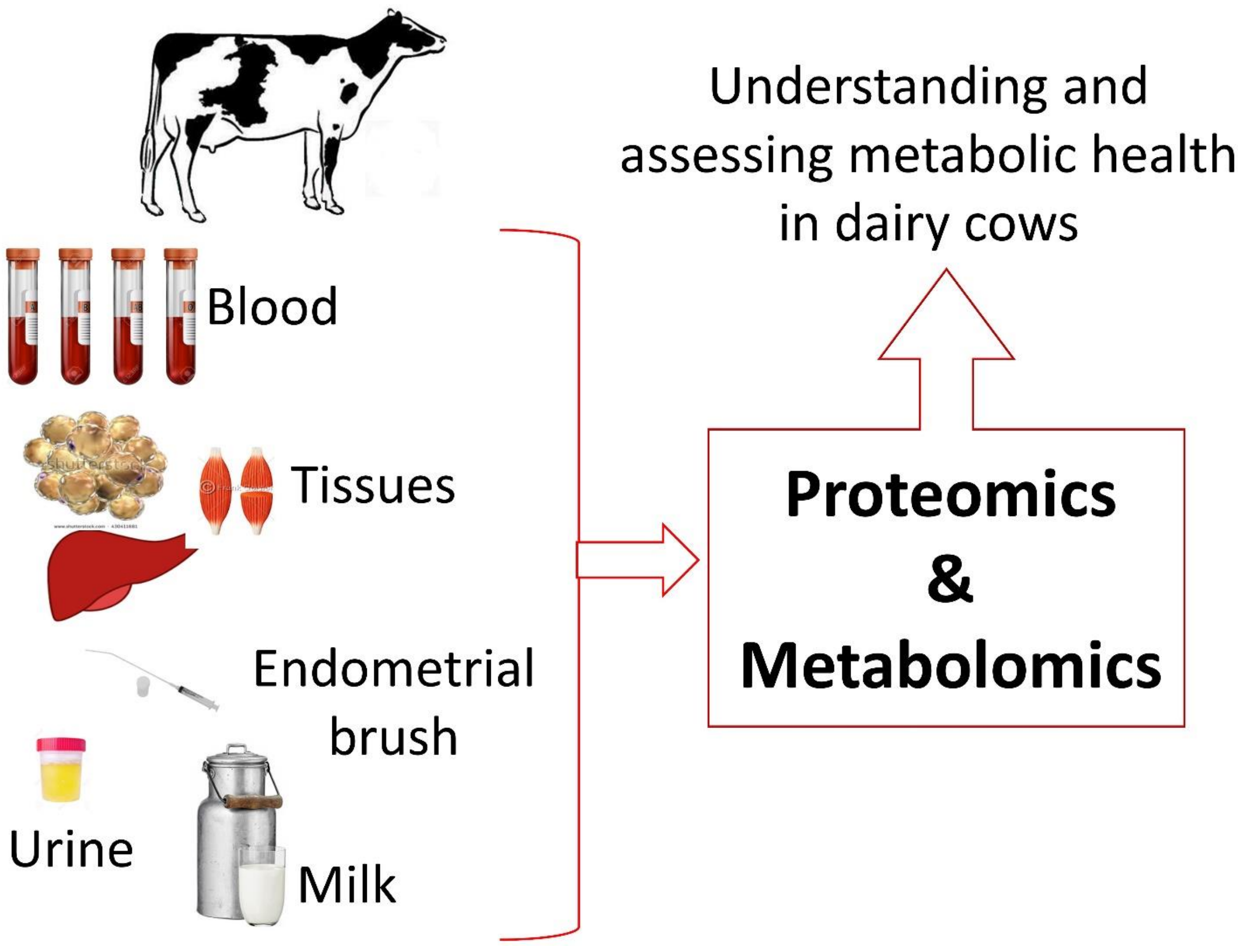
Altered blood proteins in hypocalcaemia
Increase
• Serpin peptididase inhibitor
• Endopin 2B
• Albumin
• Fibrinogen Alpha Chain
• Amyloid beta A4 proteins
• VGF
• A2M protein
• Fibrinogen Beta Chain
• IgG –C(H)
• Albumin
• Apolipoprotein A-II
• Serum amyloid A
• Vit-D binding protein precursor
• Paraoxonase,
Decrease
• β-hydroxybutyrate
• acetone
• pyruvate
• lysine
• Glucose
• alanine
• glycerol
• Phosphocreatine
• γ-aminobutyrate
Altered blood metabolites in hypocalcaemia



Hepatic proteins differentially expressed during ketosis
Increased
• Myosin light chain
• Tropomyosin
• MGC128326
• Myoglobin
• Peroxiredoxin -5, -6
• Glutathione S-transferase alpha-1
• Flavin reductase
• α-enolase
Decreased
• Acetyl-CoA acetyltransferase 2
• 3-hydroxyacyl-CoA dehydrogenase
↑ Lipolysis
↓ Fat synthesis
Adipose tissue
NEFA
Acetyl CoA
Ketone bodies
↓ Appetite
Rumen
Acetate Butyrate Propionate
TCA cycle
Oxalacetic acid
Liver
Milk synthesis
Udder
Glucose
↑ Gluconeogenesis


160
140
120
100
80
60
40
20
0
proteomics publications
metabolomics publications
number of publications/year
2000
2005
2010
2015
Year

1348


Understanding and assessing metabolic health in dairy cows
Blood
Tissues
Endometrial brush
Urine
Milk
Proteomics
&
Metabolomics