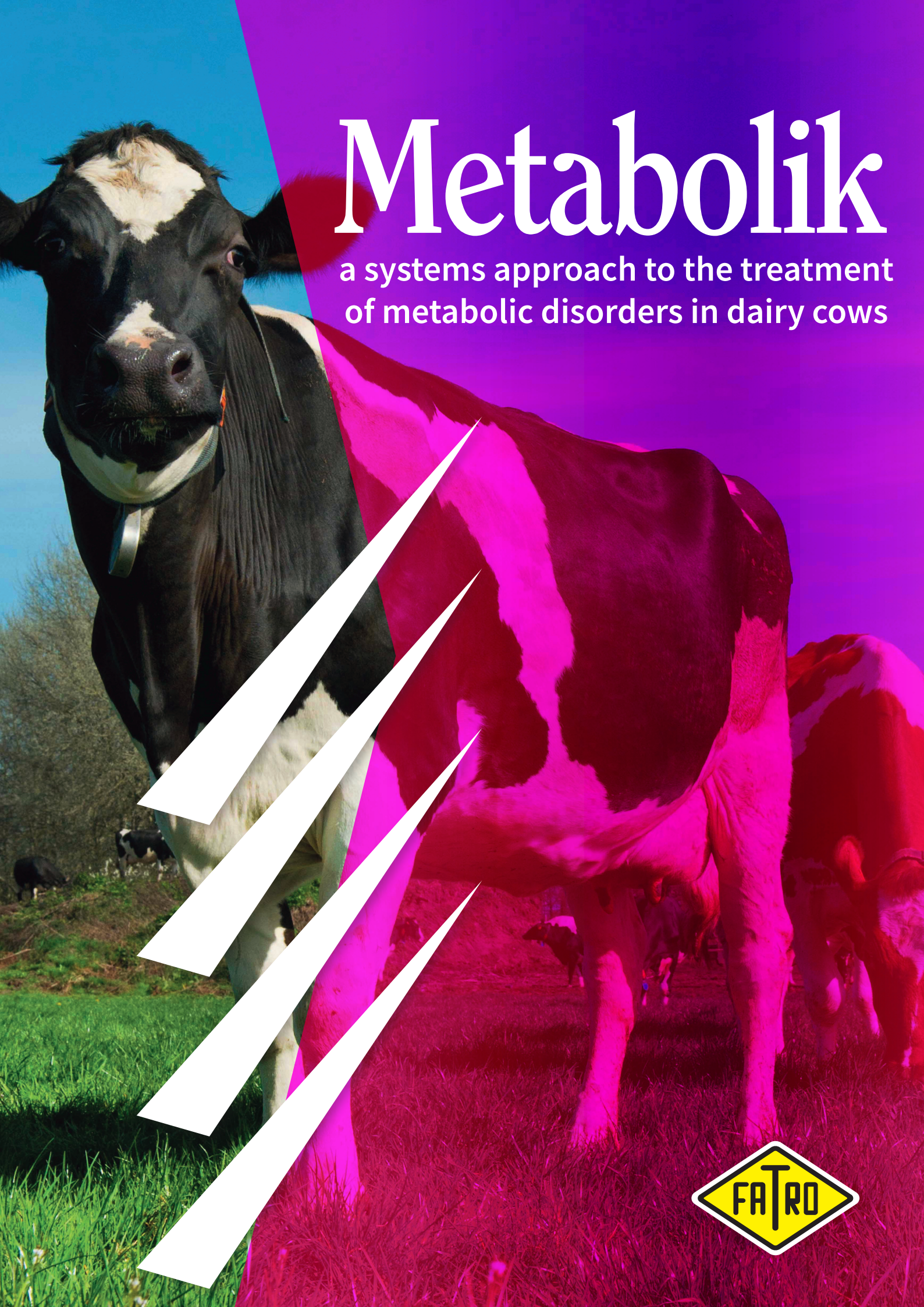
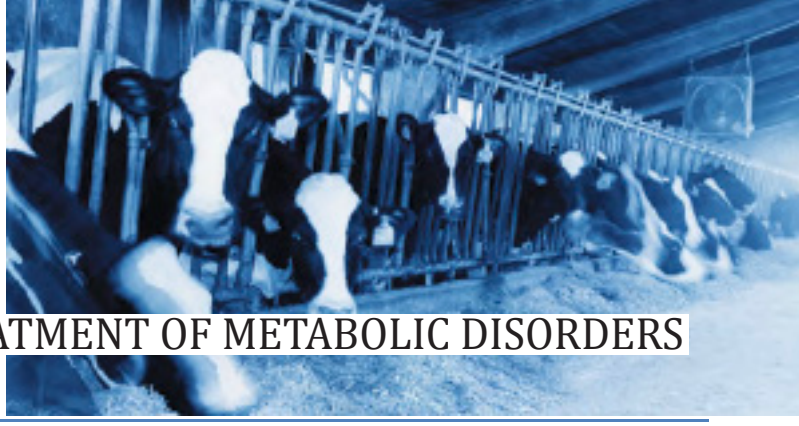


Metabolik

a systems approach to the treatment of metabolic disorders in dairy cows

FATRO

Metabolik



A SYSTEMS APPROACH TO THE TREATMENT OF METABOLIC DISORDERS IN DAIRY COWS

The approach that veterinary medicine has used so far for the treatment of metabolic disorders in dairy cows has been to focus the attention on each single disease. The traditional methodology, from diagnosis to treatment, has always been based on the supposition that the perturbation of one or a few biological variables, such as an hematologic or biochemical value, were responsible for a particular disease and that fixing these few perturbed variables could resolve the pathological condition. This reductionist approach is now, and has always been, the dominant methodology in all biological sciences, including veterinary medicine, and has led to many advances in understanding and treating diseases. It is based on the assumption that it is easier to understand things by taking them to pieces and studying the individual components. But although reductionism can make things more simple and intelligible, it looks only at individual isolated pieces of information and often fails to capture the complexity of a physiological or pathological state [1]. This is particularly true in the case of metabolic disorders. Most of the problems affecting cows during the transition period are strictly interconnected and usually a disease is a manifestation of a more complex pathological process that involves many interrelated biological factors. Undoubtedly veterinary medicine will continue to rely primarily on a reductionist methodology, but to improve the prevention and therapy of metabolic diseases, it will also have to understand the complex interactions within biological systems and to use a more holistic approach [2, 3].

Increasing awareness of the complex dynamics of living systems has led to development of systems biology, a new science that attempts to study biological systems as a whole. It is an inter-disciplinary field of research that focuses on complex interactions between the components of biological systems, and how these interactions give rise to emergent properties that characterize the function and behavior of that system [4]. This new approach is particularly useful with diseases that involve multiple organs and etiological factors and proposes a completely different way of conceiving illness. Diagnosing and treating diseases translates into identifying and manipulating global perturbed networks, rather than focusing only on unique failing components [5].

Systems biology is becoming an increasingly important tool in clarifying the mechanisms underlying the fundamental biological processes perturbed in diseases and for this reason has led to the development of "systems medicine": the application of systems biology approaches to medical research and medical practice.

Systems medicine can overcome the current limitations of disease complexity and drug therapy through the analysis and targeting of disease-perturbed networks and opens new perspectives in the development of novel therapeutic tools to improve health and productivity of dairy cows [3]. Complexity of pathological processes that characterize metabolic disorders during the transition period of dairy cows limits the therapeutic success that can be achieved by even the most potent and highly selective drug. In these situations, an effective approach should be directed to most, if not all, the impaired metabolic pathways underlying the clinical signs of the disorders, through the use of multicomponent drugs targeting interconnected elements within biological networks, each contributing a fraction of the perturbations that cause the disease. This reflects the way that living organisms function to constantly adapt to physiological or pathological changes.

In this view, Metabolik, a multicomponent drug for the treatment and prevention of metabolic disorders in dairy cows, has been formulated to tackle with the complexity of periparturient diseases, offering advantages that far outmatch the reductionist

approach that has commonly guided the development of drugs in veterinary medicine.

METABOLIC DISORDERS IN DAIRY COWS

The transition from pregnancy to lactation represents the most critical point in the productive cycle of dairy cows. In this period dairy cows experience considerable physiological changes and dramatic modifications in metabolism to support pregnancy and subsequent production of large quantities of milk. This transition is very challenging because numerous and simultaneous adaptations take place. Most health disorders occur during this time and the way they are managed is of great importance as they are closely linked to lactation performance, reproductive efficiency and significantly affect herd profitability [6]. There are several disorders that affect dairy cows during the first month after parturition, among which the most frequent and important are the following: acidosis, ketosis, fatty liver, left displaced abomasum (LDA), milk fever, downer cow syndrome, laminitis, retained placenta, metritis, and mastitis. All these diseases are not independent events, but rather a complex set of interrelated disorders that ineluctably elicit changes that predispose cows to other diseases, so that cows suffering from one postpartum pathological condition are at greater risk for contracting others. For example milk fever increases the odds of developing mastitis, retained placenta, metritis, dystocia, left displaced abomasum, ketosis and udder edema. Acidosis increases the risk of contracting laminitis, LDA, milk fever, fatty liver and mastitis. The ketosis/fatty liver complex is commonly associated with milk fever, LDA, laminitis, metritis, retained placenta, mastitis and udder edema [7, 8]. Many researchers have tried to find a common denominator that could link all these disorders, but without success, because there is no unique causal agent, but multiple organs, multiple etiological factors, and multiple biological networks are involved in the pathogenesis of these diseases (Fig 1).

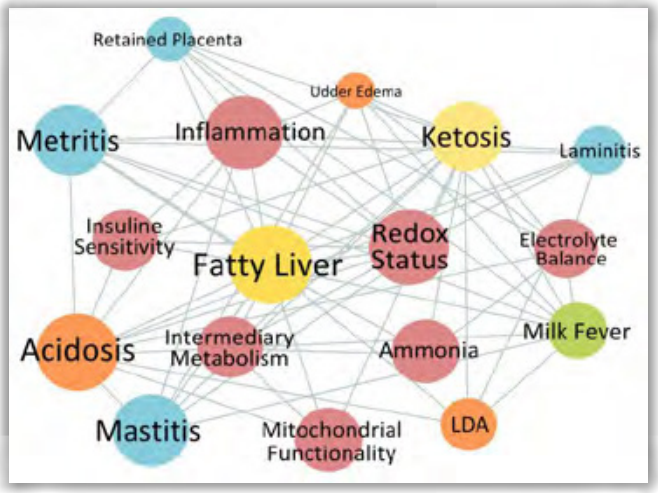


Fig 1: Multifactorial interaction networks in metabolic disorders.

One of the main characteristic of the transition to lactation is that it dramatically increases requirements for energy, glucose, amino acids, and other nutrients. Because of feed intake depression and high milk production, transition cows necessitate more energy and nutrients than they are able to consume and suffer a pronounced negative energy balance (NEB). Cows mobilize body fat to meet the increased demand for energy, and in almost all high-producing cows, large amounts of fatty acids are released into the blood and accumulated in the liver as triglycerides, leading to varying degrees of fatty liver. Fatty liver is a common postpartum condition affecting up to 50% of dairy cows in early lactation [9]. Fatty liver is associated with reduced health status, well-being, productivity and reproductive performance [6, 10]. Although the precise pathogenesis of postpartum diseases is unknown, decreased metabolic functions of the liver due to fatty infiltration are believed to be closely related to the development of these disorders. Triglycerides accumulation in hepatocytes decreases ureagenesis [11] and gluconeogenesis [12]. Toxic substances such as bile constituents accumulate in the liver of cows with fatty liver due to a reduction in bile flow [9] and impair detoxication processes. Synthesis of lipoproteins, which are important in lipid packaging, secretion and metabolization, also decreases [13]. Fatty liver is also associated with impairment of the immune system and increased risk and severity of infectious diseases such as mastitis and metritis [14]. Declining fertility in dairy cows is also suggested to arise from increased accumulation of triacylglycerol in the liver [15]. The intense mobilization of body fats and fatty liver could be the common finding that explains why periparturient diseases are strictly interrelated with each other. Fatty infiltration of the liver in early lactation surely plays a major role in the etiology and pathogenesis of

several diseases and it is extremely important to correctly manage this condition, but it is becoming more and more clear that other factors contribute to the perturbations that cause diseases during the transition period, among which oxidative stress is considered to be very important.

The use of oxygen in energy metabolism is the most powerful step in the evolution of life because has allowed living organisms to increase the production of energy, but during aerobic metabolism toxic substances are also produced, the so called reactive oxygen species (ROS). ROS are intermediate products of oxidative metabolism. They are normally generated in all living aerobic organisms and, because of their high predisposition to interact with other molecules, they are capable of causing notable damage to cells and tissues. They can be both free radicals, such as superoxide anion and hydroxyl radical, or other highly reactive substances, capable of transforming themselves into, or giving rise to free radicals, like for example hydrogen peroxide and singlet oxygen. ROS are continually produced in cells and then removed by the antioxidant defense systems in the organism. They are formed during processes of energy production in mitochondria, in many enzymatic reactions, in detoxification processes and during phagocytic activity of immune cells. ROS are highly reactive molecules, capable of interacting with most of the molecules present in living organisms. Once the constituent elements in the organism are attacked, they are transformed in their turn into free radicals, giving rise to chain reactions which enormously amplify the negative effects of ROS. The mechanisms of cellular damage induced by oxidative stress are many and complex and can contribute to the development of numerous pathological and degenerative disorders. ROS can damage cell membranes and lipids in living organisms through a process called lipid peroxidation, they can attack proteins and cause structural alterations which lead to loss of enzymatic activity, or can cause molecular damage to DNA, causing genetic mutations or cellular death.

Living organisms have developed numerous systems to defend themselves from the oxidative attack of ROS. At the level of cell membranes, the principal defense mechanism consists of vitamin E, a potent liposoluble antioxidant able to eliminate free radicals and interrupt the chain reactions of lipid peroxidation. Within the cell, at the level of the cytoplasm and mitochondria, there are defense systems which operate in aqueous phase to destroy ROS which are formed during cell metabolism. Amongst these, we find vitamin C and certain enzymes, such as superoxide dismutase, catalase and glutathione peroxidase.

The damage caused by ROS assumes particular significance under so-called oxidative stress conditions, situations often associated with inflammation, infection and disease, in which an imbalance is created between production of ROS and the defense systems in the organism. Many researchers have discovered that, during the transition period, cows experience oxidative stress [16]. The high energy demand and intense metabolic processes that characterize the transition period are indeed accompanied by a strong increase in oxygen consumption. This results in excessive production of ROS which may overwhelm natural cellular antioxidant defenses and lead to oxidative stress. This seems to be an important factor for the increased susceptibility of cows to production diseases and other health problems in the postpartum period [16–19]. It is thus evident that timely detection and early intervention to reduce oxidative stress is important for prevention and treatment of periparturient disorders, but recent research has also highlighted the role of another common factor contributing to the development of metabolic disorders in the postpartum, which is strongly interrelated with oxidative stress, and that is inflammation.

There are many events during parturition and early lactation that can elicit an inflammatory reaction: traumas associated with calving, bacterial contamination of the uterus in the postpartum, higher risk of infectious diseases, and different environmental, social, or nutritional stressors. Also the sudden shift to diets with greater energy density at the onset of lactation can contribute to systemic inflammation, because of the increased lysis of rumen Gram-negative bacteria and the translocation of endotoxin into the peripheral circulation [20].

Moreover, the increased circulating concentrations of non-esterified fatty acids and oxidative stress significantly contribute to systemic inflammation, and it has been seen that dairy cows always display an inflammatory response during transition, even without clear signs of microbial infections or other diseases [21].

The physiological response to local or systemic disturbances in homeostasis caused by infection or tissue injury not only involves a local inflammation but also a systemic response, the so called acute phase reaction: a non-specific defense mechanism aimed at fighting harmful agents, removing damaged tissues and promoting healing. The acute inflammatory response is triggered by the release of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα), interleukin-1β (IL-1β), and interleukin-6 (IL-6), which are chemical messengers able to act on a wide range of cell types, both at the site of injury and at distant sites in the organism. Cytokines determine several endocrine and metabolic changes and alter the bio-chemical processes of many tissues. Protein synthesis by the liver is drastically altered, resulting in an increase of some blood proteins, the positive acute phase proteins (e.g. C-reactive protein, serum amyloid A and haptoglobin) and in the decrease of normal blood proteins, the negative acute phase proteins (e.g. albumin, transferrin, retinol-binding protein, antithrombin and transcortin) [22]. For these reasons systemic inflammation and cytokines are thought to play an important role in the pathogenesis of metabolic disorders. For example it has been proved that TNFα exacerbates the anorexic status that occurs at parturition, reduces insulin sensitivity, activates lipolysis, decreases fatty acid oxidation, promotes liver triglyceride accumulation and impairs gluconeogenesis [23, 24]. All these are important factors in the development of ketosis, fatty liver and other metabolic problems, so it is not unusual that cows with a stronger inflammatory response during the first month of lactation have also an impaired liver function, are at greater risk for experiencing transition disorders, produce less milk and show impaired fertility [25].

Another metabolic disturbance that dairy cows usually experience during early lactation and that is thought to contribute to the morbidity of periparturient disorders is hyperammonemia (sub-clinical ammonia intoxication). In this period, to maximize milk production, cows are commonly fed diets containing high levels of crude protein, which is metabolized to ammonia in the rumen which, if not converted to microbial protein, is absorbed into the blood stream. Feeding urea-based supplements, especially with low quality forage diets, may also lead to hyperammonemia. Moreover, dairy cows mobilize large amounts of body proteins in early lactation to supply amino acids for energy and glucose production, leading to increased release of ammonia. The liver plays an important role in ammonia detoxification and dysfunction of this organ leads to higher susceptibility to ammonia toxicity. During the transition period, the urea cycle has limited adaptive capacity [26] and fat accumulation in the liver decreases the capacity to detoxify ammonia [11, 27]. Both these conditions may precipitate ammonia toxicity in early lactation. Hyperammonemia causes disarrangement in intermediary metabolism [28], decreases the ability of the liver to convert propionate to glucose [29], and reduces insulin release [30, 31]. Moreover, increased levels of urea and ammonia affect proper functions of the reproductive organs, and result in decreased fertility in dairy cows [32, 33]. For all these reasons, to successfully managing health and productivity of dairy cattle during early lactation, special attention must also be paid to the control of sub-clinical ammonia intoxication.

All mammals show some degree of insulin resistance during late pregnancy, which is useful for the partition of nutrients toward the fetus and the mammary gland for milk production. Nevertheless cows, during the transition period, may develop a type of insulin resistance of the adipose tissue similar to that found in the metabolic syndrome in humans. Insulin resistance of the adipose tissue results in increased fatty acid flux to the liver and subsequent fat deposition in hepatocytes. In turn, fatty liver, which has been linked to the development of insulin resistance, leads to a vicious cycle that exacerbates and contributes to the development of metabolic disorders. Adipose tissue acts as an endocrine organ, secreting biologically active molecules in response to various stimuli, such as stress or lipid overloading.

These signaling molecules, among which we find adipokines, cytokines and acute phase reactants, are linked with insulin resistance and inflammation in fat play a crucial role in the development of this condition [34]. The accretion of fat in the cow during the dry period and the consequent release of biologically active molecules by the adipose tissue may be responsible for the increased susceptibility of over-conditioned cows to metabolic disorders. There is also evidence that pregnancy itself is a condition of moderate inflammation, in which adipose tissue and placenta contribute to the local and systemic increase of inflammatory molecules, and that this low-grade inflammation eventually leads to insulin resistance in late pregnancy [35].

The control of insulin resistance has been proved to improve metabolic health in transition cows. Cows administered thiazolidinediones, which are activators of the peroxisome proliferator activated receptor-gamma (PPAR-γ) and in this way can potentiate the action of insulin in peripheral tissues, show reduced fat mobilization from adipose tissue, higher dry matter intake, decreased liver fat accumulation, reduced body condition score loss, and improved fertility [36]. Therefore, in the prevention and treatment of metabolic disorders during the transition period, particular attention must also be paid to the management of insulin resistance.

METABOLIK

Metabolik is a multicomponent drug developed for the treatment and prevention of metabolic disorders of dairy cows. It contains l-carnitine, thioctic acid, specific B-group vitamins, biologically significant amino acids and sugars. These compounds carry out a broad-scale action on several biochemical processes of the organism and below, we will see how, taken together, they are capable to address the extremely intertwined and complex biochemical networks perturbed in periparturient disorders.

L-CARNITINE

Carnitine is one of the main components of Metabolik and, even alone, is able to counteract many of the metabolic alteration typically found during the transition period: fatty acid accumulation in the liver, oxidative stress, hyperammonemia and inflammation. Carnitine is an essential metabolite which plays a number of critical roles in the intermediary metabolism of all animals. It is found in living organisms both as the free carnitine and as the ester of a wide variety of acyl compounds. Of the two stereoisomers of carnitine, l- and d-carnitine, only l-carnitine (levocarnitine) is naturally occurring and biologically active, and for this reason Metabolik has been formulated to contain only pure l-carnitine.

L-Carnitine Role in Metabolism

The best known and most important biological function of carnitine is in the transport of fatty acids from cytoplasm into the matrix of mitochondria, where they are oxidized for energy production via the β-oxidation pathway. To be metabolized, free fatty acids must firstly be bound to CoA to give acyl-CoA, which is the activated form of fatty acids. The inner membrane of mitochondria is impermeable to acyl-CoA , therefore the acyl group on CoA must be transferred to carnitine and the resulting acyl-carnitine transported into the mitochondrial matrix.

This translocation occurs via a series of reactions catalyzed by three carnitine-dependent enzymes that together represent the carnitine shuttle system:

1. Acyl group esterified to CoA is transferred to carnitine by carnitine-palmitoyltransferase 1 (CPT-1) located on the outer mitochondrial membrane

2. Acyl-carnitine is shuttled inside by a carnitine-acylcarnitine translocase (CACT), which mediates the transmembrane exchange of acyl-carnitine for carnitine..

3. Acyl-carnitine is converted to acyl-CoA by carnitine-palmitoyltransferase 2 (CPT-2) located on the inner mitochondrial membrane. The fatty acid is now esterified to CoA within the mitochondrial matrix and the free carnitine diffuses back to the cytosol across the membrane by the action of carnitine-acylcarnitine translocase.

In addition to CPT-1 and CPT-2, carnitine is also a substrate for many other carnitine-dependent acyltransferases, which have different intracellular

distributions and different chain-length specificities. Carnitine-acyltransferases catalyze the exchange of acyl groups between CoA and carnitine, buffer potentially toxic acyl-CoA metabolites, facilitates mitochondrial efflux of excess carbon fuels, modulates the ratio of acyl-CoA/CoA in various intracellular compartments and in this way play numerous crucial roles in metabolism. Because of these key regulatory functions, carnitine effectively protects mitochondrial function and is indispensable for a sound energy metabolism.

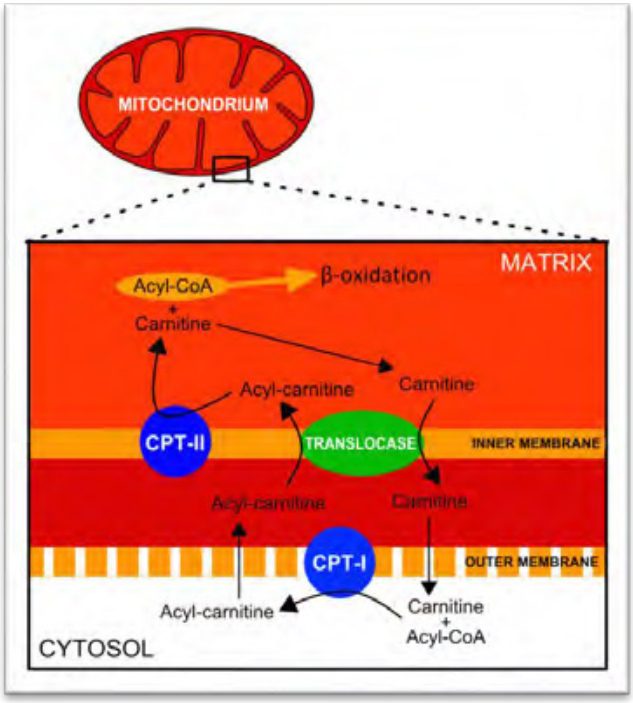


Fig 2: The carnitine shuttle

Ruminant liver is not able to efficiently export fatty acid as very low density lipoproteins (VLDL) [37], on the contrary hepatic β-oxidation of fatty acids is stimulated by exogenous carnitine [38], so the administration of carnitine, for its capacity to improve fat catabolism, is an important tool in the control fatty liver and related disorders. Carnitine also decreases the synthesis of fatty acids (lipogenesis) in the liver, through the diversion of acetyl-moieties towards acetyl-carnitine that is subsequently released by the liver into the circulation [39]. In-vitro studies using bovine liver slices have shown that carnitine enhances the oxidation of fatty acids and inhibits the synthesis of triglycerides [40, 41]. It has been proved that carnitine decreases liver lipid accumulation in cows with experimentally induced negative energy balance [42].

In addition, carnitine performs a number of other essential intracellular and metabolic functions. It transfers fatty acids produced by the peroxisomal beta-oxidation to the mitochondria, favoring their oxidation which cannot be complete in peroxisomes. It improves recycling of CoA by removing short-chain acyl groups accumulating in mitochondria, and in this way it raises the levels of free CoA, which is then available to support the continuation of ß-oxidation and the Krebs cycle. Carnitine provides also a protective effect against ketosis, because it promotes fatty acid complete oxidation [43], acts as an acetyl buffer, increases the use of ß-hydroxybutyrate by peripheral tissues, and improves urinary excretion of acyl-carnitines [44][45]. Moreover, carnitine has been shown to increase hepatic gluconeogenesis by stimulating the flux of metabolites through pyruvate carboxylase [46], and by increasing the availability of amino acids through the inhibition of the branched-chain α-keto acid dehydrogenase, an action that reduces the breakdown of branched chain amino acids and promotes protein synthesis [47].

L-Carnitine and Oxidative stress

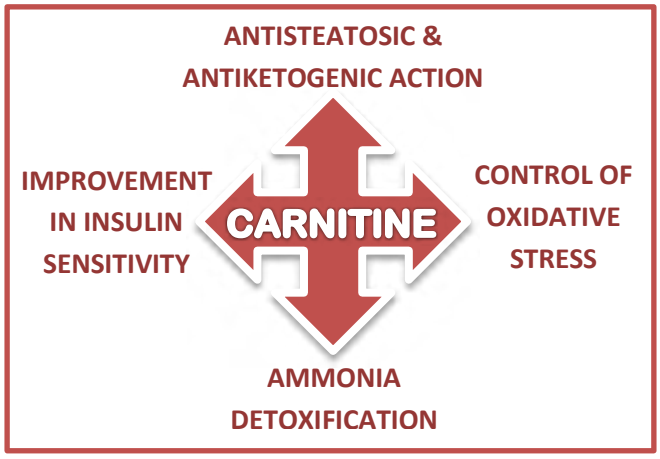
Reactive oxygen species (ROS) are involved in the pathogenesis of several metabolic disorders, therefore the control of oxidative stress in dairy cows represents an obligatory therapeutic target in the management of periparturient disorders. L-carnitine has been shown in many species to be effective in pathological conditions characterized by increased oxidative stress and there is evidence that it plays a critical role as modulator of cellular stress response in health and disease states [48]. L-carnitine is capable to reduce oxidative stress in different ways: by direct radical scavenging activity, as it has been proven in vitro [49, 50] and in vivo [51]; by its ability to stabilize mitochondrial membranes and in this way to reduce the generation of ROS through the electron transport chain [52] ; by preventing the accumulation of end-products of lipid peroxidation [53]. Furthermore l-carnitine has a metal chelating activity that decreases the concentration of cytosolic iron, an element that increases free radical generation [54].

Anti-inflammatory Effects of L-Carnitine

Several findings have consistently shown that L-carnitine can reduce the inflammatory process in many pathological conditions in which inflammation is thought to play an important role. In animal models of hypertension, L-carnitine supplementation was able to counteract the increase of IL-1β, IL-6, and TNF-α in plasma and heart tissue [55]. L-carnitine treatment reduced the acute-phase proteins C-reactive protein and serum amyloid A, and increased plasma levels of total protein and negative acute-phase proteins (albumin and transferrin) in haemodialysis patients [56, 57]. In a model of carrageenan-induced inflammation in aged rats, which has been shown to be highly predictive for testing antiinflammatory drug activity, L-carnitine was capable of restoring the age-related changes in the functions of inflammatory cells and protected tissue destruction in inflammation by decreasing the superoxide anion production [58]. L-carnitine, through the enhancement of the mitochondrial function, can reduce oxidative stress, which is a major contributor to the release of pro-inflammatory cytokines during conditions that impair cellular energy homeostasis [59]. It has been also proposed that l-Carnitine can modulate the immune response and it has been proved that its deficiency is responsible of an impaired immune response and implicated in the pathophysiology of endotoxin-mediated multiple organ failure [60].

L-Carnitine and Ammonia Detoxification

L-carnitine also plays a role in ammonia detoxification and its administration has been proven to reduce blood ammonia levels, prevent ammonia toxicity and improve disorders of ammonia metabolism in humans and animals [61–64]. L-carnitine has also been shown to prevent hyperammonemia in ruminants. Intravenous administration of L-carnitine in ewes given an oral urea drench significantly lowered plasma ammonia levels [65]. Furthermore, supplementation of L-carnitine alleviated hyperammonemia in ruminants fed high levels of non-protein nitrogen [66]. The mechanism by which L-carnitine protect against ammonia toxicity is poorly understood, but it has been proposed that it might act by inducing ureagenesis [63], by improving the structural and metabolic integrity of mitochondria [67] or by preventing the inhibition of ureagenesis by acyl-CoA derivatives [68]. A mechanism that also provides a link between fatty acid metabolism and urea synthesis, during conditions of negative energy balance [69].



L-Carnitine and Insulin Sensitivity

Carnitine requirements increase under conditions of metabolic stress, as those found during the transition period in dairy cows, and this can lead to carnitine insufficiency, mitochondrial dysfunction and impairment in insulin sensitivity, which can be reversed by carnitine supplementation [43]. Carnitine supplementation has been proved to restore insulin responsiveness in multiple models of glucose intolerance and a significant effect on whole body glucose uptake has also been observed in normal subjects. Mechanisms for this metabolic effect of carnitine have not been completely clarified, but several carnitine actions has been proposed to be linked to it: regulation of acetyl and acyl trafficking between intracellular compartments, regulation of the activity of the pyruvate dehydrogenase complex, modulation of the expression of genes encoding glycolytic and gluconeogenic enzymes and components of the insulin signaling cascade, stimulation of insulin-like growth factor (IGF) signaling pathway [70]. The activation of the IGF axis would also explain the positive effect of carnitine supplementation on reproductive performance [71]. Experiments in pregnant sows and other animal species have showed that carnitine increases the concentrations of IGF-1 and IGF-2 in blood [72, 73] and this increase is considered to be responsible for the positive effects on reproduction seen in animals, because IGF-1 is a key hormone favoring placenta development and intra-uterine nutrition [70].

THIOCTIC ACID (LIPOIC ACID)

Thioctic acid (TA), also known as lipoic acid, is a disulfide compound that is found naturally in mitochondria as a cofactor for dehydrogenase enzyme complexes, which are essential for energy production and glucose and protein metabolism. Aside from its enzymatic role, TA has gained increasing clinical interest for its potent antioxidant and redox modulation activities and has been proved beneficial in a number of oxidative stress models and pathological conditions associated with increased oxidative stress.

TA contains two thiol groups, which may be oxidized or reduced. As the antioxidant glutathione, TA is part of a redox pair, TA and its reduced form, dihydrothioctic acid (DHTA), but unlike glutathione, for which only the reduced form is an antioxidant, both the oxidized and reduced forms of TA are potent antioxidants [74]. TA is unique among natural antioxidants in its ability to fulfill all of the requirements of an ideal antioxidant. Its reduced and oxidized forms are able to scavenge a broad array of reactive oxygen and nitrogen species, including hydrogen peroxide, singlet oxygen, hydroxyl, nitric oxide and superoxide radicals [75]. TA exhibits a further antioxidant activity by chelating transition metals, such as iron, copper, and cadmium, which otherwise catalyze the generation of free radicals [76]. TA also plays an important role in the antioxidant network of biological systems, network that enables antioxidants to be recycled and regenerated in a coordinated way. When antioxidants scavenges a free radical, they become oxidized themselves and not able to perform their function anymore, but TA can recycle them and extend their biological lifespan, as has been proved for vitamin C, vitamin E, coenzyme Q10 and glutathione [75]. TA has also been shown to increase de novo synthesis of glutathione in cells, by reducing cystine to cysteine [77]. Further, it has been demonstrated that in vivo TA is reduced metabolically to DHTA and thus subject to metabolic regeneration. In addition, unlike all other natural antioxidants, which work only in water (as vitamin C) or fatty environments (as vitamin E), TA is both fat- and water-soluble [78]. Therefore it can act in all parts of the cell, readily cross biological membranes and disperse in extracellular and intracellular tissue components. Due to all these unique features TA is commonly referred to as the universal or ideal antioxidant, and recently it has become evident that it plays additional important roles in the control of oxidative stress related diseases, through a redox modulating action on signaling and transcription [79].

The classical definition of oxidative stress, as an imbalance between oxidants production and cell antioxidant defenses, now seems to provide a reductionist view of oxidative stress. In contrast to the conventional idea that ROS mostly induce damage through oxidation of biological structures, it is now clear that ROS, which are also generated in healthy cells, can regulate a variety of critical molecular mechanisms at a concentration much below that required to induce oxidative damage. It is now clear that several biological molecules involved in cell signaling and gene regulation systems are sensitive to ROS, whose generation in living organism is a tightly regulated process modulated through compartmentalized redox pathways rather than through global balances. The maintenance of the redox status is, indeed, crucial for cellular homeostasis and its dysregulation is associated with an alteration of numerous signaling pathways that can contribute to disease emergence or progression. For this reason oxidative stress has also been defined as a disruption of redox signaling and control [80].

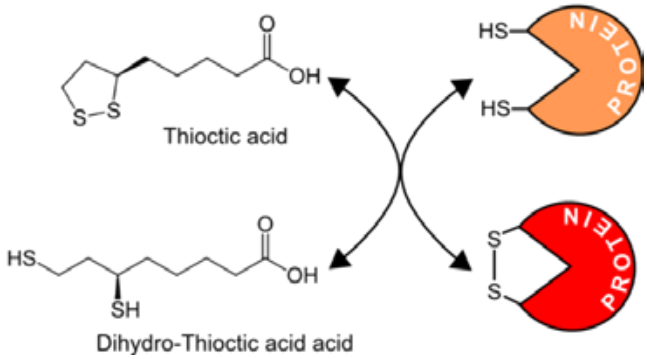


Fig 3: Role of thioctic acid in thiol-based redox regulation and signaling

Proteins containing thiol groups (thiol of cysteine and thioether of methionine) are particularly susceptible to reversible oxidation-reduction and represent important targets in oxidative signaling. Oxidation of these critical thiol groups, which are widely distributed in biological systems, can alter the structure and the activity of signaling, structural, and

regulatory proteins and in this way control all the aspects of cell biology. Therefore, the disruption of thiol redox is thought to highly contribute to the pathologies linked to oxidative stress, and new prospects are offered for applying specific antioxidant therapy in the treatment of these conditions. TA can regulate several redox circuits because of its ability to widely distribute into all subcellular compartments and is a critical component of the antioxidant network for its ability to regenerate other antioxidants and increase intracellular GSH levels. Furthermore TA is critical for the modulation of thiol redox status of proteins via thiol/disulfide exchange reactions and in this way can rearrange the redox signaling pathways disrupted by oxidative stress [79].

CYANOCOBALAMIN

Cyanocobalamin is a compound with vitamin B12 activity, commonly used in pharmaceuticals for its higher stability, which in living organisms is rapidly converted into the active co-enzyme forms of vitamin B_{12}. Vitamin B_{12} (cobalamin) is a water-soluble vitamin and essential cofactor of two important enzymes: methionine synthase and methylmalonyl-CoA mutase. The first catalyzes the regeneration of methionine (Fig 5) and is necessary for the synthesis of proteins, nucleotides and the universal methyl donor S-adenosylmethionine (AdoMet or SAM) which plays a pivotal role as a methyl donor in a myriad of biochemical reactions. The second catalyzes the conversion of methylmalonyl-CoA into succinyl-CoA (Fig 4), a key molecule of the TCA cycle. This reaction is an essential step for the metabolism of odd-chain fatty acids, some amino acids (valine, isoleucine, methionine and threonine) and for the entry of propionic acid into the TCA cycle and subsequent conversion to glucose. Propionic acid from rumen fermentation of dietary carbohydrates is the major glucose precursor in ruminants, therefore the activity of vitamin B12 is of great importance in these animals. Ruminants are generally supplied with adequate vitamin B_{12} from ruminal microbes, but in early lactation, the status of vitamin B_{12} is not adequate and this fact has been found to limit the lactation performance of cows [81, 82]. Therefore, with the administration of Vitamin B12 to periparturient dairy cattle, it is possible to support the replenishment of oxaloacetate in the TCA cycle from propionate via methylmalonyl-CoA mutase activation, to increase gluconeogenesis efficiency and to induce glucose-stimulated insulin secretion, which consequently inhibits lipolysis and has positive effects on the metabolic status of cows. Intramuscular administration of vitamin B_{12} has indeed been shown to increase gluconeogenesis from propionate in liver slices of sheep [83], to decrease fat accumulation in the liver of cows [84] and to improve milk and milk protein yields when given in early lactation [81].

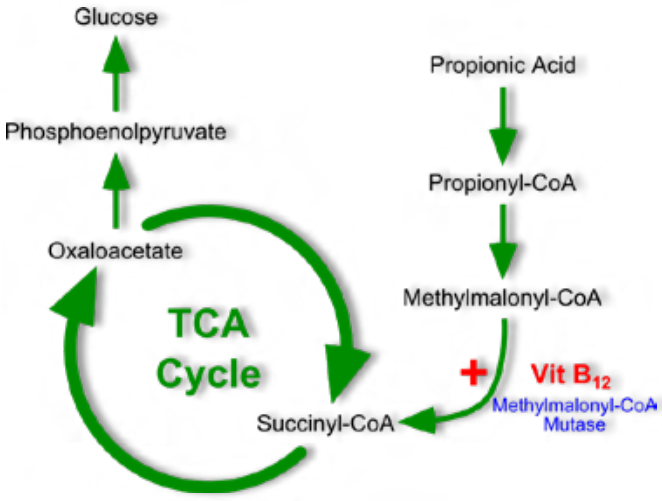


Fig 4: Role of vitamin B12 in gluconeogenesis from propionate

PYRIDOXINE

Pyridoxine is one of the naturally occurring form of vitamin B_6, along with pyridoxal, pyridoxamine and their phosphorylated derivatives. All vitamers of vitamin B_6 have equal activity when administered parenterally. Vitamin B_6 is an essential cofactor in a wide range of biochemical reactions (about 150 enzymes involved in the metabolism of proteins, fats, and carbohydrates, some examples in Fig 5) and supports more vital functions than any other vitamin. It is particularly involved in protein metabolism and takes part in almost all reactions of amino acid metabolism.

Vitamin B_6 is normally synthesized by microorganisms in the digestive tract of ruminants, however, stressed cattle may have very low plasma concentrations of this vitamin [85], while both pregnancy and lactation are known to increase vitamin B_6 requirements. Furthermore, the administration of pyridoxine produces significant biological effects also in animals

not deficient, showing functions which are beyond its classical role as coenzyme [86]. It has been seen that inflammation results in an increased need for vitamin B_6 and that a higher vitamin B_6 intake protects against inflammation [87]. Researchers have found that vitamin B_6 can suppress inflammation preventing the degradation of Iκ-B (Inhibitor of κB), which therefore inhibits the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), a transcription factor which is a first responder to harmful cellular stimuli and responsible for the transcription of cytokines and proteins involved in inflammation [88]. Moreover, vitamin B_6 has been recently found to be a powerful antioxidant, even more potent than vitamins C and E [89, 90]. Vitamin B_6 can directly scavenge ROS [91, 92], chelate redox metal ions [93], increase GSH synthesis [94], and block the formation of advanced lipoxidation end-products (ALEs)[95], which may worsen hepatocyte injury during fatty liver.

Therefore, the multiple anti-oxidative and anti-inflammatory activities of vitamin B_6, along with its safety, posture it as a useful drug for treatment of metabolic conditions in which oxidative stress and inflammation are involved.

ACETYL-METHIONINE

Acetyl-Methionine is a bioavailable source of methionine, an essential amino acid that contains both a methyl and a sulfur group and plays a key role in protein synthesis and several cellular functions. Methionine is required for the synthesis of S-adenosylmethionine (SAM, also called AdoMet), which is the major methyl donor in biological systems and an essential precursor of polyamines. Methionine also provides cysteine for the synthesis of glutathione (GSH), which protects cells from oxidative damage and plays a vital role in detoxification. Hence methionine is strictly linked both to cellular methylation and redox buffering, making it a central metabolite for cellular homeostasis and hepatocyte function. SAM and GSH stores are known to be critical in the maintenance of mitochondrial function and hepatocellular survival, and methionine deficiency has been linked to hepatic lipid accumulation, overexpression of inflammatory cytokines, fibrosis, and oxidative liver injury due to the depletion of SAM and GSH in mitochondria [96][97].

Methionine is most often deficient in dairy cow fed diets based on legume forages, corn silage, corn grain, and soybean meal, which have little methionine within their protein [98], moreover pro-oxidant conditions, as found during the transition period, inhibit methionine recycling [99] and may reduce methylation reactions, contributing to the development of periparturient diseases and impairing immune system activation.

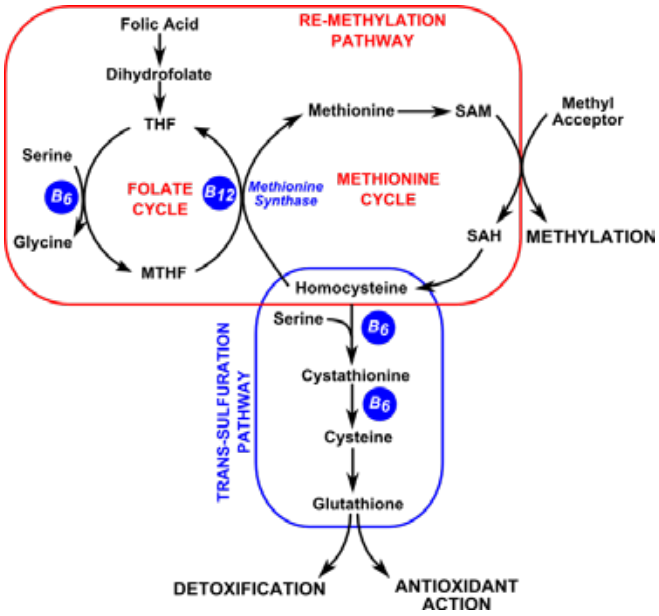


Fig 5: One carbon metabolism and methionine role in methylation, detoxification and redox buffering

LYSINE

Lysine is an essential amino acid and, together with methionine which serves as a methyl donor via S-adenosyl methionine, the precursor for the synthesis of endogenous carnitine. As well as methionine, lysine is a limiting amino acid in dairy cows and its supplementation improves both milk yield and milk composition [100]. It has been found that lysine deficiency increases stress-induced anxiety and that lysine supplementation blocks the anxiogenic effects of stress [101, 102]. This effect involves the hypothalamic-pituitary-adrenal axis and is probably linked to lysine acting as a partial serotonin-receptor-4 (HTR4) antagonist and concurrently as a partial benzodiazepine-receptor agonist [103]. The anti-stress effect of lysine is strengthened when concomitantly provided with arginine. Pigs fed with a

lysine plus arginine fortified diet showed a reduced anxiogenic response to transportation and decreased plasma cortisol levels [104]. Stress predisposes and exacerbates metabolic disorders of dairy cows. The relationship between environmental stress and metabolic disorders is not well understood, but it apparently involves interactions between humoral and neural pathways.

Therefore lysine supplementation, particularly when associated with arginine, can help cows cope with stressors and reduce incidence and severity of periparturient disorders. Furthermore, lysine can both enhance intestinal calcium absorption and improve the renal conservation of the absorbed calcium, contributing to improve calcium balance during the transition period [105].

UREA CYCLE AMINO ACIDS

In mammals, the major pathway for ammonia detoxification and removal of surplus nitrogen from the organism is the conversion of ammonia into urea in hepatocytes through the urea cycle (also called ornithine cycle). Liver ability to detox ammonia depends on the integrity of hepatocytes and on the availability of amino acids involved in the urea cycle: arginine, ornithine and citrulline. The administration of these amino acids has been proven valuable in reducing hyperammonemia in many animal species, including ruminants. In an experiment, approximately 33% of rats poisoned with a lethal dose of ammonium acetate survived after treatment with a urea-cycle mixed amino acid solution, whereas in the control group survival rate was only 1% [106]. An injection of a solution of urea cycle amino acids in steers infused with ammonium chloride reduced plasma ammonia concentration compared to the control group and promoted efficient improvement in clinical condition [107]. Furthermore, arginine directly protects against ammonia intoxication by stimulating N-acetyl-glutamate synthetase, which is a critical step required for efficient functioning of the urea cycle [108].

The urea cycle comprises enzymes that generate urea, but also overlaps with the arginine-citrulline cycle, which produces nitric oxide (NO). NO is an ubiquitous signaling molecule and extraordinarily important bioregulator, synthesized from arginine by nitric oxide synthase (NOS). Therefore, beside their importance in ammonia detoxification, urea cycle amino acids, as nitrogenous precursor for the synthesis of nitric oxide, regulate several vital metabolic pathways. Arginase, which hydrolyzes arginine to ornithine and urea, and nitric oxide synthase compete for arginine and relative changes in their enzymatic activities can shift arginine utilization from the catabolic (urea production) into the functional pathway (NO production). NO, as a signaling molecule, regulates glucose, fat and amino acid metabolism in mammals. It stimulates glucose uptake as well as glucose and fatty acid oxidation in skeletal muscle, heart, liver and adipose tissue [109]. It is therefore necessary to maintain arginine homeostasis through supplementation of urea cycle amino acids for good health under many metabolic disorders [110]. For example, liver inflammation and injury results in a severe deficiency of arginine due to a massive release of arginase from injured hepatocytes and disturbed NO production [111], while stress conditions inhibit ammonia detoxification, redirecting arginine from ureagenesis to NO production [112].

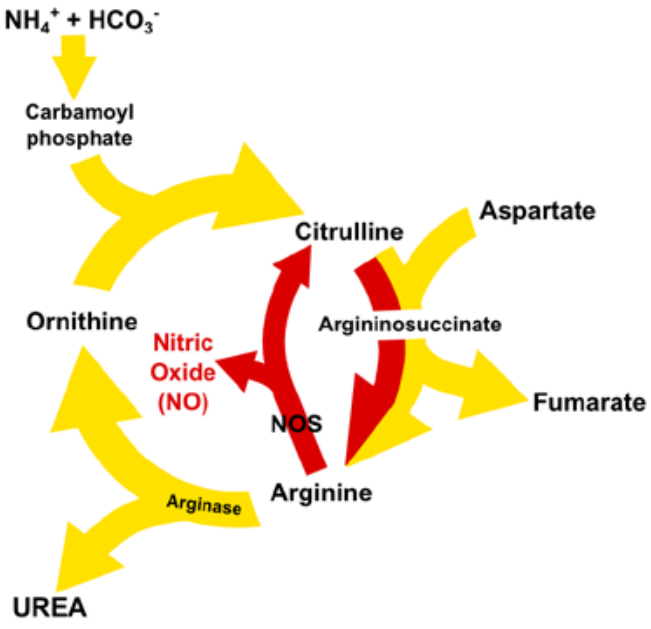


Fig 6: Role of arginine, citrulline and ornithine in urea (yellow) and nitric oxide (red) synthesis.

Arginine is also a well-known stimulator of growth hormone (GH), insulin-like growth factor-1 (IGF-1) and insulin release [113, 114]. All actions that may help improve the metabolic status of cows. Furthermore it has been proved that arginine supplementation has beneficial effects in attenuating hepatic injury induced

by *Escherichia coli* lipopolysaccharides in piglets. It is possible that the protective effects of arginine on the liver are associated with a decreased release of liver pro-inflammatory cytokines and free radicals through inhibition of TLR4 (Toll-like receptor 4) signaling [115], which plays a fundamental role in pathogen recognition and activation of innate immunity.

GLUTAMIC ACID

Glutamic acid, found as the anion glutamate at physiological pH, is an amino acid that has a crucial role in cellular metabolism. It participate in key biochemical reactions that produce intermediates involved in fundamental processes of cellular metabolism, such as the Krebs cycle, amino acid metabolism, and gluconeogenesis. Glutamate plays a central role in nitrogen flow, serving as both a nitrogen donor and nitrogen acceptor. Glutamate, through the action of glutamate dehydrogenase, can be reversibly converted to α-Ketoglutarate, a key intermediate in the Krebs cycle, or to glutamine, through glutamine synthase. Amino and amide groups from these two amino acids can be freely transferred to other carbon skeletons by transamination and transamidation reactions. These are key anapleurotic processes that can replenish the Krebs cycle and link amino acid and glucose metabolism, therefore have central importance in both energy production and biosynthesis.

Along with ureagenesis, the synthesis of glutamine from glutamate and ammonium ions via glutamine synthase is the most important mechanism by which mammals can detoxify ammonia. In the liver, glutamine synthetase is found in hepatocytes close to centrilobular vein, while urea synthesis enzymes and glutaminase are mostly localized in periportal hepatocytes. Thus, following the direction of blood flow, the two ammonia detoxification systems are structurally and functionally interlaced, so that perivenous glutamine synthetase serves as a high affinity scavenger of any ammonia escaped from periportal ureagenesis [116]. Glutamine synthesis is particular important during acidosis, when ureagenesis is inhibited to spare bicarbonate ions. The liver, in fact, may also play a role in the metabolic regulation of systemic pH, because hydrogen ions released from NH_4^+ during ureagenesis neutralize the excess bicarbonate produced by the breakdown of amino acids. The synthesis of glutamine from glutamate provides the most important nontoxic storage and transport form of ammonia. Glutamine is produced in high amounts by skeletal muscle during periods of negative energy balance as a consequence of intense amino acid catabolism. Glutamine is then transported to the kidneys where it is hydrolyzed to release ammonia and glutamate. Ammonia spontaneously ionizes to ammonium ion (NH_4^+) and is excreted in the urine. This process, along with detoxification of ammonia, serves as a regulation of acid-base homeostasis. For example, sheep increase potential for glucosamine production by skeletal muscle and for uptake of glucosamine by the kidney during acidosis [117]. The glutamate derived from glutamine degradation in the kidney releases one more ammonia in the urine and is converted to α-ketoglutarate, which can enter the Krebs cycle and produce glucose via oxaloacetate. In this way, although the liver remains the main site of gluconeogenesis, the kidney plays a significant role in glucose homeostasis, especially during fasting and periods of negative energy balance. Therefore, glutamic acid supplementation during the transition period may help restore many metabolic pathways, among which: liver ammonia detoxification, extra-hepatic ammonia detoxification, neoglucogenesis, and maintenance of acid-base equilibrium.

ASPARTIC ACID

Aspartate is an important amino acid and metabolite of intermediary metabolism, useful in maintaining liver function and metabolic efficiency in dairy cows during the peripartum. Aspartate can be reversibly converted to oxaloacetate and in this way replenish the Krebs cycle or enter gluconeogenesis:

- Aspartate + α-ketoglutarate ↔ oxaloacetate + glutamate

Aspartate is involved in transamination reactions, important for amino acid metabolism and ammonia transport and detoxification and it participates in ureagenesis, since waste ammonia can enter the urea cycle directly via this amino acid (Fig 6). Reduced levels of aspartic acid leads to an increase in serum ammonia levels [118]. In liver diseases, when activity

of major ammonia detoxifying enzymes for the production of urea and glutamine are impaired, aspartate has been used in conjunction with ornithine with beneficial effects [119, 120]. Aspartate increases glutamine synthase flux in the perivenous scavenger hepatocytes and supports the Krebs cycle [121], while ornithine improves the flux through the urea cycle enzymes, localized in the periportal hepatocytes [122]. In this way it is possible to improve liver capacity for ammonia detoxification even in conditions of impaired liver function. Aspartate is also a component of the malate-aspartate shuttle, which is the principal mechanism for the transport of reducing equivalents, in the form of NADH (nicotinamide adenine dinucleotide), across the impermeable inner mitochondrial membrane. The malate-aspartate shuttle transfers NADH from the cytosol into mitochondria, enabling oxidative phosphorylation and ATP production, and NAD^+ out of mitochondria into the cytosol, sustaining metabolism of glucose and lactate. Therefore, aspartate, by stimulating the Krebs cycle flux and regulating the redox state of the NAD couple, may increase the metabolic fitness of the cells and ameliorate metabolic diseases [123].

GLYCINE

Glycine is simple non-essential amino acid that plays crucial roles in numerous biological functions, as a contributor to the one-carbon pool, a component of glutathione, and a substrate in purine and protein synthesis. Glycine, besides its metabolic function, is one of the main inhibitory neurotransmitter and it also exerts important signaling roles outside the nervous system where, by binding to a specific receptor, it has anti-inflammatory, cytoprotective and immune-modulatory effects. The glycine receptor is a ligand-gated chloride channel present on a wide variety of cells. By binding to its receptor, glycine causes an intracellular influx of chloride and hyperpolarization of the cell membrane, which inhibits calcium fluxes and consequently leads to a decreased secretion of pro-inflammatory cytokines [124, 125]. Glycine has also direct cytoprotective effects through the blocking of aspecific membrane pore formation, which prevents plasma membrane leakage and cell death and probably through other unknown protective mechanisms. [125–127]. Oral or intravenous administration of glycine has been found to have many beneficial effects in a variety of diseases. Glycine has a significant protective effect in animal models of fatty liver, counteracting the development of steatosis, hepatic inflammation, and fibrosis [128]. In rats with cholestasis, glycine showed beneficial effects by lowering TNFα levels, liver enzymes and liver necrosis [129]. Glycine ameliorated liver injury induced by bile duct ligation, through attenuation of oxidative stress and apoptosis [130]. Glycine improved liver regeneration after partial hepatectomy [131]. Glycine also reduced hepatic damage and improved survival rate in animal models of endotoxic shock, by regulating the production of pro-inflammatory and anti-inflammatory cytokines in the liver and lungs. [132–134]. Therefore glycine is a relevant strategy to modulate many factors and processes implicated in the pathogenesis of periparturient disorders, including fatty infiltration of the liver, cytokine release and inflammation, oxidative stress, endotoxin-induced damage, and various kinds of liver injury.

FRUCTOSE AND SORBITOL

In the treatment of metabolic disorders it is usually useful to increase blood glucose levels in order to provide energy, reduce lipid mobilization from adipose tissue and decrease hepatic fatty acid uptake and ketogenesis. Intravenous glucose injection is a rapid and direct way of supplying blood glucose but the response is usually short and must be integrated by longer acting treatments. Metabolik provides two physiologically significant monosaccharides as a source of energy and glucose: fructose and sorbitol.

Fructose is rapidly converted to glucose by the liver and provides a quickly available source of energy [135]. In ruminants it also prompts a much more prolonged insulin response than that induced by glucose [136], offering a more sustained inhibitory effect on fatty acid mobilization.

Sorbitol conversion to glucose, on the contrary, does not occur instantaneously and hence it is able to provide a long-lasting source of energy without perturbing the regulatory mechanisms of glucose homeostasis. Sorbitol is also known to possess antiketogenic properties and it was found to decrease

ketogenesis in liver slices more efficiently than glucose, fructose and most other compounds [137, 138].

CONCLUSIONS

Metabolik is a multicomponent drug able to reverse major molecular mechanisms responsible for the metabolic disorders of dairy cows, including oxidative stress, mitochondrial dysfunction, cytokine overexpression, impaired insulin signaling, and ammonia toxicity. For this reason, Metabolik is indicated as a preventive or therapeutic tool in most disease that affect dairy cows during the transition from the dry period to peak lactation, and in all conditions of reduced performance or metabolic stress.

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Metabolik is marketed outside of Spain under the name of Metabolase. To consult the data sheet: https://cimavet.aemps.es.
To access the product information on the Fatro Ibérica website: https://fatroiberica.es/productos/metabolik/