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EVALUATION OF GLUCOSE TOLERANCE TEST AND SURROGATE INDEXES OF INSULIN SENSITIVITY TO DETECT INSULIN RESISTANCE SYNDROME IN TRANSITION DAIRY COWS

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Index

1. Abstract

Healthy multiparous Holstein-Fresian cows (n=101, parity ≥ 2) from 3 large-scale dairy herds in Italy were subjected to an intravenous glucose tolerance test (GTT) 14-1 d before (Week -1) and 3-9 d after calving (Week +1). A single blood collection was repeated 10-16 d after calving. Several plasma metabolites and insulin were determined at basal samples (T0); Revised Quantitative Insulin Sensitivity Check Index (RQUICKI) and Homeostasis Model Assessment (HOMA) for the estimation of peripheral insulin sensitivity were also calculated at T0. Insulin and glucose were also measured 10 (T10) and 80 (T80) minutes after glucose infusion. T80/T0 glucose ratio in Week -1 was used for cows' classification in two categories: GTT class 1 = cows with T80/T0 > 1.2; GTT class 0 = cows with T80/T0 ≤ 1.2. First milk test productions, diseases and mastitis cases were recorded. Cows in GTT class 0 and 1 in Week -1 differed both for glucose concentration at T0 and glucose at T80 ($P < 0.01$). Cows in GTT class 1 had a relative risk of 2.18 of having NEFA higher than 0.5 mEq/l pre-partum ($P < 0.05$). There was a significant herd effect over NEFA, Glucose, Insulin, Albumins, Urea, GOT-AST and GPT-ALT at T0 pre-partum ($P < 0.05$); post-partum herd effect was confirmed for Glucose and RQUICKI ($P < 0.05$). In the pre-partum weeks interestingly different levels of plasma insulin at T10 were evidenced within GTT class with lower hormone concentrations in class 1 ($P < 0.01$). Those cows also reported post-partum higher HOMA values ($P < 0.01$) and lower RQUICKI ($P < 0.05$), which reflect decreased insulin sensitivity. In conclusion, our study demonstrates a promising opportunity for application of surrogate indices of insulin sensitivity and GTT in field trials to detect insulin resistance syndrome in dairy cows in the future. The T80/T0 ratio derived by GTT seemed to be useful in detecting a low insulin secretion as a likely complication of altered glucose uptake following glucose load in transition cows.

2. Riassunto

101 bovine di razza Holstein-Fresian, pluripare (> 2 parti) e in buono stato di salute, selezionate all'interno di 3 grandi aziende di bovine da latte in Italia, sono state sottoposte al test da carico intravenoso del glucosio a 14-1 giorni prima (Settimana -1) e a 3-9 giorni (Settimana +1) dopo il parto. Un singolo campione di sangue è stato ripetuto a 10-16 giorni dopo il parto. Diversi metaboliti e la concentrazione di insulina sono stati determinati in corrispondenza dei campionamenti basali (T0); inoltre a T0 sono stati calcolati l'indice RQUICKI, Revised Quantitative Insulin Sensitivity Check Index, e HOMA, Homeostasis Model Assessment per la valutazione della sensibilità insulinica periferica. L'insulina e il glucosio sono stati anche misurati a 10 (T10) e 80 (T80) minuti dopo l'infusione del glucosio. Il rapporto tra le glicemie rilevate a T80 e T0 nella Settimana -1 è stato utilizzato per la classificazione degli animali in due categorie: la classe GTT 1 per gli animali con T80/T0 > 1.2; la classe GTT 0 per quelli con T80/T0 ≤ 1.2. Sono state inoltre raccolte le produzioni di latte al primo test, i casi di patologie e di mastiti. Le bovine in classe 0 e classe 1 per GTT differivano tra loro sia per la glicemia basale che per la glicemia a T80 ($P < 0.01$). La bovine in classe 1 di GTT avevano un rischio relativo pari a 2.18 di avere NEFA più elevati di 0.5 mEq/l pre-parto ($P < 0.05$). E' stato riscontrato un significativo effetto azienda su NEFA, Glucosio, Insulina, Albumine, Urea, GOT-AST e GPT-ALT a T0 pre-parto ($P < 0.05$); post-parto l'effetto azienda è stato confermato per Glucosio ed RQUICKI ($P < 0.05$). Nelle settimane pre-parto sono stati osservati livelli diversi di insulina plasmatica a T10, in particolare concentrazioni inferiori dell'ormone sono state messe in evidenza in classe 1 di GTT ($P < 0.01$). Le stesse vacche hanno successivamente riportato valori più elevati di HOMA ($P < 0.01$) e valori inferiori di RQUICKI ($P < 0.05$) post-parto, che riflettono una diminuita sensibilità insulinica. Per concludere, il nostro studio dimostra per il futuro una buona possibilità di applicazione degli indici surrogati di sensibilità insulinica e del GTT in prove di campo per individuare forme di insulino resistenza nella vacca da latte. Il rapporto T80/T0 ricavato dal GTT sembra utile nel rilevare una scarsa secrezione di insulina quale probabile complicazione dell'assorbimento del glucosio successivo alla sua somministrazione durante il GTT nelle bovine in transizione.

3. Introduction

The periparturient period is characterized by metabolic and endocrine changes resulting from negative energy balance and the insulin resistance phenomenon by extra-hepatic tissues (Sano et al., 1991; 1993), which helps to primarily direct the flow of nutrients to the fetus and mammary gland during last few weeks of gestation and first periods of subsequent lactation (Baird, 1981). Insulin resistance syndrome is also assumed as being a decisive factor involved in the pathogenesis of metabolic disorders of dairy cows such as ketosis and fatty liver (Hayirli, 2006).

3.1 Glycaemia: comparison between ruminants and non-ruminants

Glycaemia usually shows very constant and similar values across different animal species, with slight variations within a reference range of 70-100 mg/dl in humans and most domestic animals, if measured after few hours of fasting. After a prolonged fasting period the glycaemia is steadily preserved as well through a sensitive and complicated regulation mechanism. This regulation system is necessary because both hyperglycaemia and hypoglycaemia are noxious to the organism. Lasting high levels of glucose, as reported in diabetes, can cause multiple effects due to its osmotic pressure in the extracellular fluid, with cellular dehydration and loss of glucose in the urine accompanied by osmotic diuresis. In the diabetic patients persistently high glucose is also responsible for blood vessel wall damage, and consequently for a higher risk of heart failure, ictus, kidney diseases and blindness. Nevertheless animals can bear and put up with prolonged state of hyperglicaemia, the hypoglycaemia can be lethal with increasing seriousness and severe alterations when glucose gets down 50 mg/dl: headache, dizziness, shuddering, convulsions, loss of consciousness and death. All these consequences are caused by an insufficient supply of glucose to the brain that cannot loosely utilize alternative energetic sources to meet its requirements because of the presence of the haemato-encephalic barrier.

Unlike all the other species and young ruminants, postprandial plasma glucose concentrations range from 40 to 60 mg/dl in adult ruminants, under normal physiological conditions (Hsu and Crump, 1989). In ruminants, dietary carbohydrates and proteins are converted to volatile fatty acids (VFA) and some other biomolecules (e. g. ammonia and microbial protein) in the reticulo-rumen, by microbial degradation and fermentation. Only a small amount of glucose passes through the reticulo-rumen and is absorbed from the intestine, while the majority of glucose is provided via gluconeogenesis in the liver (Young, 1976). Hence, plasma glucose concentration in ruminants is lower than that in non-ruminants. Besides ruminants can tolerate larger fluctuations of glycaemia than non-ruminants and are less sensitive to the effects of hypoglycaemia. For example, glycaemia in sheep reared on pastures ranges between 30 and 60 mg/dl according to seasons. This may be explained by some important physiological

modifications of glucose requirements that occur throughout lactation and pregnancy without apparent disorders in ruminants. Typically these changeable situations may include alterations of the feed quality along the whole year on pastures, the fetus demand of energetic substrates during gestation, and the rapid onset of lactose and milk production after calving. Ruminants can face with these priorities because they use VFA (acetic, propionic and butyric acids), abundantly produced by ruminal fermentation, as alternative energetic sources. Among these VFA, only propionic acid has a strategic rule in gluconeogenesis. Since gluconeogenesis is supported by components that favor propionic production, the feed composition can indirectly influence the plasma glucose level (Wilson et al., 1983).

3.1.1 Regulation of glycaemia

The regulation of glucose occurs on multiple levels by cooperation of endocrine and nervous systems in order to arouse endocrine, neurological and behavioral responses that all together are able to preserve glycaemia from variations (Aguggini et al., 2000). The process of glucose homeostasis exhibits oscillatory behavior involving the following principal mechanisms:

1. Automatic response of the pancreatic islets which secrete glucagon and insulin under condition of hypo or hyperglycaemia, respectively. When insulin binds on the cellular insulin receptor, it leads to a cascade of cellular processes that promote the uptake of glucose from the blood stream, and its usage or, in some cases, its storage in the cell. Insulin is an anabolic hormone and acts to preserve nutrients. It has multiple roles in metabolism of carbohydrates, lipids and proteins. Glucagon is the most important counter regulatory mechanism to stop the activity of insulin beyond a certain limit.
2. Intervention of the central nervous system (CNS), which stimulates hunger and food research and intake, when the glucose reserves are depleting. On the contrary of all the remaining CNS, some hypothalamic neurons are sensitive to glucose concentration and require insulin to consume glucose. This fact explains the insatiable hunger state in diabetics suffering from hyperglycaemia but also deficiency of insulin or inefficiency of insulin action. On the other hand, insulin produces satiety only if glycaemia is maintained elevated, otherwise it itself causes hypoglycaemia and thus stimulates appetite. In part insulin secretion is controlled by vagus nerve. Either the view of food, food sensing by olfactory and taste receptors or conditioned stimuli associated with food determine an insulin release with reinforcement of hunger and food interest.
3. Catecholamine release from adrenal medullary in danger and emergency situation. Epinephrine is immediately released in large amount and rapid rush and has acute hyperglycaemic effects, including hepatic glycogen lisis and lipolisis in adipocites and muscle.
4. Adrenal glycocorticoids secretion in several stress situations as trauma, hemorrhagies, diseases, poisonings, anaphylaxis, emotional stresses and other general conditions with severe

homeostatic alterations. In these cases the hypothalamus secretes the CRF (Corticotropin Releasing Factor) which in turn stimulates the pituitary gland to release ACTH (Adrenocorticotropic Hormone) that promotes glycocorticoids synthesis and excretion in the blood. Similarly to epinephrine, their effects are hyperglycaemic because they suppress protein synthesis and foster hepatic gluconeogenesis in order to preserve the CNS functionality and be able to completely utilize and oxidize fatty acids.

5. Thyroid gland activity, which basically exerts a hyperglycaemic effect due to an increased protein and lipid catabolism, improved intestinal absorption, sympathetic tone and synergic action with catecholamine and food consumption.

6. Somatotropin hormone activity: it has hyperglycaemic effects and is an insulin antagonist too, because it depresses glucose uptake and consumption from tissues and preferably facilitates the use of adipose reserves to sustain energetic demand of protein synthesis, particularly during growth and development, gestation and lactation.

3.1.2 Endocrine Pancreas:

As an endocrine gland, the pancreas secretes peptide hormones, such as insulin from β cells, glucagon from α cells, somatostatin from δ cells, and pancreatic polypeptide from F cells (Hsu and Crump, 1989). These cells are located in the clusters known as islets of Langerhans diffused among exocrine parenchymal tissue and they constitute 1-3% of the total pancreatic mass with approximately 2500 cells in each islet and one million islets in humans. The proportions of β, α, δ, and F cells are about 60%, 30%, 8% and 2% respectively (Hsu and Crump, 1989; Hadley, 1996) and they strictly control each other hormones secretion within the islet.

3.1.3 Insulin: synthesis and structure

Insulin biosynthesis is a complex event that is characterized by formation of two insulin precursors: preproinsulin, which is composed of acidic (A,) and basic (B) chains and a peptide chain connecting the A and B chains (C peptide) in a single polypeptide of 97 amino acids, and proinsulin that is formed after cleavage of 23 residues of C peptide and formation of disulphide linkage between the A and B chains in the RER. After the proinsulin in transfered to the Golgi apparatus, C-pepetide is removed in the secretory granules and proinsulin and C-peptide are both stored in the cytosol (Hadley, 1996). They are released from granules at the same time and therefore C-peptide can be measured to monitor insulin endogenous production in patients subjected to insulin therapy (Swenson and Reece, 2002). Ultimately insulin consists of two peptide chains, A and B, composed of 21 and 30 amino acids respectively and linked by two disulphide bridges. There are only minor differences in chemical structures of insulin secreted by different mammalians, that don't influence its biological effects if administered in heterologous species but can cause antigenic responses after prolonged treatments (Ganong,

1991). For instance, threonine located in position 30 of the B chain in human insulin is replaced with alanine in bovine insulin (Hsu and Crump, 1989).

3.1.4 The process of insulin secretion:

Once insulin is synthesized, it is secreted in two different phases after glucose enters the β cells. As for the first phase, preformed insulin release is triggered rapidly within 3-5 minutes since sudden blood glucose increase. This initial hormone secretion can be tenfold but halves and last only for 5-10 minutes. The second phase is a slow release of newly formed vesicles that are triggered regardless of the glucose level (Guyton and Hall, 2000). It starts after 15 minutes and achieves a new plateau within 2-3 hours at a level that is usually higher than the initial phase.
Insulin release occurs by exocytosis. After an increase of glucose in the blood, glucose is captured by his specific transport proteins, the insulin-independent GLUT-2, and flows into the cell. Next, glucose metabolism via glycolysis generates ATP inside the cell. Elevated ATP/ADP ratio inhibits the ATP sensitive potassium ion channels on the β cells membrane, eventually leading to membranes depolarization and influx of extracellular Ca2+ ions through the voltage–gated Calcium channels. Thus there is a transient vesicles fusion to the cell membrane and their insulin content is excreted in the bloodstream (Kumar et al., 2005; Guyton and Hall, 2006).

3.1.5 Insulin receptors and tissues signal trasduction:

The insulin transduction pathway is an important biochemical pathway beginning at the cellular level affecting homeostasis. This insulin signal transduction pathway is composed of trigger mechanisms that serve as signals throughout the cell. The insulin receptor is a dimer of 2 α and 2 β subunits. The α subunit is located on surface of the cell and the insulin binds to it at the cell membrane, whereas the β subunit is located inside the cell and has a tyrosine kinase domain (Kahn, 1994). The α subunit acts as an enzyme that activates insulin functions and after insulin attachment causes autophosphorylation by ATP of the β subunit, which triggers tyrosine kinase activity in the cell. Thus, the hormone-receptor complex is internalized and intracellular secondary messengers are propagated in a chain reaction through enzymes phosphorylation and dephosphorylation cascades. These messengers are specific for the final biological effects of insulin, which for simplicity can be distinguished in mitogenic and metabolic functions. For instance, phosphorylation and activation of guanosine triphosphate (Ras complex) and Mitogen-activated protein kinase (MAPK) are responsible for expressing mitogenic functions like cell growth, proliferation and gene expression; phosphorylation of phosphatidylinositol-3-kinase (PI-3K) leads to crucial metabolic functions such as synthesis of lipids, proteins and glycogen. Most importantly, the PI-3K pathway leads to the distribution of glucose for important cell functions by binding to various glucose transporters (GLUT vesicles), and stimulates the translocation of vesicles containing GLUT 4 on cell membranes, thus increasing

the glucose entry rate inside cells (Kumar et al., 2006). Differences in possession of different type of GLUT implicate tissue dependency from insulin for uptake of glucose. For instance, GLUT 1 is predominant in brain, placenta, mammary gland and erythrocytes; GLUT 2 in the liver, kidney and pancreas; GLUT 3 in brain and placenta; GLUT 4 in adipose tissue and skeletal and heart muscle; and GLUT 5 in small intestine (DeFronzo et al., 1992). Among these, only GLUT 4 needs of insulin for uptake of glucose. Instead the liver and mammary gland are not insulin-sensitive organs. Therefore the effects of insulin are specific only for certain tissues: in particular insulin is very important in the uptake of glucose by muscle and adipose tissue (Zhou et al., 1999).

3.1.6 Stimulus for insulin release in non-ruminants:

There are several factors that can stimulate both insulin synthesis and release (Berne and Levy, 1993). In non ruminants the most important stimulus is glucose elevation in the blood, followed by other nutrients like sugars (galactose, mannose, glyceraldehydes), aminoacids (especially arginine, leucine, alanine, lysine) free long-chain fatty acids and minerals (potassium and calcium). Regarding aminoacids, they have a different effect than glucose and can hardly increase insulin production if administered in absence of hyperglycaemia. Anyway, aminoacids are very powerful agonist of glucose in inducing insulin release under hyperglycaemic conditions. Furthermore numerous hormones can promote insulin activity, like gastrointestinal hormones (gastrine, secretine, cholecystokinin, pancreatic polypeptide, gastric inhibitory peptide and glucagone), in addition to drugs and β-adrenergic and parasympathetic activity (vagal stimuli and acetylcholine).

On the other side, analogous but opposed factors can suppress insulin release: decreased glucose level in the blood, gastrointestinal hormones (galanin, somatostatin, pancreastatin), sympathetic and α-adrenergic stimuli (catecholamines) and other specific compounds (e.g. IL-1, PGF2-α). Even some psychic or sensory perceptions, like the view of food or its presence in the mouth, can anticipate and strengthen the last direct action of glucose on endocrine pancreas. These factors can either induce insulin release via vagal stimulation of hypothalamic hunger center (ventro-lateral hypothalamus) or suppress its release via sympathetic nervous system and ventro-medial hypothalamus centers (Aguggini et al., 2000). All the CNS neurons are permeable to glucose with no need for insulin mediation, except for the ventro-medial hypothalamus. Still later the presence of ingesta in the gastrointestinal tract, and their quality, will further promote insulin secretion through gastrointestinal hormones and vagal tone.

3.1.7 Stimulus for insulin release in ruminants:

Owing to distinct differences in nutrient metabolism between ruminants and non-ruminants, the magnitude of insulin secretion in response to nutrients varies greatly (Brockman and

Laarveld, 1986). It is well known that fatty acids (FA) with 3- to 8-carbon chains increase insulin secretion in ruminants (Horino et al., 1968). Moreover, valerate and butyrate were reported to be responsible for maximum elevation of plasma insulin and were more potent for insulin secretion than glucose in ruminants, but failed to stimulate insulin secretion in rabbits and pigs. However a certain haematic glucose concentration is necessary in ruminants to meet the basal requirements and thoroughly use fatty acids, preventing the formation of keton bodies. Given to low plasma glucose levels, ketonaemia is very frequent in ruminants. Thereby the insulin secretion is mostly regulated by haematic VFA and keton bodies because they are the most abundant energetic source and can easily enter the haemato-encephalic barrier and they can be utilized by brain (Aguggini et al., 2000). In conclusion, glucose has a secondary role in insulin control in ruminants.

3.1.8 The role of insulin in glucose metabolism:

Insulin forces entry of glucose into cells and its storage in the form of glycogen, most prominently in muscle and adipose tissue, via modification of action of numerous enzymes. Inward movement of glucose is speeded up by two mechanisms: an increased rate of exocytosis and decreased endocytosis of GLUT 2 in hepatocytes, and stimulation of glucokinase activity, which phosphorylates glucose to glucose 6-phosphate (G6P) (Katzung, 1995). The rapid glucose phosphorylation tends to keep a low intracellular glucose concentration. Thus, the gradient of glucose is normally directed inward but, again, insulin can greatly speed up this process. In the same way, insulin facilitates entry of glucose into adipose tissue through GLUT 4, where glucose is then oxidized for esterification of free FA during lipogenesis. This phenomenon is highly specific and mostly limited to glucose and other few similar sugars, which can compete for their transportation (Ganong, 1991). At the same time both in the liver and muscle, insulin can stimulate glycogenesis and glycolysis by following similar pathways except for uptake of glucose. The liver utilizes GLUT 2, whereas muscle utilizes GLUT 4. Glycolisis is stimulated by insulin through activation of phosphofructokinase and pyruvate kinase, which direct the flow of glucose towards pyruvate and lactate (Berne and Levy, 1993). Glycogen synthesis is mediated by the action of glycogen synthase. In ruminants, insulin also stimulates glycogen synthase, but glucokinase activity is little or absent in the liver (Brockman and Laarveld, 1986). Instead, hexokinase is involved in the uptake of glucose in the ruminant liver (Brockman, 1984). However, hexokinase has a lower affinity for glucose compared with glucokinase (Berne and Levy, 1993). For this reason, the ruminant liver normally takes up only small amounts of glucose. Insulin then suppresses glucose production from non-sugar substrates by inhibiting key enzymes (e.g. pyruvate carboxylase and phosphoenolpyruvate carboxykinase) for gluconeogenesis (O'Brien and Granner, 1990) and glycogen phosphorylase for glycogenolysis.

3.1.9 The role of insulin in lipid metabolism:

In adipose tissue and muscle, insulin enhances FA esterification and triglyceride (TG) synthesis by providing FA substrates inside cells. In the small vessels of the adipose tissue, insulin stimulates lipoprotein-lipases (LPL) and TG division and lets free FA enter into adipocytes. The free FA is re-esterified with glycerophosphate, derived either from glycolysis, glycerol or other FA. Precursors for lipogenesis are different between ruminants and non ruminants: glucose is the major precursor for lipogenesis in adipose tissue of non-ruminants, whereas acetate is the major precursor in ruminants (Prior and Scott, 1980). Also in the liver, insulin stimulates lipogenesis and inhibits ketogenesis (Brockman, 1978, 1979). Further, NEFA mobilized from adipose tissue is the primary source of hepatic lipogenesis in ruminants (Emery et al., 1992). Nevertheless, unlike in non-ruminants, the ruminant liver is not a primary organ for lipogenesis (Ingle et al., 1972). In any case, oxidation of glucose is indispensable to generate $NADPH_2$ and α-glycerophosphate for FA synthesis from lactate and acetate in ruminants (Prior and Scott, 1980). The effect of insulin is decisive on carboxylation of mitochondrial acetyl-CoA generated by glycolysis in non-ruminants or directly derived from acetate in ruminants. Thanks to insulin intervention, acetyl-CoA is then converted into malonyl-CoA by acetil-CoA carboxylase (Brockman, 1978, 1979). Malonyl-coA represents the primary step in FA formation. Additionally, insulin can inhibit lipolysis by slowing down the activity of lipase and protein kinase A and lowering the level of cAMP. Moreover, insulin control of lipogenesis and lypolysis in adipose tissue guarantees lower circulating non-esterified fatty acids (NEFA), reduces their uptake from the liver and improve peripheral tissue ketone utilization. In summary, it is clear that insulin mainly favorites the utilization of carbohydrates as energetic font. Instead, it depresses fat usage by tissues, except for CNS.

3.1.10 The role of insulin in the protein and mineral metabolism:

Insulin intervenes on protein metabolism through activation of amino acids uptake inside the cell, decreased proteolysis and breakdown of proteins inside the lysosomes, increase of special DNA sequences transcription, mRNA translation and protein synthesis. The enzymes responsible for carbohydrates, lipids and proteins storage are the most induced by insulin activities. Owing to her anabolic influence, insulin is even concerned in the growth process and cooperates with other anabolic hormones like somatotropine (growth hormone, GH) and androgens in a different but synergic manner (Guyton and Hall, 2000). For instance, each hormone has a stronger capacity for the regulation of certain amino acids uptake by cells. In the case of insulin, it promotes valine, leucine, thysorine and phenylalanine transfer.

As to mineral metabolism, insulin facilitates the entry of potassium ions into the muscle and miocardic cells because it enhances the activity of the Na^+-K^+ ATPase pump in the cell membranes. The consequence is a decrease of potassium in the extracellular fluid and cells

iperpolarization. On the contrary, potassium ions depletion, like in patients affected by primary iperaldosteronism, tends to reduce insulin secretion and develop diabetic symptoms.

3.2 Insulin resistance phenomenon:

Insulin resistance is a generic term which describes "the state where a physiological level of insulin produces a less than normal biological response" (Kahn, 1978). This may be due to defects located either at the pre-receptor level, or at the receptor and post-receptor levels. Actually insulin resistance can be sketchily defined as insulin responsiveness or insulin sensitivity. The former is evaluated as the response of insulin to glucose, the latter as tissue responsiveness to insulin. First, pre-receptor level defects include decreased insulin production, increased insulin degradation, or both. Secondary, the molecular mechanisms in defects at the receptor level include decreased number of receptors and decreased binding affinity. Ultimately, in post-receptor defects the intracellular signaling steps of insulin action and translocation of GLUT may be impaired. Kahn (1978) has stated that, in general, hypoinsulinaemia is a common feature in pre-receptor defects; reduced insulin responsiveness is typical of receptor level defects; and reduced insulin sensitivity is linked to post-receptor defects. The consequence of these alterations is a multi-factorial complex of symptoms, such as hyperglicaemia, ketonaemia and ketosis, metabolic acidosis, glucosuria, diuresis, dehydration, exc., (McCance and Huether, 1994) that is common to many physio-pathological events in humans and all domestic animals, included ruminants. In humans, these disorders are well characterized into different types of diabetes. In ruminants, they are also seen in ketosis syndrome and haepatic lipidosis during particular stages of productive life (Drackley et al., 1992). In all cases, this syndrome recognizes similar aetiological and pathogenetical factors (Hayirli, 2006).

3.2.1 Risk factors of insulin resistance syndrome:

Hereafter, I will focus on description of disorders and factors that are relevant for ruminant animals, and particularly in high producing dairy cows.

<u>Gestation:</u>

Insulin resistance and a decrease in peripheral tissues sensitivity to insulin are commonly observed during late gestation (Hay et al., 1988; Petterson *et al.*, 1994). In this phase, fetal glucose uptake is approximately 50% of glucose production in ewes (Prior and Christenson, 1978) and insulin-mediated uptake of glucose by skeletal muscle and adipose tissues and inhibition of lipolysis are decreased (Schlumbohm et al., 1997). Uptake of glucose and numbers of GLUT 4 in heart muscle and white and brown adipose tissues were lower in pregnant rats compared to non-pregnant rats (Nieuwenhuizen *et al.*, 1998). The glucose transfer from placenta to fetus is dependent on the difference of concentration of this molecule between maternal and fetal plasma (Simmons et al., 1979) and is mediated by specific transport proteins GLUT-1 and GLUT-3. The number of these carriers increases along the advance of pregnancy in

order to enhance the transfer of glucose across placenta (Hay, 1995). Reduced insulin sensitivity by peripheral tissues during late pregnancy assures adequate transfer of glucose from dam to fetus as an insulin-independent process. This mechanism is affected by increased serum concentrations of the hormones estradiol, progesterone and prolactin during late gestation. It appears that oestrogen enhances the action of insulin during non-pregnancy and lactation and progesterone suppresses insulin actions during late pregnancy. This was suggested by Ryan and Enns investigation on the effects of these hormones on insulin action in isolated cells from adipose tissue of pregnant, non-pregnant, and virgin rats (1988). Addition of estradiol to culture medium increased maximum insulin binding; addition of progesterone decreased glucose transport and maximum insulin binding; and addition of prolactin and placental lactogen decreased glucose transport without changing maximum insulin binding. Besides, maternal insulin concentration tends to decrease towards the end of gestation and is inversely proportional to placental lactogen levels in ewes (Blom et al., 1976; Vernon et al., 1981). Otherwise, the insulin response to a glucose load is significantly lowered in these pregnant animals (Van der Walt et al., 1980). Lomax and colleagues hypothesized that the decline in insulinemia in the last third of gestation in ruminants might be linked to a reduced capability of pancreas to react to insulinotropic agents like glucose (1979).

Nutrition - energy density of diet:

Malnutrition and feed restriction reduces the glucoregulatory actions of insulin. Feed restriction results in hypoinsulinaemia in donkeys (Forhead and Dobson, 1997) and a decrease in islet numbers and islet size, which may cause lower insulin secretion (Tse *et al.*, 1998). Further, an experiment conducted in rats by Reis and colleagues (1997), reported that basal serum insulin and plasma glucose concentrations and glucose clearance rate were lower in malnourished rats than in well-nourished rats during the oral GTT. Moreover, insulin secretory response to glucose addition to cell media containing pancreas islets isolated from malnourished rats was lower compared to those isolated from well-nourished rats. Similarly, in dairy cattle, decreased plasma insulin prior to parturition and in the first weeks after calving may reflect regression of the pancreas as a result of depression in DMI. Inadequate feeding during the dry period can cause a variety of problems in the postparturient cow, including susceptibility to infections, infertility, and adverse effects on milk production. A common recommendation is that the dairy producer should try to maximize DMI intake in close-up dry cows, to prepare the cow for a higher feed intake immediately after calving and, in turn, reduce metabolic disorders (Grummer, 1995).

In a study of 2003, Holtenius and colleagues reported that plasma glucose disappearance rate, and thereby the effectiveness and concentration of insulin, was related to feeding regimen offered during the prefresh transition period of cows fed to consume 6, 9 and 14.5 kg DM,

providing 71 (Low energy diet, L), 106 (Medium energy diet, M), or 177 MJ (High energy diet, H) of metabolizable energy (ME) per day. The diets provided in average 75, 110, and 178% of the energy requirements for maintenance and pregnancy according to the Swedish feeding recommendations (Spörndly, 1999). These diets were introduced when the cows were dried off from the previous lactation, at least 8 wk before expected parturition. After parturition all cows were fed another total mixed ration ad libitum. They found a markedly higher plasma insulin concentration and a lower glucose clearance rate in prepartum H cows, compared to M and L cows. They suggested that this could reflect a prepartum positive energy balance (EB) but also a greater insulin resistance. Also, there were no differences in DMI in early lactation, but during wk 6 to 12 postpartum DMI was lower among H cows, which was linked to a prolonged negative energy balance in this group. At the same time body weight loss was greatest in these cows and mainly occurred in lactation weeks 1 to 4, whereas the milk yield did not differ between treatments (Agenäs et al., 2003).

However, the magnitude and duration of malnutrition required to develop metabolic disorders in dairy cows are largely unknown. Relatively few studies have evaluated the effects of restricted feeding during the dry period on the health and metabolism of cattle. Cows subjected either to 30% feed restriction or reduction in DMI as they approach parturition are known to develop postpartum hepatic disturbances (Veenhuizen *et al.*, 1991; Hayirli and Grummer, 2004). In contrast, Holcomb et al. (2001) found no negative effects of restricted feeding except lower milk fat percentage in early lactation. Others believe that the low energy density of diets during the transition period is associated with improved DMI and energy balance (Douglas et al., 1998; Rabelo et al., 2003). On the other hand, overfeeding during the dry period might lead to appetite depression and an increased rate of health disorders and poor milk production (Rukkwamsuk et al., 1999).

Most researchers have indicated that higher energy concentration of the diet precalving could improve voluntary intake, increase BW gain (VandeHaar et al., 1999; Dewhurst et al., 2000), and reduce the mobilization of adipose tissue and plasma NEFA concentrations (Ingvartsen and Andersen, 2000; Hayirli et al., 2002), which would improve the maternal regulation of physiological responses and enhance productive performance during the transition period (Contreras et al., 2004). Very recently (2012), Gao and colleagues ascertained that feeding lower energy diets to cows during the last three weeks prepartum had several significant effects both on the cow and her calf. They assigned 30 Holstein dairy cows to one of three diets during the last 21 days prior to calving. The diets consisted of a low energy group (net energy of lactation (NEL) = 5.25 MJ/kg of DM); medium energy group (NEL = 5.88 MJ/kg of DM); and high energy group (NEL = 6.48 MJ/kg of DM). Unfortunately, the authors did not report how much each cow was fed and estimate their energy intake. Cows fed the low energy diet had a large increase in plasma NEFA concentration from 21 to 7 days prepartum and their calf health was

profoundly affected. Their results suggest that maternal energy density during the last 21 d prepartum negatively affected growth, development, immunity, and antioxidant capability of neonatal calves and the energy balance of the mothers in the immediate postpartum period.

Nutrition - fat feeding (hyper-insulinemia):

During high-fat feeding lipid availability in muscle and liver and oxidation of fat are elevated but incomplete. Thus, fat feeding is accompanied by increased plasma NEFA and ketons concentration. Ketogenic diets also cause acute hyper-insulinaemia and reduced insulin sensitivity. In general, hyper-insulinaemia downregulates insulin actions and signal trasduction at the receptor and postreceptor levels (Berne and Levy, 1993; Sebokova et al., 1995). In fact, hyper-insulinaemia jeopardizes the ability of insulin to suppress hepatic glucose production in the liver (Oakes et al., 1997) and impair glucose uptake by peripheral tissues. Glucose uptake is limited by alterations of cell membrane fluidity, reduced number of GLUT 4 and decreased glucokinase activity in the liver and muscle. These mechanisms were confirmed in rats fed increasing dietary fat in vivo and in vitro experiments (Watari et al., 1988; Ruth and Kor, 1992; Ruth, 1992). High-fat diets were associated to important reduction in insulin binding. Insulin anti-lipolitic effect is also reduced and FA is released from adipose tissue (Sparks and Sparks, 1995). The increased NEFA concentration in lipid induced insulin resistance was demonstrated in humans by Laville and colleagues (1995): insulin was not able to reduce plasma NEFA and lipid oxidation, following intravenous infusions of labeled glucose and palmitate compared to a control group. Furthermore, adverse effects of supplemental fat (more than 4%) on DMI of lactating dairy cattle are well documented. Mechanisms by which high level of supplemental fat adversely affect DMI include interference with acceptability of diet, reduced gut motility, decreased fermentation and degradation of fiber, and alteration of hormonal status (Devendra and Lewis, 1974; Palmquist and Jenkins, 1980; Allen, 2000). In 2002, Hayirli demonstrated a liner decrease in DMI in response to increasing level of dietary EE concentration and during the prefresh transition period. The extent of feed and energy intake depression is dependent upon fat type (Allen, 2000). Fat sources with more unsaturated fatty acids reduce intake to the greatest extent and fatty acids that are highly saturated have less effect.

Obesity and fat mobilization:

A number of studies reveal that obesity is associated with increased likelihood of metabolic disturbances. Hyper-insulinaemia (McCann et al., 1986) and insulin resistance (Mahler, 1981; McCann and Reimers, 1985; Bergman et al., 1998) are common metabolic signs of obesity in non-ruminants and ruminants. Unlike obese non-ruminant species, obese ruminants have poor appetites (Garnsworthy and Topps 1982; Treacher *et al.*, 1986; Hayirli *et al.*, 2002b). Therefore, obesity in non-ruminants is associated with hyper-glycaemia and hyper-insulinaemia, whereas

obesity in ruminants is associated with hypo-glycaemia and hypo-insulinaemia. Feeding behavior has been linked to ATP concentration within cells in the liver with satiety occurring as fuels are oxidized and ATP is produced, and hunger occurring as oxidation decreases and ATP is depleted (Allen, 2000). According to the Hepatic Oxidation Theory (HOT), formulated by Allen and colleagues (2000; 2005), it is important to realize that "the pattern of oxidation of fuels (minute to minute) is what affects feeding behavior because the amount of oxidation over longer periods of time (hours or days) is relatively constant". Because fatty acids are readily oxidized in the liver, the supply of NEFA from mobilization of body fat reserves likely suppresses feed intake in the transition period. As a consequence of obesity, the degree of fat mobilization is dependent upon the amount of fat reserves available for mobilization as well as changes in insulin concentration, tissue sensitivity to insulin, and stress. Cows with excessive body condition generally mobilize fat very rapidly through transition because their tissues are more insulin resistant and they have greater fat stores to mobilize. Recent research indicates that allowing cows to consume more energy than required during the far-off dry period results in increased NEFA concentrations in early lactation (Holtenius et al., 2003). Hormones released during stress increase fat mobilization, elevating plasma NEFA concentration further. Free FA competes with glucose for utilization by insulin-sensitive tissues (Boden, 1977; Koopmans *et al.*, 1996) and has toxic effects on peripheral tissues (Spector and Fletcher, 1978). Noshiro and colleagues (1997) tested insulin action on insulin-sensitive lean rats and insulin-resistant obese Zucker rats. The adverse effect of NEFA concentration was more pronounced in obese rats than in lean rats. The adverse effect of elevated NEFA concentration on adipose tissue insulin sensitivity was also demonstrated (VanEpps-Fung and colleagues, 1997; Bergman and Mittelman, 1998). In summary, elevated NEFA concentration causes inhibition of insulin-stimulated glucose uptake by peripheral tissues, decreases the number and translocation of GLUT 4, and disturbs intracellular insulin signalling pathways in the liver and peripheral tissues with an increased risk for receptor downregulation (Garvey *et al.*, 1986).

Genetics:

Until recently, general dairy cow breeding policy has resulted in a cow that produces more milk and has a greater propensity to mobilize body fat in early lactation (Buckley et al., 2000; Roche et al., 2006; McCarthy et al., 2007). Intensively selected cows in early lactation experience a subsequent failure to replenish body energy stores. This challenge has also been associated with reduced reproductive performance (Beam and Butler, 1999; Buckley et al., 2003; Roche et al., 2007). Low insulin levels are associated genotypically (Gutierrez et al., 2006) and phenotypically (Ingvartsen and Friggens, 2005) with high milk yield. Although insulin has no direct effects on galactopoiesis, low insulin decreases the uptake of glucose by insulin-sensitive tissues, such as skeletal muscles, and enhances glucose availability for the mammary gland,

which is insulin-insensitive (Zhao et al., 1996; Nishimoto et al., 2006; van Knegsel et al., 2007). Therefore, insulin is a very interesting candidate molecule concerning the paradox between yield, metabolic diseases, and reproductive performance. There is further evidence of a tendency for higher body weight in cows with genetic merit for low milk fat content compared to high fat content (Agenäs et al., 2003). Cows of North American (NA) and New Zealand (NZ) origin represent 2 strains of high and low genetic merit for milk production (Roche et al., 2006). NA cows produce more milk, have a greater propensity to mobilize body condition in early lactation and partition less energy to improving body condition in mid and late lactation (Horan et al., 2005; Roche et al., 2006; Mc-Carthy et al., 2007; Macdonald et al., 2008). These strain differences should be due, at least in part, to an altered response of tissues to insulin and a resultant increased nutrient supply, particularly of glucose and fatty acids, to the mammary gland to support milk production. Cows of NZ origin fed a TMR have a greater insulin response to a glucose challenge than either NZ cows grazing pasture or NA cows fed TMR or pasture (Chagas et al., 2003), indicating a potential interaction between strain of Holstein-Friesian (HF) cow and diet on insulin resistance in early lactation. These data are consistent with the hypothesis that insulin dynamics may be involved in the effect of genetic strain, nutrition, or both on nutrient partitioning (Chagas et al., 2007a). In 2009, Chagas and colleagues confirmed that differences in milk production between NA and NZ cows in early lactation can be explained by a greater glucose fractional turnover rate in NZ cows compared with those of NA origin. In 2009, further data by Kay and colleagues from New Zeland supported the hypothesis for potential strain differences in recoupling of the somatotropic axis, insulin resistance, and energy partitioning, and may help explain the physiology behind the previously reported greater milk production and body condition score loss in NA HF. They offered 0, 3, or 6 kg of concentrate DM/cow per day for an extended lactation to fifty-six genetically divergent NZ and NA HF cows grazing pasture (605 ± 8.3 d in milk; mean ± standard error of the mean). During early lactation, NEFA and GH concentrations were greater and IGF-I concentrations were less, and increased at a slower rate in NA HF. During the extended lactation period, NA HF had greater NEFA and GH concentrations; there were strain × diet interactions for insulin and leptin, and a tendency for a strain × diet interaction for glucose. These interactions were primarily due to greater plasma insulin, leptin, and glucose concentrations in the NZ HF fed 6 kg of concentrate DM/cow per day, a result of excessive body condition in this treatment. In a similar study conducted in New Zeland (White et al., 2012), expression of pyruvate carboxylase (PC) mRNA in liver biopsies from 27 NZ and 27 NA HF cows was monitored at 0, +1, and +4 wk relative to calving. Pyruvate carboxylase is a rate-limiting enzyme for hepatic gluconeogenesis. The responses of NZ and NA cows to the transition to lactation and concentrate supplementation appeared to be similar; however, NZ cattle had a higher basal expression of PC. In the same recent years, McCarthy et al. (2009) compared the hepatic expression of genes

of the growth hormone (GH)-IGF (or somatotropic) axis in the NA HF and the NZ HF strains of dairy cow at early and mid lactation. They found that early-lactation adaptations to negative energy balance may have more severe effects in the NA strain compared with the NZ strain because the NZ strain had greater expression of IGF-1. In the immediate postpartum period, the somatotropic axis in the liver becomes uncoupled, whereby elevated plasma GH concentrations fail to stimulate an increase in hepatic IGF-1 synthesis (Thissen et al., 1994; Fenwick et al., 2008; Lucy, 2008). IGF-1 plays a critical role in stimulating the anabolic and mitogenic activity of GH in various tissues (Laron, 2001). Numerous reports have suggested that nutritionally compromised cows have reduced systemic concentrations of insulin and IGF-1 (Patton et al., 2006; Lucy, 2008). It has been reported that irreversible glucose loss leads to some degree of uncoupling of the somatotropic axis as plasma concentrations of IGF-1 decline concomitantly with a reduction in hepatic mRNA abundance of IGF-1 and GH receptor (GHR; Meier et al., 2008). Previous studies have demonstrated that systemic concentrations of the metabolic hormone IGF-1 in early lactation are positively associated with the subsequent calving-to-service interval and ultimately the pregnancy outcome in dairy cattle (Taylor et al., 2004; Patton et al., 2007; Wathes et al., 2007). McCarthy's results were consistent with other recent authors' findings of greater IGF-1 plasma concentrations in NZ HF during the post-transition period (30 to 90 d post-calving; Patton et al., 2008).

3.3 Physiological metabolic modifications in the transition dairy cow:

The transition from late gestation to early lactation is a period of dramatic physiological and metabolic adaptation for the dairy cow. During late gestation the nutritional demands of the fetus and uterus increase exponentially, while intake is often reduced by the endocrine changes that induce parturition and parturition itself. Other several factors which can impact on intake have been investigated and discussed for many decades (Ingvarsten and Andersen, 2000). Factors affecting and regulating DMI of lactating dairy cattle are numerous, complex, and span from cellular to environmental conditions (Forbes, 1996; Roseler et al., 1997a, b; Allen, 2000). These factors can be categorized broadly as animal factors (i.e., age, body condition, breed, physiological stage, and milk yield level), dietary factors (i.e., ingredient and nutrient compositions of diets and physical and agronomic characteristics of feed stuffs), managerial factors (i.e., production, feeding, and housing systems), and climatic factors (i.e., temperature, humidity, and wind) (NRC 1987). Factors affecting DMI and depression in DMI during the prefresh transition period are largely unknown. It is possible that factors affecting DMI in lactating dairy cattle and other ruminants also influence DMI in prefresh transition dairy cattle (Hayirli et al. 2002).

After calving, increases in nutrient requirements for milk synthesis outpace increases in intake (Bell, 1995). For the dairy cow, the most severe nutritional imbalances typically occur during transition (Grummer, 1995). Development of negative energy balance prior to parturition and its continuation through early lactation are due to significant DMI depression (Bertics et al., 1992; Hayirli et al., 2002b) and the lag time between peaks of intake and milk yield, respectively (Baird, 1981; Grummer, 1995). Dairy cattle loose body weight (BW) and body condition score (BCS) to compensate for the energy deficit (Coppock, 1985). Lactogenesis is accompanied by alterations of metabolism, which include increased lipolysis and decreased lipogenesis in adipose tissue, decreased glycogenesis and increased gluconeogenesis and glycogenolysis in the liver, decreased use of glucose and increased use of lipid as energy sources by body tissues, and increased mobilization of protein reserves from muscle tissue (Bauman and Currie, 1980; Collier *et al.*, 1984; Reynolds *et al.*, 2003). Several euglycemic clamp studies (Debrass et al., 1989; Prior and Christenson, 1978; Sano et al., 1991) ascertained that insulin resistance begins before parturition and continues during early lactation. Thus, during the periparturient period, insulin resistance may be an important factor in the initiation of catabolic activities (Holstenius, 1993). Milk synthesis is drawn by glucose availability for lactose production. For a cow producing 35 kg of milk per day with 4.9% lactose, about 2.9 kg glucose is used for lactose secretion in the mammary gland, of which 2.7 kg is provided via gluconeogenesis in the liver (Young, 1976). Propionate accounts for most of glucose released from the liver (about 70%) during the final two weeks of gestation (Reynolds et al., 2003).

Although propionate is very rapidly taken up by the liver, when it is absorbed faster than it can be utilized to produce glucose, it will likely be oxidized, generating ATP and a satiety signal to the brain. The capacity of the liver to produce glucose is affected by glucose demand because limiting enzymes in the liver are up-regulated to meet the need. Because of this, propionate is less likely to be oxidized (and decrease feed intake) at peak lactation when glucose demand is high, than in late lactation when glucose demand is lower. Although propionate might be expected to have little effect on feed intake of fresh cows because they have high glucose demand, decreasing oxidation of propionate *per se*, propionate also stimulates oxidation of acetyl CoA. Fresh cows have a large supply of acetyl CoA in the liver from partial oxidation of NEFA. Some acetyl CoA is exported as ketones, but it is also readily oxidized when propionate is taken up by the liver, quickly generating ATP and a satiety signal (Allen and Bradford, 2006). This is an apparent contradiction: propionate is a primary fuel used to produce glucose, which is needed to increase insulin and decrease NEFA, thereby alleviating the depression in feed intake by NEFA oxidation in fresh cows, but propionate itself suppresses feed intake by stimulating oxidation of acetyl CoA in fresh cows. That is, the metabolic profile during early lactation includes low concentrations of serum insulin, plasma glucose and liver glycogen and high concentrations of serum glucagon, adrenaline and GH, plasma β-hydroxybutyrate (BHB) and NEFA, and liver triglycerides (Herbein *et al.*, 1985; Vazquez-Anon *et al.*, 1994). These changes predispose dairy cows to hepatic lipidosis and ketosis. This metabolic pattern is also reported in cases of induced or spontaneous hepatic lipidosis and ketosis (DeBoer *et al.*, 1985; Veenhuizen *et al.*, 1991; Drackley *et al.*, 1992). Lower mitochondrial glycerol-phosphate acyltransferase activity (Rukkwamsuk *et al.*, 1998, 1999b) diverts FA from esterification into β-oxidation in order to protect the hepatocytes against further accumulation of TG during NEB (Bruss, 1993). Because liver contributes a small fraction of total body fat synthesis (Ingle *et al.*, 1972), FA mobilized from adipose tissue is the primary sources of hepatic TG in ruminants (Emery *et al.*, 1992; Bruss, 1993; Grummer, 1993). NEFA are either directly transported to mammary gland (Annison *et al.*, 1967) or are taken up by the liver in relation to their concentration in plasma (Heimberg and Wilcox, 1972). In the liver, FA is re-esterified to TG that can be stored or exported as VLDL, or FA is oxidized either completely to carbon dioxide in tricarboxylic acid (TCA) cycle or incompletely to ketone bodies. When the export of TG as VLDL from the liver cannot keep pace with increased NEFA uptake and TG synthesis by the liver, hepatic lipidosis becomes significant (Grummer, 1993).

3.4 Post-partum metabolic disorders related to energetic metabolism:

Hepatic lipidosis and ketosis occur as a result of inability to keep pace with homeorhetic changes during the periparturient period, as previously described, and are common lipid-related metabolic disorders in the fresh high producing dairy cow (Herdt *et al*., 1983, 1988; DeBoer *et al*., 1985; Veenhuizen *et al*., 1991; Drackley *et al*., 1992; Grummer, 1993; Vazquez-Anon *et al*., 1994). The aetiologies of hepatic lipidosis and ketosis are similar and liver function is impaired in both cases (Strang *et al.*, 1998; Zhu *et al.*, 2000).

Hepatic lipidosis refers to accumulation of lipids in hepatocytes (Pearson and Maas, 1990). Many cows experience hepatic lipidosis and ketosis of varying degrees of severity during the periparturient period (Grummer, 1993). The severity of hepatic lipidosis is related to the degree of mobilization of adipose tissue fat reserves (Roberts *et al.*, 1981). Hepatic lipidosis may compromise production (Gerloff *et al.*, 1986a), immune function (Ropstad *et al.*, 1989; Kaneene *et al.*, 1997), and fertility (Reid *et al.*, 1979a,b) and increases the likelihood of ketosis (Drackley *et al.*, 1992). Major factors causing hepatic lipidosis include increased supply of long-chain FA by adipose tissues and diet, impairment of TG incorporation into VLDL, and defects in VLDL transport (Gruffat *et al.*, 1996).

Ketosis occurs after hepatic lipidosis in high-producing dairy cows (Reid, 1980) and is characterized by hypo-phagia, decreased milk production, increased BW and BCS loss, lethargy or hyper-excitability, hypo-glycaemia, hypo-insulinaemia, hype-rketonaemia, hyper-lipidaemia, and depleted hepatic glycogen (Veenhuizen *et al.*, 1991; Drackley *et al.*, 1992). Moreover, liver TG concentration is negatively correlated with plasma glucose and serum insulin concentrations and positively correlated with plasma NEFA and BHBA concentrations (Studer *et al.*, 1993). Brockman (1979) summarized direct and indirect antiketogenic effects of insulin, which include decreasing liver NEFA uptake through stimulating lipogenesis and inhibiting lipolysis in adipose tissue; enhancing peripheral tissue ketone utilization; and altering enzyme activities and availability of substrates that are involved in ketogenesis in the liver. The impact of insulin on enhancing utilization of BHB and acetoacetate by extra hepatic tissues was demonstrated in normal and alloxan induced diabetic sheep (Jarret et al. 1976). Insulin decreases activity of carnitine palmitoyl transferase-I (CPT-I) and increases affinity of CPT-I for malonyl-CoA (Grantham and Zammit 1988). Moreover, insulin inhibitory effect on ketogenesis is also related to its stimulatory effect on activity of acetyl-CoA carboxylase and formation of malonyl-CoA that inhibits activity of CPT I (Zammit 1981, 1990, 1996).

3.4.1 Relationship between insulin resistance and immunosuppression:

Metabolic disorders (milk fever, displaced abomasum, ketosis, and fatty liver syndrome) and mammary gland (mastitis and mammary gland edema) and reproductive diseases (veterinary assisted dystocia, retained placenta, and metritis) seemingly occur as a complex during the periparturient period (Curtis et al., 1985; Correa et al., 1990; Erb and Grohn, 1998). Moreover, these health problems compromise lifetime milk yield and reproductive efficiency (Erb et al., 1985; Deluyker et al., 1991; Rajala-Schultz et al., 1999). Elevated plasma ketones (greater than > 20 mg BHB per deciliter) are a key element in the development of most disorders, other than the sole ketosis, because they compromise immune potency through suppressing mitogenic response of lymphocytes (Targowski et al., 1985; Franklin et al., 1991; Sato et al., 1994). There are also indications that reduced insulin sensitivity and low insulin result in lipolysis and elevated peripheral concentrations of NEFA during last weeks before calving, which, in turn, increase the risk of production diseases such as ketosis, retained fetal membranes (Dyk et al., 1995), abomasal dislocation (Holtenius et al., 2000; Doll et al., 2009), and hepato-lipidosis and have a detrimental effect on granulosa cell function (Vanholder et al., 2005). Furthermore, various studies provide indications that inflammatory processes contribute to lipolysis, ketogenesis and hepatic disorders in dairy cows (Bionaz et al. 2007; Bradford et al. 2009, Hiss et al. 2009). It is also known that inflammatory diseases such as metritis and mastitis and inflammatory mechanism associated with subacute ruminal acidosis (Plaizier et al. 2008), very frequent in the fresh cow, possibly affects insulin response (Bigner et al. 1996) and reduce considerably insulin sensitivity (Kushibiki et al. 2000, 2001, 2003). In dairy cows currently there is little evidence that, compared to monogastrics suffering from the metabolic syndrome, chronic inflammatory processes in adipose tissue may contribute by means of adipokines and cytokines to the development of reduced insulin sensitivity (Saremi et al. 2011). Glucocorticoid treatment (Sternbauer et al. 1998, Kusenda et al. 2012) as well as endogenous cortisol release caused by stress challenges or pain (Rizk et al. 2012) affect glucose and fat metabolism due to reduced insulin sensitivity and depression of insulin mediated suppression of lipolysis. Therefore, the cow in the transition stage may enter a vicious cycle supported by concomitant alterations of energetic metabolism, diseases and inflammatory states that progressively influence and favour each other.

3.5 Nutritional strategies for preventing and limiting NEB and insulin resistance:

Minimizing DMI depression or increasing nutrient density of the diet during the transition period would be an essential strategy to alleviate the severity of negative energy balance, maintain body reserves, increase nutrients available for rapid fetal growth, ease metabolic transition from pregnancy to lactation, and acclimate rumen microorganisms to lactation diets (Van Saun, 1991; Grummer, 1995; Nocek, 1995). Increasing dietary concentration of non-fibre carbohydrates during the last 21 days of gestation (NFC) (1.58–1.63 Mcal NEL/kg DM or 38–44% NFC) increases energy density of the diet by providing greater amounts of glucogenic precursors like propionate (Flipot *et al.*, 1988; Minor *et al.*, 1998; Dann *et al.*, 1999; Rabelo *et al.*, 2003) and maximizes DMI and energy intake by decreasing gut fill (Forbes, 1996). This feeding regimen stimulates the microbial flora (Forbes, 1996) and serum insulin secretion, which promotes papillae growth (Driksen *et al.*, 1985). Increased growth of papillae enlarges the surface area and VFA absorptive capacity of the rumen epithelium (Driksen *et al.*, 1985). In turn, this could prevent accumulation of VFA and normalize pH of the reticulo-ruminal fluid.

In addition, factors affecting DMI during the prefresh transition are linked to limiting fat mobilization. Nutritional factors (e.g., feeding more energy as fermentable carbohydrates) that alleviate severity of hepatic lipidosis also affect ketogenesis (Hayirli and Grummer, 2004). The effects of this feeding regimen on the development of ketosis are mediated through insulin potential antiketogenic effects (Schultz, 1971; Schalm and Schultz, 1976). Despite a minimal energy density of the diet should be guaranteed in the prepartum phase, attention must be paid to avoid an excessively high speed of propionate production in the rumen. Because feed intake of fresh cows is likely controlled primarily by hepatic oxidation (HOT theory; Allen, 2000; Allen et al., 2005; Allen and Bradford, 2006; Allen and Bradford, 2010), it is necessary to manipulate the rate of propionate production to extend meal length, supplying other glucose precursors that stimulate oxidation of acetyl CoA to a lesser extent, and providing alternate energy sources for tissues to spare glucose and elevate insulin concentrations, decrease fat mobilization and the period of time feed intake is suppressed by oxidation of NEFA in the liver.

Diets with moderately high forage fiber concentrations might benefit fresh cows (Voelker Linton and Allen, 2007). Forage fiber increases rumen fill, decreasing the risk of abomasal displacement, and increases acetate production, sparing glucose utilization by extrahepatic tissues. While research is needed to evaluate effects of concentration and fermentability of starch on feed intake response, starch sources with moderate ruminal fermentability and high digestibility in the small intestine such as dry ground corn, compared to cracked corn and high moisture corn, will likely provide more glucose precursors by increasing feed intake. Hepatic oxidation of ketogenic amino acids can contribute to satiety according to HOT and urea production from excess ammonia produces a carbon skeleton that can be oxidized. However, greater dietary protein concentration can also increase feed intake by reducing propionate

production. Increasing protein concentration could dilute diet starch concentration and decrease energy spilling by ruminal microbes, thus converting a greater fraction of fermented digesta into microbial cells and less into VFA. Formulating diets to maintain gut fill with ingredients that are retained in the rumen longer, and have moderate rates of fermentation and high ruminal digestibility will likely provide more energy over time when feed intake decreases at calving or from metabolic disorders or infectious disease. This will help maintain plasma glucose and prevent even more rapid mobilization of body reserves.

Feed intake of dry cows must be limited by feeding diets with high forage NDF concentration. Diets with high concentrations of grain, non-forage fiber, and finely chopped forages fed through the transition period should be avoided. Increased amounts of ruminal digesta also decrease risk of displaced abomasum and increase buffering capacity, decreasing risk of acidosis. Some long fiber particles are necessary to form a mat and increase digesta retention in the rumen, but the length of cut, digestion characteristics and maturity of forage type can vary greatly and influence feed intake. Grass silage or hay is likely more beneficial than wheat straw because the fiber is more digestible and it provides energy for a longer time when feed intake decreases at calving (Beauchemin et al., 1994).

Withal, mobilization of body fat during mid to late lactation must be prevented. It is advisable to feed a more filling, less fermentable diet as milk yield declines. As lactation progresses past mid lactation, faster propionate production by highly fermentable diet can depress feed intake. This will provide a more consistent supply of fuels, reducing insulin and partitioning more energy to milk rather than body condition.

3.5.1 Diet Supplementation in the transition period:

Supplementations of choline (Hartwell *et al.*, 2000; Piepenbrink and Overton, 2003), inositol (Gerloff *et al.*, 1986b) and methionine (Bertics and Grummer, 1999), which was intended to improve TG export by enhancing lipoprotein synthesis, failed to alleviate hepatic lipidosis in dairy cattle. Therefore, until factors impeding hepatic VLDL-TG export are identified, limiting fat mobilization from adipose tissue will play a key role in prevention of hepatic lipidosis. Studer and colleagues (1993) reported similar changes in transition dairy cows supplemented with propylene glycol. The antilipolytic effect of niacin supplementation during the periparturient period on alleviation of hepatic lipidosis is controversial. Minor and colleagues (1998) found a tendency for decreased liver TG, whereas Skaar and colleagues (1989) found no effect on hepatic lipidosis. Niacin likely needs to be supplemented at higher concentrations than currently recommended unless provided in a protected form. Chromium increases insulin sensitivity and supplemental chromium has been demonstrated to decrease plasma NEFA concentration and plasma glucose to serum insulin ratio in lactating dairy cows, no effect on hepatic lipidosis was reported in cows supplemented with chromium (Yang *et al.*, 1996; Besong

et al., 2001; Hayirli *et al.*, 2001; Pechova *et al.*, 2002), which was intended to increase insulin action on extrahepatic tissues to inhibit lipolysis. However chromium supplements are not allowed for use in lactating dairy cattle diets by the European Union and other international food inspection agencies. Supplemental fat should not be fed through the transition period because it can depress feed intake by increasing the supply of fatty acids to be oxidized. An exception might be the use of supplemental conjugated linoleic acid (CLA) to suppress fat production in the mammary gland, benefiting fresh cows by sparing glucose.

3.6 Tests for measuring insulin resistance:

Although several methods had been developed and validated to evaluate insulin sensitivity in human medicine, none of these methods can be universally used in all patients (Lais et al., 2003). Some of these methods include: fasting plasma insulin, homeostatic model assessment, quantitative insulin sensitivity check index, glucose-to-insulin ratio, continuous infusion of glucose with model assessment, indices based on oral glucose tolerance test, insulin tolerance test, and the so called "gold standard" methods, the hyperinsulinemic euglycemic clamp and the frequently sampled-intravenous glucose tolerance test. Each method is characterized by specific procedures, cut-off values for defining insulin resistance, advantages and limitations, and suitability for use either in clinical practice or in research settings according to its handiness, accuracy and validity. In humans tests on insulin resistance are generally performed after an overnight fast to ensure a comparable and stable metabolic state regarding pancreatic baseline insulin secretion, glucose disposal and gluconeogenesis (Muniyappa et al. 2008; Borai et al. 2011). However, in contrast to monogastrics, ruminants are not in a fasting metabolic state after an overnight fast and additionally restricting access to feed will quickly result in increased lipolysis, ketogenesis and fat accumulation in hepatic tissue in early lactating dairy cows (Oikawa and Oetzel 2006, Quiroz-Rocha et al. 2010). In ruminants little information is available about the necessity and optimal duration of fasting and interpretation on test results yet. The hyperinsulinemic euglycaemic glucose clamp (HEC) technique and intravenous glucose tolerance test (IVGTT) are the most commonly used tests in research in dairy cows to evaluate insulin resistance or glucose intolerance.

3.6.1 Hyperinsulinemic Euglycemic Glucose Clamp (HEC):

The glucose clamp technique was originally developed by DeFronzo et al. (1979) and is seen as the reference standard for directly determining metabolic insulin sensitivity. In humans, after an overnight fast, during the clamp insulin is continuously infused resulting in a new steady state insulin concentration (SSIC). To maintain plasma glucose, parallel glucose is infused at varying infusion rates depending on results of frequently measured plasma glucose concentrations. Assuming that hepatic gluconeogenesis is almost completely suppressed by insulin infusions, the steady state glucose infusion rate (SSGIR) reflects glucose disposal to peripheral tissues, in particular muscle and adipose tissue. The ratio between SSGIR and SSIC reflects glucose disposal per unit of insulin (insulin sensitivity index, ISI). The HEC procedure allows the effects of insulin to be measured without the ensuing hypoglycemia. The amount of insulin required to achieve the maximum response indicates insulin responsiveness, whereas the amount of insulin required to reach the half-maximal response indicates insulin sensitivity (Kahn, 1978). Glucose infusion rate is equal to metabolic rate of glucose utilization (Sano *et al.*, 1991, 1993). The major limitations of HEC are that it is time consuming, labourious, very

expensive and requires an experienced operator to manage technical difficulties. Thus, use of HEC is restricted to experimental studies with limited number of animals.

3.6.2 Frequently Sampled Intravenous Glucose Tolerance Test (IVGTT):

The GTT is a more practical and cheaper test than the euglycaemic clamp technique for determining glucose intolerance. It is based on a bolus infusion of glucose and frequent blood sampling in short intervals to register glycemia, insulinemia and changes in blood NEFA concentrations. These paremeters give information on pancreatic insulin release, glucose disposal and insulin dependent suppression of blood NEFA concentrations (Grünberg et al., 2011). In the GTT, basal and peak concentrations, plasma disappearance rate, half-life, time to reach basal concentration and ratio of plasma glucose to serum insulin are parameters for evaluation of glucose tolerance (Hayirli et al., 2001). Response measures to glucose challenges often involve area under the curve (AUC) calculations to estimate response over time after the challenge. Smaller glucose AUC might suggest greater glucose clearance and thus less insulin resistance. However, information obtained from GTT is not as easily interpreted as that generated from euglycaemic clamps. For instance, during the GTT, it is not known whether increased plasma glucose disappearance rate is due to increased glucose utilization or decreased insulin production. In this case, the molar ratio of serum insulin to glucose or the ratio of their disappearance rates is a better indicator of insulin resistance than plasma glucose disappearance rate alone (Subiyatno et al., 1996). Another limitation of IVGTT is hyperglycemia which may result in renal glucose losses and increased insulin independent glucose disposal to the mammary gland in lactating cows so that insulin dependent glucose utilization of peripheral tissues may be overestimated. Schoenberg and Overton (2011) combined the IVGTT with a single injection of insulin in low dose to estimate insulin dependent glucose disposal and suppression of blood NEFA.

The GTT has been performed in various experiments in ruminants in the last years with particular attention to the few weeks prior to calving and immediately after. These studies have been used to show that insulin can alter plasma concentrations of several metabolites but, because of the low circulating concentration of insulin in early lactation, it may exert less control over metabolic processes in proximity of calving and under pathologic conditions. For example, artificial hyperlipidemia was used in dairy cattle to mimic the increases in NEFA during late pregnancy, and successfully illustrated the negative effects that rising NEFA have on insulin signaling and glucose clearance during a GTT in the transition period (Pires et al., 2007). Bossaert and colleagues (2008) infused dextrose intravenously in 23 dairy cows at -14, 14 and 42 d around calving to assess their glucose-induced insulin responses at different time points relative to calving and to relate this to the metabolic status and the time of first ovulation. Furthermore, IV glucose infusion has been used to evaluate the profile of metabolic hormones

change, such as GH, Ghrelin, insulin and epinephrine, after a glucose challenge in ten multiparous dairy cows 35 d in milk (Roche et al., 2008). GTT was used in association to Insulin Tolerance Test (ITT), to determine the glucose-induced insulin responsiveness and insulin sensitivity in cows showing different forms of periparturient ketone pattern with and without puerperal metritis, and their interrelation with different metabolic and hormonal parameters in dairy cows (Kerestes et al., 2009). 31 Holstein cows were involved in this experiment between 18 and 22 d before, and GTT was performed again on d 7 and 60-70 after calving. In a similar trial, the effects of periparturient propylene glycol supplementation on the same parameters and on the time of the first postpartum ovulation and pregnancy rates were analyzed through GTT and ITT (Kerestes Ph.D. dissertation, 2010).

Likewise, additional researches have been carried out by use of GTT with the purpose of testing diets, nutrients and various therapeutic or other compounds effects on glucose metabolism. For instances, GTTs were conducted by Hayirli and colleagues (2001) on d 10 prepartum and d 28 postpartum in 48 cows in order to study the effect of supplemental chromium as chromium-methionine (Cr-Met) on production and metabolic parameters from 28 d before expected calving date through 28 d of lactation. Glucose curves in response to GTTs have been compared to those of Xylitol Tolerance Tests in four non-lactating cows, where xylitol appeared to cause similar secretion of insulin to that caused by glucose and have similar disappearance rate (Mizutani et al., 2003). Xylitol is a sugar alcohol that is often used for treatment of ketosis in dairy cattle in Japan. Therefore the experiment proved that xylitol had value for treatment of ketosis. IV treatments with 50% dextrose solution decreased phosphorus concentration in serum, compared with the control treatment and suppress BHB and NEFA concentrations for < 12 hours in 24 clinically normal multiparous cows enrolled 5 to 10 d after parturition in the study (Wagner and Schimek, 2010). Recently, a study was completed in order to determine the effects of different dietary energy levels on responses to glucose and insulin challenges during the dry period in multiparous cows (Schoenberg et al., 2012). The insulinotropic effect of a high plane (HP) compared to a low plane (LP) of nutrition beginning 48 d before expected parturition was confirmed by the lower glucose AUC, than for those fed the LP diet, during GTTs (0.25 g of dextrose/kg of body weight (BW)) performed first on d 14 of treatment and then, following 24 h of feed removal, on d 17. In both nutrition planes, the AUC was higher during the GTT in the feed-deprived state than in the GTT during the fed state, suggesting slower clearance of glucose during negative energy balance either pre- or post-feed deprivation. Beyond this, after each GTT, cows were subjected to the HEC procedure, which attested prior GTT results. Even NEFA AUC in response to glucose and insulin challenges could be measured: in this way it is feasible to assess differential responses to treatments (such as dietary energy level or insulin-sensitizing agents) in glucose versus lipid metabolism (Shoenberg and Overton, 2010).

3.6.3 Surrogate insulin sensitivity indices: HOMA and QUICKI

In humans surrogate insulin sensitivity indices were developed to test patients with diabetes for insulin resistance on a simple and cheap basis. Again after an overnight fast, a single blood sample is taken for determination of blood glucose and plasma insulin. In healthy humans, the fasting condition represents a basal steady state where glucose is homeostatically maintained in the normal range such that insulin levels are not significantly changing and hepatic gluconeogenesis is constant. The different indices provide similar but not same results so that more than one index should be used when diabetic patients are tested (Muniyappa et al., 2008). Indices are calculated from plasma concentrations of glucose, insulin, NEFA, or glycerol using the following equations:

1) Homeostasis model assessment (HOMA; Matthews et al. (1985)):
HOMA = Glucose (mmol/L) x Insulin (μU/mL).
HOMA-IR uses a fasting sample and is derived from the use of the insulin glucose product, divided by a constant: HOMA-IR = {[Glucose (mmol/L) x Insulin (μU/mL)]/22.5} x 0.5 (Radziuk, 2000; Singh and Saxena, 2010). The denominator of 22.5 is a normalizing factor and derives from the product of normal fasting blood glucose (4.5 mmol/L) and insulin (5 μU/mL) in typical "healthy humans" (Muniyappa et al., 2008). For using this index in other subjects, basal levels of the respective animal have to be considered. The higher HOMA-IR, the higher is IR and the lower is insulin sensitivity. Its log-transformation (log (HOMA)) and the reciprocal score (1/HOMA) provide better correlations compared to HOMA in evaluation of IR in humans (Yokoyama et al., 2003; Muniyappa et al., 2008).

2) Quantitative insulin sensitivity check index (QUICKI; Katz et al. (2000)):
QUICKI = 1/ [log (Glucose (mg/dL)) + log (Insulin (μU/mL))];

3) Revised quantitative insulin sensitivity check index (RQUICKI; Perseghin et al. (2001)):
RQUICKI = 1/ [log (Glucose (mg/dL)) + log (Insulin (μU/mL) + log (NEFA (mmol/L))].

4) Modified quantitative insulin sensitivity check index replacing NEFA by glycerol (QUICKI-glycerol; Rabasa-Lhoret et al. (2003)):
QUICKI-glycerol = 1/ [log (Glucose (mg/dL)) + log (Insulin (μU/mL) + log (Glycerol (μmol/L))].

5) Revised quantitative insulin sensitivity check index including BHB (Balogh et al., 2008):
RQUICKI-BHB = 1/ [log (glucose (mg/dl)) + log (insulin (μU/ml)) + log (NEFA (mmol/l)) +log (BHB (mmol/l))].

A low RQUICKI index value indicates decreased insulin sensitivity.

Recently Holtenius and Holtenius (2007) suggested usage of RQUICKI as an estimate for insulin sensitivity also for dairy cattle. However, in ruminants glucose metabolism is significantly different from that in monogastrics, blood samples cannot be taken in fasted metabolic states and the metabolic state in dairy cows in early lactation (hypo-insulinemia, hypoglycaemia) differs substantially from that in diabetic patients (hyper-insulinemia, hyper-glycemia) so that a thorough evaluation of accuracy and reliability of surrogate indices appears necessary before these tests are widely used. So far, RQUICKI index and GTT results were compared (Balogh et al. 2008; Kerestes et al. 2009) with controversial findings. Balogh et al. (2008) obtained significant correlations between some parameters derived by GTT and the RQUICKI and RQUICKI-BHB, whereas Kerestes et al. (2009) did not find any significant correlation between GTT variables and the RQUICKI. Nevertheless, such indices appear promising and are already used in research studies (Al-Trad et al., 2009; Bossaert et al., 2009; Stengärde et al., 2010, Schoenberg et al., 2011) since they are cheap and easy to perform and therefore applicable in large numbers of animals. Moreover, pre-sampling fasting procedure is not feasible in ruminants. Energetic metabolism during early lactation is essentially different between dairy cows and diabetic patients and therefore it substantially influence glucose utilization. For instance, hypoglycaemia and hypo-insulinemia are typical of fresh cows after calving, whereas hyperglycaemia and hyper-insulinemia are frequent consequences of diabetes. Thus, currently the numeric comparison of test results between studies is of limited value and establishment of general reference values for surrogate insulin sensitivity indices is difficult.

4. Materials and Methods

4.1 Herds

This trial involved 3 Holstein-Fresian dairy farms in the province of Padova (Veneto), selected for rearing genetically high producing cows and having similar dry cow and transition cow management. One herd (Herd A) was composed of about 270 lactating animals, the second herd (Herd B) was composed of 340 lactating animals and the third (Herd C) had 1.100 lactating cows. The survey was carried out during spring and summer seasons in 2011. Herd C was started first beginning in March and its sampling was ended up in late April; Herd A and B were investigated simultaneously beginning in early April and their sampling was ultimated in late July. All the three farms were equipped with cooling systems of various kinds for preventing heat stress in the summer. The speed of animals' enrollment in the trial in each herd was limited by dry cows availability in those months due to fertility efficiency seasonal variations. All farms used to offer a total mixed ration (TMR) ad libitum to both non-lactating and lactating cows and TMR was delivered to animals between 9.00 and 11.00 am and refreshed on a daily basis, after animals exit from milking parlour in the morning milking session. Cows were milked twice a day at 6.00 and 15.00 in the milking parlour. TMR was based on grass hay, alfalfa hay, soybean, concentrated feed and silage. Some hay was left in the bunk out of the TMR for non lactating cows. Besides, all three farms used to dry-off cows around 60 days before expected calving date and adopted a steaming-up phase in late dry period (close-up period) with higher energy and protein levels than far-off. The length of close-up period could be variable, ranging from 20 to 10 days, according to individual animals and herds, and space availability due to animals stocking density. Cows were chosen and moved to the close up pen by the herd manager on the basis of their appearance and days left to predicted calving date, given estimated gestation length of 275 days. Herd C tended to house cows in the close up pen for less days (about 10) according to their nutrition program. Also the energy density and composition of far-off and close-up diets could vary between farms but nutritional strategies were kept constant during the trial in each herd. Cows were introduced to a new lactation diet, with differences in nutrients composition between herds, immediately following parturition. Both dry-off and close-up cows were housed in permanent straw bedding pens in all farms, whereas immediately after calving they were moved to a new bedding pen for a few days for better health check. Fresh cows were monitored once daily by the herd private vet according to their routine protocols and treated for diseases. Cows that did not report apparent unhealthy state were moved to free stall facilities in larger groups within 10 days in milk. In all farms cows were never moved to a maternity unit for calving but calved in the close-up pen. Fresh cows

were neither routinely checked for fever nor ketosis in the blood by ketons strips in any herd. Fresh cows were checked for clinical signs of diseases, including retained placenta and puerperal metritis, and even animals' appetite, attitude and milk production were taken into account by veterinarians for treatment decisions.

Table 1: Composition and contents of the different rations in Herd A

	Herd A		
Item	Dry cow	Close-up	Fresh cow
Ingredients (% of DM)			
Corn silage	20,93	-	32,02
Wheat straw	13,44	11,61	2,06
Hay	53,52	-	10,68
Soybean meal	10,65	-	8,96
Mineral mixture	1,45	2,47	2,22
Water		-	
Alfalfa hay	-	11,52	12,23
Ground corn silage	-	-	11,94
Ground corn	-	-	10,28
Cotton seeds	-	-	4,20
Sunflower meal	-	-	4,16
Flax seeds	-	-	1,26
Fresh cow TMR	-	74,40	-
Chemical analysis, % of DM			
DM (% as fed)	59,28	59,89	53,75
NEL (Mcal/kg of DM)	1,18	1,39	1,60
NDF (%)	54,87	40,51	34,18
NFC (%)	22,58	33,45	40,55
Starch (%)	7,23	19,60	26,29
Crude Protein (%)	11,63	14,07	15,20
Ether extract (%)	1,77	3,44	3,32
Crude ash (%)	9,06	9,51	6,87

Table 2: Composition and contents of the different rations in Herd B

	Herd B		
Item	Dry cow	Close-up	Fresh cow
Ingredients (% of DM)			
Corn silage	14,97	30,69	26,34
Wheat straw	14,89	7,17	-
Hay	54,14	26,06	-
Soybean meal	5,90	9,65	-
Molasses	3,24	1,56	-
Mineral mixture	1,60	4,42	-
Water			
Ground corn silage	-	-	7,48
Ground corn	-	13,76	-
Sunflower meal	5,27	6,70	-
Grass silage	-	-	5,22
Dry hay and raw material mixture	-	-	60,95
Chemical analysis, % of DM			
DM (% as fed)	62,63	51,54	55,42
NEL (Mcal/kg of DM)	1,22	1,45	1,59
NDF (%)	54,52	40,49	31,84
NFC (%)	23,74	34,35	40,92
Starch (%)	5,02	20,00	26,22
Crude Protein (%)	12,01	14,20	16,77
Ether extract (%)	1,89	2,63	3,25
Crude ash (%)	7,73	6,42	7,33

Table 3: Composition and contents of the different rations in Herd C

	Herd C		
Item	Dry cow	Close-up	Fresh cow
Ingredients (% of DM)			
Corn silage	35,67	34,56	44,80
Wheat straw	17,74	17,18	-
Hay	36,86	35,71	4,34
Soybean meal	8,78	8,50	8,27
Mineral mixture	0,96	2,30	2,25
Water			-
Alfalfa hay	-	-	14,49
Ground corn	-	-	14,62
Ryegrass silage	-	-	4,90
Flaked soybean	-	-	6,32
Flax seeds	-	1,75	-
Chemical analysis, % of DM			
DM (% as fed)	55,76	54,46	54,94
NEL (Mcal/kg of DM)	1,22	1,26	1,53
NDF (%)	53,06	51,82	33,39
NFC (%)	27,89	28,33	42,12
Starch (%)	13,74	13,39	27,92
Crude Protein (%)	10,71	11,04	14,07
Ether extract (%)	2,06	2,62	3,55
Crude ash (%)	6,23	6,12	6,88

4.2 Animals

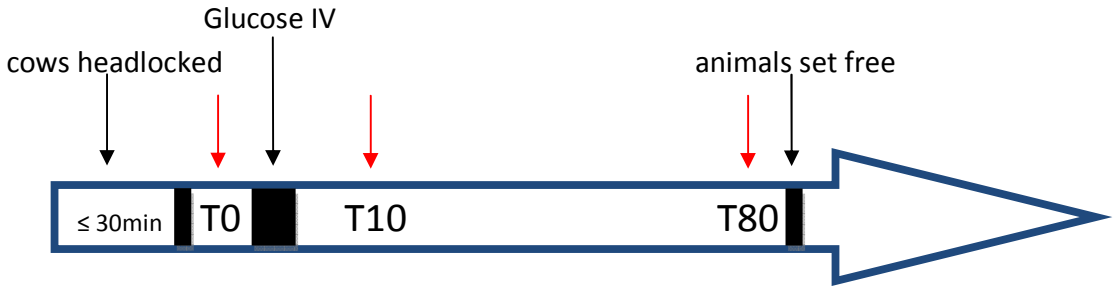
Forty-six cows entering second lactation (parity 2) and fifty-five multiparous (parity 3 to 10) cows producing mean ± SEM: 10.917 ± 179 kg of fat corrected milk in the previous 305-day lactation were randomly selected for the study among animals in the close-up pens of the three herds. Cows were within 14 days before expected calving date, considering normal gestation length of 275 days and whole physical appearance of calving proximity at the time of selection. Cows that were going to calve later than 14 days from the enrollment date were tested again in order to obtain all samples from pregnant cows in the range of 14 pre-partum days. These cows had a high genetic merit for milk yield. Their milk production in the previous lactation reported an average of mean ± SEM 11.480 ± 278 kg of fat corrected milk at 305-day lactation (305EVM) for herd A, 10.306 ± 323 for herd B and 11.082 ± 288 for herd C. The final number of cows that entered in the trial was 101 and they were evenly partitioned between each farm: 32 animals were in herd A, 35 animals in herd B and 34 animals in herd C. They were apparently in a general organic healthy status and were not submitted to any treatment for diseases at least in the previous month at the moment of selection.

4.3 The Intravenous Glucose Tolerance Test (IVGTT)

Glucose tolerance test was performed between days 14-1 before delivery in late gestation (Week -1), and after calving, on days 3-9 (Week +1). A single blood sample was collected again on days 10-16 of lactation (Week +2) to evaluate further incidence of ketosis in the transition period and monitor the evolution of insulin and other metabolites, which might be very fluctuant in the first two weeks of lactation. The GTT was not performed at 10-16 days in milk.

First the animals were selected and restrained by headlocks of the close-up pens up to 30 minutes before beginning of tests and remained captured during the whole individual test. GTT were accomplished serially and thereby some selected cows had to remain locked for a longer time. When a lot of tests were scheduled in a certain day, we managed to postpone the capture of some animals. Thus, we let the animals never be restrained longer than one hour before GTT. Most tests were performed between 8 and 12 am, sometimes after TMR delivery was already completed, and it was not possible to completely control or restrict feed access to animals due to obvious requirements of productive cows in a field trial. Some feed was always available in mangers, both prior and after daily TMR distribution, even during restraint. Simultaneously, an initial blood collection (T0) through a 2.5 ml syringe and 21-gauge x 1 ½" needle (0.80 x 40 mm) for basal glucose determination by glucose meter, and a couple of 9 ml-evacuated tubes containing lithium heparin as anticoagulant (Vacuette®, Greiner Bio-One, Kremsmuenster, Austria) were sampled from one jugular vein from each cow. These samples were immediately followed by a glucose infusion in the same jugular vein over an average time of 5 min. A 14-gauge x 1 ½" needle (2.0 x 40 mm) was inserted in the vein and connected to an

urinay defluxor (Haemotronic® S.p.A., Mirandola MO, Italia) for rapid infusion of 300 ml of 50% glucose solution, approximating a glucose dose of 0.20 to 0.25 g/kg of BW (Glucosio 50 g/100 mL; solution for infusion, glucose monohydrate, AltaSelect Srl, San Giovanni Lupatoto - Verona, Italy). The body weight was estimated based on the average size and condition score of the cows in the herd and varied between 600 and 700 kg; great variability in body weights was reduced by excluding heifers from the research. The proper amount of glucose solution was prepared before IV administration start. Proper needle insertion in the vein was continuously checked during glucose challenge. Whether cows were nervous and therefore IV administration was not correctly accomplished, the blood measurements were excluded from the statistical analysis. Stress was avoided as much as possible; cows were rigorously blocked against the headlocks only during IV infusion. They generally appeared relaxed and were let move their head and rest in standing position while waiting for subsequent samples till 80 minutes. Moreover, haematological samples were taken before any other manipulations, like daily health check, took place to avoid stress because it can modify some hormones and metabolic parameters. Also, milking cows were submitted to any pharmacological therapies after the completion of the last sample at 80 min since GTT to avoid distorted findings. Therefore, there was the chance that a tested cow was treated for diseases last time the previous day, about 24 h before GTT, or during the morning milking session in case of intra-mammary treatment for mastitis. All animals treatments, included hour and date and pharmacological compound administered, were registered in our field sheets and weighed up before inclusion of that specific GTT in the trial. However, certain interference from herd treatments must be taken into consideration and will be discussed in the appropriate chapter. Zero time was set immediately after the end of glucose infusion. Additional blood samples, both in Lithium-Eparine tubes and syringes for glucometer determination, were obtained on 10 (T10) and 80 min (T80) relative to the glucose infusion, on the contro-lateral vein of jugular vein punctured at T0.



Blood tubes were stored on ice at less than 4°C in field until centrifuged (10 min at 3.500 x g, Labofuge machine) within 1 h of sampling, after the end of field operations, at the Laboratory of the Department of Veterinary Clinical Science of the University of Padova. The plasma fraction was transferred to a sterile polystyrene tube with a 3-mL plastic pipette. Two aliquots of plasma were obtained from each vacutainer and they were stored at −20°C until analysis. After centrifugation, highly and moderately clotted and haemolytic samples were excluded from subsequent analysis.

4.4 In field: data collection and analysis

Data collection

Data about individual anamnesis were recorded for each cow. We had a work sheet predisposed to record the following data: parity, exact dates of sampling relative to calving, glucose readings by glucose meter and relative times at sampling on T0, T10 and T80, exact time of the end of IV infusion, health records and dates and description of treatments submitted to cows. Milk fever, retained placenta, metritis, mastitis, left abomasal displacement (LDA) and other specified unhealthy status were checked on the sheet. The health status of the cows was estimated subjectively by observation of individual milk yield, attitude and appetite, conversation with the herd manager and vet, and regular clinical inspections. Other details like dystocia, twins, partum induction were also recorded. Induced calving were less than 5%. Body condition score (according to Edmonson procedure (1989) based on 5 points scale) was evaluated by the same subject during animal selection in order to estimate the average herd body weight of dry cows for administration of adequate volume of IV solution. Milk production at 1st APA test and days in milk (DIM) at 1st test, mature cow milk equivalent on 305-d (EVM305), calculated as a prevision from 1st milk test in the current lactation, EVM305 in the previous lactation, and somatic cells count (SCC) at 1st APA test were later obtained for each cow from the APA district of Padova. BHB was also measured twice by a glucose meter post partum in order to immediately provide the herd managers with field BHB values that could help them in making health evaluation and treatments decision about every single cows.

Below I attach the template sheet for field records of each single cow:

FARM	
COW ID TAG	
COW ID CHAIN	
Lactation N°	

1. (Week -1) 14 – 1 DAYS PREPARTUM: DATE_____

BCS	

	T0	time iv	T10	T80	T80/T0	NOTES
GLUCOSE						
Time of sample						

CALVING DATE: _____

2. (Week +1) 3 - 9 DAYS POSTPARTUM: DATE_____

BHBA at Time 0	

	T0	time iv	T10	T80	T80/T0	NOTES
GLUCOSE						
Time of sample						

3. (Week +2) 10 – 16 DAYS POSTPARTUM: DATE _____

GLUCOSE	
BHBA	

HEALTH RECORDS

	YES	NO	NOTES	date
RIT PLACENTA				
MILK FEVER				
METRITIS				
LDA				
MASTITIS				
OTHER				

Glucose determination in field:

After each sample glucose was straight measured in field. We decided to do so in order to evaluate the IVGTT as a field test that could be fully realized under field conditions on farms, with no need for further laboratory responses. Cow-side glucose measurements were conducted on whole blood using a hand-held blood glucose meter (Optium Xceed, ART19558-004 Rev.A; Abbott Diabetes Care Ltd, Witney, Oxon, UK). Optium Xceed is an instrument commonly utilized in human patients for diabetics care. As far as we know, there are no glucose meters specifically provided and validated for ruminants. However, Optium Xeed is the most diffused meter present in our herds and in other countries because it is extremely accurate and user friendly for BHB determination. In fact many herds use to monitor fresh cows for ketosis by reading BHB through glucose meter. Optium Xeed is also the most used glocometer in recent research in ruminants regarding glucose tolerance loads and heuglicaemic clamp techniques (Oetzel, 2010; Schoenberg et al., 2012). For these reasons it was one of the few glucose meters considered for comparison with laboratory analysis (McGuirck and Oetzel, 2008; Voyvoda and Erdogan, 2010). We used Optium H strips for glucose reading. These strips have been validated both for capillary and venous and arterial blood from human subjects. Glucose results were displayed in mg/dl in 5 seconds by our instrument and its assay range is 20 – 500 mg/dl. When a glucose concentration lower than 20 mg/dl was detected, we reported a glucose value of 20 mg/dl in our field sheets; similarly, when glucose concentration was indicated as higher than 500 mg/dl by the glucometer, a value of 500 mg/dl was considered as glucose present in the tested sample. The test principle is based on a reaction between glucose and chemicals on the test strip, producing a small electrical current proportional to the amount of glucose present in the blood. The optimal temperature for glucometer functioning is in the range of 15°-40°C and humidity between 10% - 90%. Regarding precision of our instrument, a study in humane medicine reported on the Optium H strips user's instructions shows that results tipically vary by no more than 3.0% to 3.6% with high (mean ± SEM 344 ± 10.2 mg/dl) and low (mean ± SEM 51 ± 1.8 mg/dl) glucose concentration respectively. Accuracy of Optium Xceed was measured by comparing its results obtained at different medical centers with those obtained using the YSI Glucose Analyser: the correlation coefficient in venous blood was 0.99 for glucose range of 70 – 540 mg/dl.

4.5 Laboratory analysis

One of the series of plasma aliquots have been transfered and stored at -20° at the Laboratory of Medical Clinic (Istituto Zooprofilattico Sperimentale delle Venezie, IZSVE) for analysis of glucose, basal NEFA and BHB (T0), insulin at T10 and insulin at T80, by use of automated equipment and commercial kits based on enzymatic, colorimetric or immunological reactions. All samples have been tested for glucose with a Cobas c 501 analyzer (Roche Diagnostics, Mannheim, Germany) and Gluc3, glucose HK test (kit no. 04404483190VB, Roche Diagnostics) by hexokinase method. The intra-assay coefficient of variation (CV) was < 1% and the inter-assay CV was < 1.3%. Standardized reference material was tested each day of assay for each parameter as a control serum to confirm calibration of the methods. On Cobas C 501 were performed also NEFA and β-OHB analysis: basal NEFA concentrations were determined by using a colorimetric method, NEFA RX Monza test (kit no. FA 115, Randox, Crumlin, UK) with intra-assay CV < 4.81% and total-assay CV < 4.51%. Basal samples were analyzed also for BHB concentration by RANBUT RX Monza test (kit no. RB 1007, Randox, Crumlin, UK), a UV kinetic enzymatic method based on the oxidation of D-3-hydroxybutyrate to acetoacetate by the enzyme 3-Hydroxybutyrate dehydrogenase with intra-assay CV < 3,78% and inter-assay CV < 5,25%. Insulin concentrations at T10 and T80 were measured by using a Immulite One analyzer (Siemens, distributed by Medical Systems S.p.A., Genova, Italy) and the commercial INS kit (Siemens Healthcare, Diagnostic Products Ltd, Gwynedd, UK), which is a solid-phase, enzyme-labeled chemiluminescent immunometric assay with intra-assay CV < 6.4% and total-assay CV < 8%. The minimum detectable level with acceptable precision has been determined as 14.7 pmol/l. The laboratory of IZSVE provided intra- and inter-assay test results that confirmed the CV values stated by the different commercial methods for all the tests used. A value equal to 14.0 pmol/l of insulin was considered for data analysis in cases of very low insulin concentrations not detectable by the immunometric assay.

At the same time a series of basal plasma aliquots (T0) was analyzed at the Department of Veterinary Clinical Science of the University of Padova for determination of Albumin, Globulin, Total Proteins, Urea, GOT-AST, GPT-ALT, Calcium, Chlorine, Phosphorus, Magnesium, Potassium and Sodium by an automated Biochemistry analyzer BT1500 (Biotecnica Instruments S.p.A., Roma, Italy). Plasma concentrations of these parameters were determined by use of Gesan reagents (Gesan Production s.r.l., Campobello di Mazara, TP, Italy): Albumin LR for albumin (REF C1200620 518 Test, colorimetric bromocresol green BCG method, intra-assay and inter-assay CV < 4.15%); Total Protein LR for total proteins (REF E4506100, biuret methods, intra-assay and inter-assay CV < 3.92%); Urea UV LR for urea (REF E4800550, Kinetic UV method Urease-GLDH, intra-assay and inter-assay CV < 3.70%); AST GOT LR for GOT-AST (REF 3700650, Kinetic UV optimized method IFCC, International Federation of Clinical Chemistry and Laboratory

Medicine, intra-assay and inter-assay CV < 3.66%); ALT GPT LR for GPT-ALT (REF 3800650, Kinetic UV optimized method IFCC, International Federation of Clinical Chemistry and Laboratory Medicine, intra-assay and inter-assay CV < 3.71%); Calcium CPC LR for Calcium (REF 1801030, Colorimetric ocresolphtalein complexone method CPC, intra-assay and inter-assay CV < 2.11%); Chloride LR for Chlorine (REF E2100650, Colorimetric method mercurious thiocyanate, intra-assay and inter-assay CV < 2.60%); Phosphorus LR for Phosphorus (REF C3300620, Colorimetric blue of molybdate method, intra-assay and inter-assay CV < 2.71%); Magnesium LR for Magnesium (REF C4400620 518 Test, Colorimetric method Xylidyl Blue, intra-assay and inter-assay CV < 4.89%); Potassium LR for Potassium (REF 4900430, Turbidimetric method, intra-assay and inter-assay CV < 2.75%); Sodium for Sodium (REF 4950180, Kinetic enzymatic method, intra-assay and inter-assay CV < 4.72%). Globulins were determined by difference between Total Proteins and Albumin concentrations.

Insulin determination at T0

A series of basal samples (T0) were shipped to the Animal Endocrinology Laboratory, Dipartimento di Scienze della Vita e Biotecnologie, University of Ferrara, for quantification of insulin by a radio-immunometric-assay in order to be able to calculate the RQUICKI indexes for each animal from basal blood collections. Free-insulin concentration in each sample was quantified with a commercial ^{125}I-IRMA kit developed for human samples (BI-Ins IRMA kit; CIS Bio International Ltd.) and previously validated for bovine plasma samples (Kerestes et al., 2009; 2010). The minimum detectable concentration of free insulin with an acceptable level of precision was determined as 1 µUI/ml (7 pmol/l). The higher sensitivity of this method compared to a enzyme-labeled chemiluminescent immunometric assay was useful for a better determination of very low insulin levels typical of cows approaching calving as was expected in T0 samples. The result was reported as 7 pmol/l for statistical analysis whether a non detectable insulin level was found by the RIA determination. Samples were assayed in single aliquotes. The intra-assay CV was between 3.36 and 4.73%, the inter-assay CV was 4.06%. Quality controls were within the 95% confidence limits of the known concentrations.

4.6 Data Management and Statistical Analysis

The goal of this trial was to investigate the responses of animals to FGTT into different herds and verify the effect of cows' ability to respond to the glucose challenge on their energy balance and health status. Moreover, we tried the applicability of the test under field conditions, by comparison with indicators of insulin resistance and glucose tolerance commonly used in human medicine.

FGTT parameters and animals classification:

We decided to evaluate T80/T0 glucose ratios above 1.2 as threshold for considering cows that had difficulties in returning to basal glucose levels, not responding to glucose load. This threshold was already studied in a preliminary trial in late gestation cows by Gianesella and colleagues (unpublished data, 2009), and it appeared to be a valid cut-point to distinguish animals with altered haematological parameters of glucose and lipid metabolism before calving. Therefore the animals were divided in two groups according to their T80/T0 ratios measured by glucose meter pre-partum: cows with a T80/T0 lower or equal to 1.2 were included in class 0 (FGTT Week-1 class 0), whereas cows with a T80/T0 higher than 1.2 were classified as class 1 (FGTT Week-1 class 1).

Further classifications of animals were used in order to estimate the prevalence of certain illnesses after calving in every farm: each cow which experienced any retained placenta, milk fever, abomasal displacement, metritis, lameness or culling before 90 DIM was considered diseased (Disease class 1), otherwise cows were considered healthy (Disease class 0); either cows for which the first milk test highlighted an average SCC higher than 200.000 or cows affected by clinical mastitis in the first 30 DIM were classified as diseased (Mastitis class 1), compared to normal cows (Mastitis class 0). A percent of the animals treated with any pharmacological and anti-ketosic treatment within 10 DIM among cows enrolled in each farm was also calculated.

Insulin Resistance indexes calculation:

Model estimations of insulin resistance were calculated from samples collected at basal times (T0) in all weeks (Week -1, Week +1, Week +2) as follows:

1) revised quantitative insulin sensitivity check index (RQUICKI), as already used by other authors in the bovine species (Holtenius and Holtenius, 2007; Kerestes et al., 2009; Bossaert et al., 2009; Balogh et al., 2009; Kusenda, 2010) according to this formula: RQUICKI = 1/[log(G T0) + log(I T0) + log(NEFA T0)], where G T0 = basal laboratory glucose (mg/dL), I T0 = basal insulin (μU/mL), and NEFA T0 = basal NEFA (mmol/L).

2) modified homeostasis model assessment (HOMA-IR), as recently done in dairy cows by other researchers (Gellrich, 2012) according to this formula: HOMA-IR = (G T0 x I T0)/22.5, where G T0 = basal laboratory glucose (mmol/L), and I T0 = basal insulin (μU/mL).

Data analysis:

The data were compared with reference values from local laboratories and checked for outliers in excel database; outliers were excluded and all variables were screened for normality by calculation of Shapiro Wilk normality test. If necessary, the variables were transformed with the natural logarithm to achieve a normal distribution. Gross means were reported in the following chapter for specific weeks of sampling and expressed as means ± standard errors of the mean (SEM).

A preliminary evaluation was accomplished by checking the Pearson correlations between pre-partum field T80/T0 ratios and plasmatic NEFA and BHB concentrations both before and after calving. A FREQ procedure in SAS (version 9.2, SAS Inst. Inc., Cary, NC) was used to better evidence the relative risk of having illnesses and increased plasma NEFA and BHB for cows in FGTT Week-1 class 1. Subclinical ketosis was defined as a serum BHBA concentration of ≥ 1.4 mmol/l (Duffield, 2000). In our trial, cows that resulted in BHB higher than 1.4 mmol/l in Week +1 or +2 were grouped in BHB class 1, whereas the others were in BHB class 0. Levels of approximately 0.3 mEq/l of pre-partum NEFA and 0.7 mEq/l post-partum have recently been identified as critical limit for predicting diseases risk after calving (Ospina et al., 2010). We used 0.5 mEq/L of NEFA Week -1 and 1.0 mEq/l of NEFA Week +1 as thresholds for classification of animals: cows exceeding these values were included in NEFA class 1, otherwise they were in NEFA class 0.

An introductory analysis was performed in order to study the hematochemical parameters and the IR indexes before calving. For this purpose cows were divided in two pre-partum groups on the basis of their enrollment time relative to parturition: cows enrolled on 14 – 7 days before calving were distinguished from cows enrolled later than 7 days pre-partum. A multi-way analysis of variance (ANOVA) was applied using PROC GLM in SAS to primarily verify any herd or week sample effects: qualitative variables included herd, pre-partum week, disease and mastitis class, EVM and parity. Cows that had a EVM305 at first milk test < 9.500 kg were classified as low productive, a EVM305 of 9.500 – 11.123 kg were classified as fair, and a EVM305 > 11.123 kg were high productive animals. Cows were also grouped in two classes according to their lactation number by dividing secondiparous animals from older cows. Multiple comparisons by Bonferroni method were applied to determine if differences were statistically significant ($P < 0,05$). Particularly interesting results are reported as least square means (lsmean) ± pooled standard errors (PSE).

Secondly, all glucose and insulin measurements and IR indexes in Week -1 were evaluated by a multi-way ANOVA to especially test the effect of the FGTT class in Week -1, as defined above,

on the hematochemical results pre-partum. The model included herd, EVM, FGTT Week -1, mastitis, parity and disease class. EVM and parity classes were built following the same definitions as previously described. A similar statistic was repeated for testing glucose and insulin measurements at T10 and T80 in Week +1. Pre-partum and post-partum hematochemical results were considered as definitely separate findings. Because basal plasma metabolites, insulin and IR indexes were measured twice after parturition, for these baseline parameters a repeated-measures approach using ANOVA was used in SAS (PROC MIXED REPEATED) including animal effect. Qualitative variables consisted of herd, EVM, FGTT Week -1, mastitis, parity, disease and postpartum week classes. The covariance structure was a compound symmetry for repeated measurements on cows in postpartum weeks with 2 levels of classification (Week +1 and Week +2). Bonferroni adjustments for estimation of significative differences between means ($P < 0,05$) were applied. The most interesting and significative effects due to FGTT and herd classes have been pointed out as lsmean ± PSE.

Afterwards having found an effect of FGTT on IR indexes, we applied the calculation of R.O.C. curves (Receiver Operating Characteristic) to evaluate the validity of FGTT performed in Week -1 in predicting the occurrence of insulin resistance phenomenon in the first couple of weeks of lactation. The analysis was carried out by use of MedCalc Software, version 12.3.0 (Broekstraat 52, 9030 Mariakerke, Belgium). According to the R.O.C. theory, the classification of animals on the basis of a cut-off equal to 1.2 for T80/T0 ratios was compared to RQUICKI and HOMA determined in post-partum weeks. The Area Under the R.O.C. Curves (AUC), the Z test and the Youden Index were calculated both for RQUICKI and HOMA. Furthermore, best cut-off values of RQUICKI and HOMA for discerning animals belonging to FGTT class 0 or 1 (Week -1) were calculated and respective Sensitivity and Specificity for each index are reported. Final accuracy of the IR indexes compared to FGTT was estimated by interpreting their AUC and referring to the scheme quoted by Bottarelli and Parodi (2003) and initially proposed by Swets (1998) about quality of diagnostic tests.

5. Results

5.1 Field data

First data available in this trial were obtained by field analysis of glucose by glucose meter at all sample times (T0 – T10 – T80), followed by individual cow calculation of respective pre-partum T80/T0 ratio and classification as "lower than 1.2" (class 0 FGTT) or "higher than 1.2" (class 1 FGTT) in glucose tolerance test class. Hereafter, the glucose measured by glucometer will be reported as Field glucose and by laboratory as Lab glucose; the T80/T0 ratio derived by glucometer readings will be reported as Field glucose T80/T0 = FGTT and the ratio derived by laboratory analysis as Lab glucose T80/T0 = LGTT. Table 4 and Fig. 1 show the distribution of cows enrolled within each farm and their main features, included the frequencies of FGTT classes in the three different herds.

Table 4: distribution of total N° of cows tested in the herds, distribution of cows enrolled less than 7 days pre-partum and 2nd parity cows in the herds, average DIM, milk production and projection of EVM at 305 d at first milk test in each herd. Pooled standard errors (PSE) are reported for means.

Herd	A	B	C	
Animals				Totals
N° lactating cows	270	340	1100	
N° tested animals	32	35	34	101
Frequency (%)	31	34	33	33
N° tested ≤ 7 d pre-partum	13	25	20	58
Frequency (%)	41	71	59	57
N° animals parity 2	14	16	16	46
Frequency (%)	44	46	47	46
First milk test	mean	mean	mean	PSE
DIM at 1st test	30,8^{a}	23,5^{b}	20,5^{b}	2,9
Milk production at 1st test (kg)	41,2^{a}	40^{a}	32,8^{b}	1,8
EVM305 1st test (kg)	10761^{a}	10740^{a}	9480^{b}	272

a, b: means with different superscripts on the same line differ (P < 0,05).

Second lactation cows were evenly distributed between farms: herd A had 44% of parity 2 cows, herd B had 46% of parity 2 cows and herd C had 47% of parity 2 cows. 71% of cows in herd B were enrolled in the trial at less than 7 d pre-partum, 59% of cows in herd C and 41% in herd A but a significant effect of pre-partum week sub-class was not present on glucose and insulin concentrations (see more details following Table 7). Cows in herd A have higher DIM at their first milk test because many of them calved in July and were tested in early September since August test is normally skipped. However, a mature equivalent with proper adjustments for DIM and calving season was determined for better comparison of herds and cows' productions.

Various series of pie chart will be used to display frequencies into different herds: herd A will be colored in green, herd B will be colored in blue and herd C in red.
The percent of cows in class 1 for FGTT in Week -1 was similar within herd A (53%), and herd B (54%); it was slightly higher in herd C (59%). The average T80/T0 glucose ratio pre-partum in herd A was 1,174 ± 0,035; 1,246 ± 0,046 in herd B and 1,291 ± 0,045 in herd C but a significant difference was not found between herds.
As regard disease incidence (Fig. 2), herd A and C seemed to have comparable percentages of illnesses and mastitis cases (disease = 56% for herd A and 62% for herd C; mastitis = 50% for herd A and 41% for herd C). Cows in herd B were declared as clinically diseased in fewer cases: 37% of cows had diseases and 34% had mastitis. Nevertheless, the herd with the higher percentage of cows treated with pharmacological and anti-ketotic therapies within 10 DIM among tested animals, was herd C (47% versus 19% of herd A and 15% of herd B; Fig. 3).

Fig. 1: distribution of cows within FGTT Week -1 class 0 (cows with pre-partum Field T80/T0 ≤ 1.2) and class 1 (cows with pre-partum Field T80/T0 > 1.2) in each herd: N° of animals and respective frequency (%) within the farm are indicated for each class.

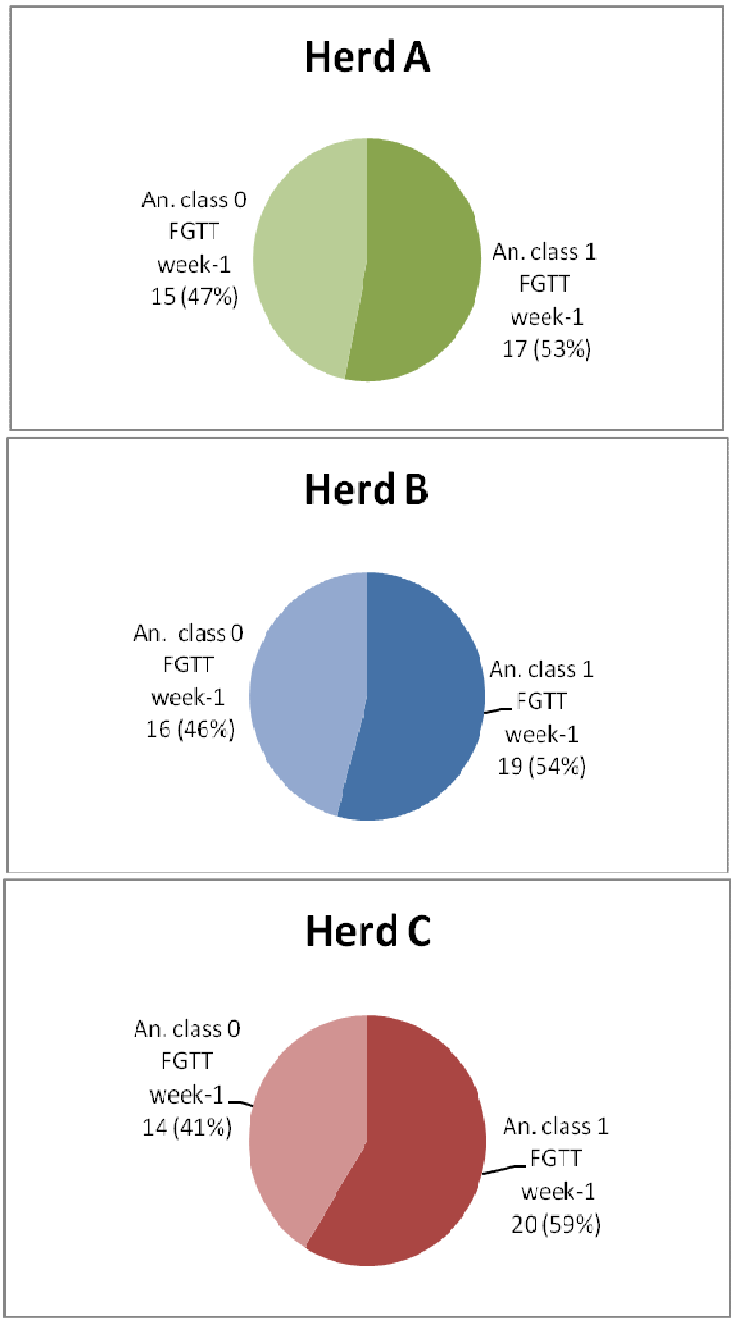


Fig. 2: distribution of cows within Disease class (charts A, C, E) and Mastitis class (charts B, D, F) in each herd: N° of animals and respective frequency (%) in class 0 and 1 within the farm are indicated for each class.

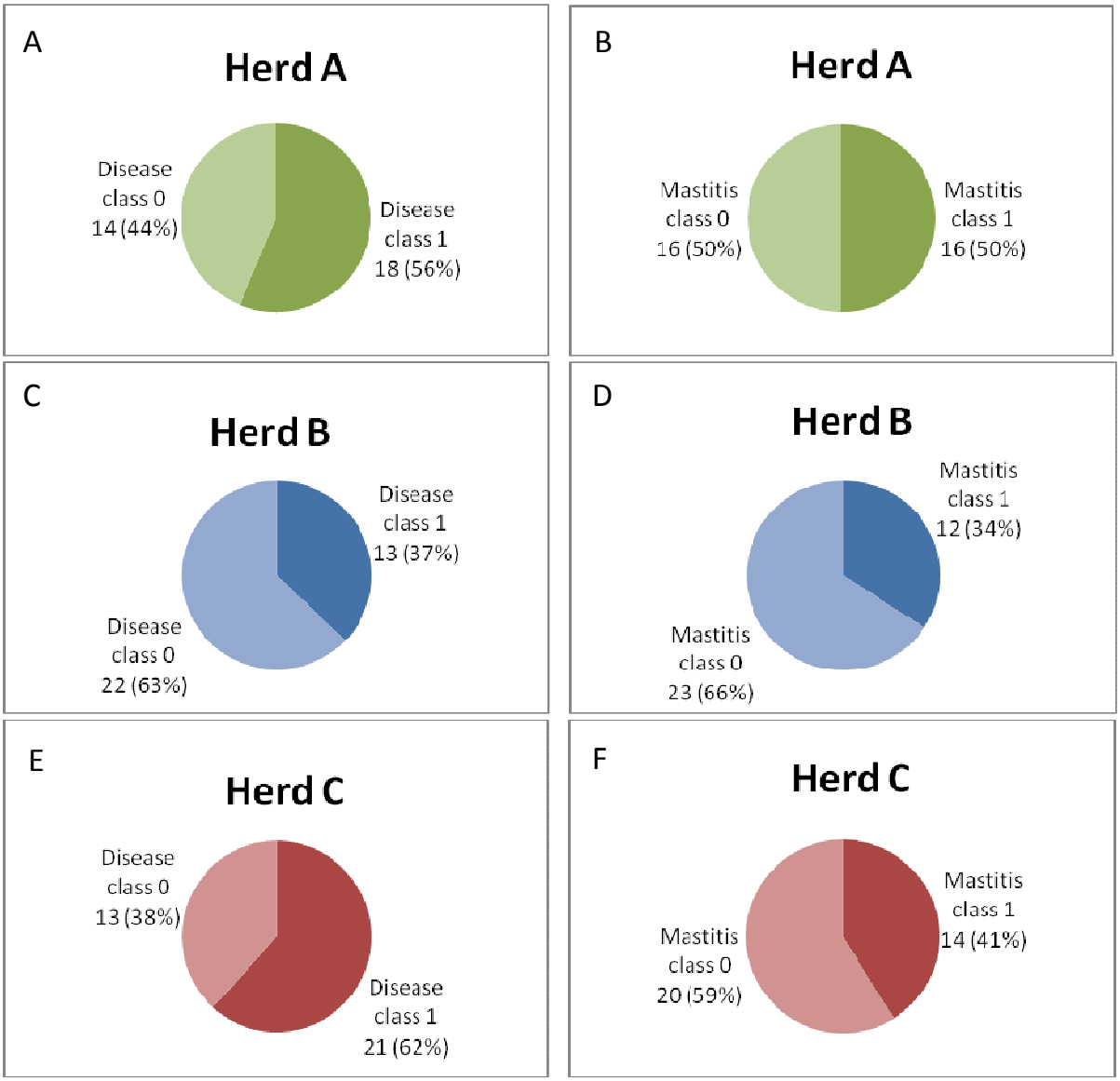
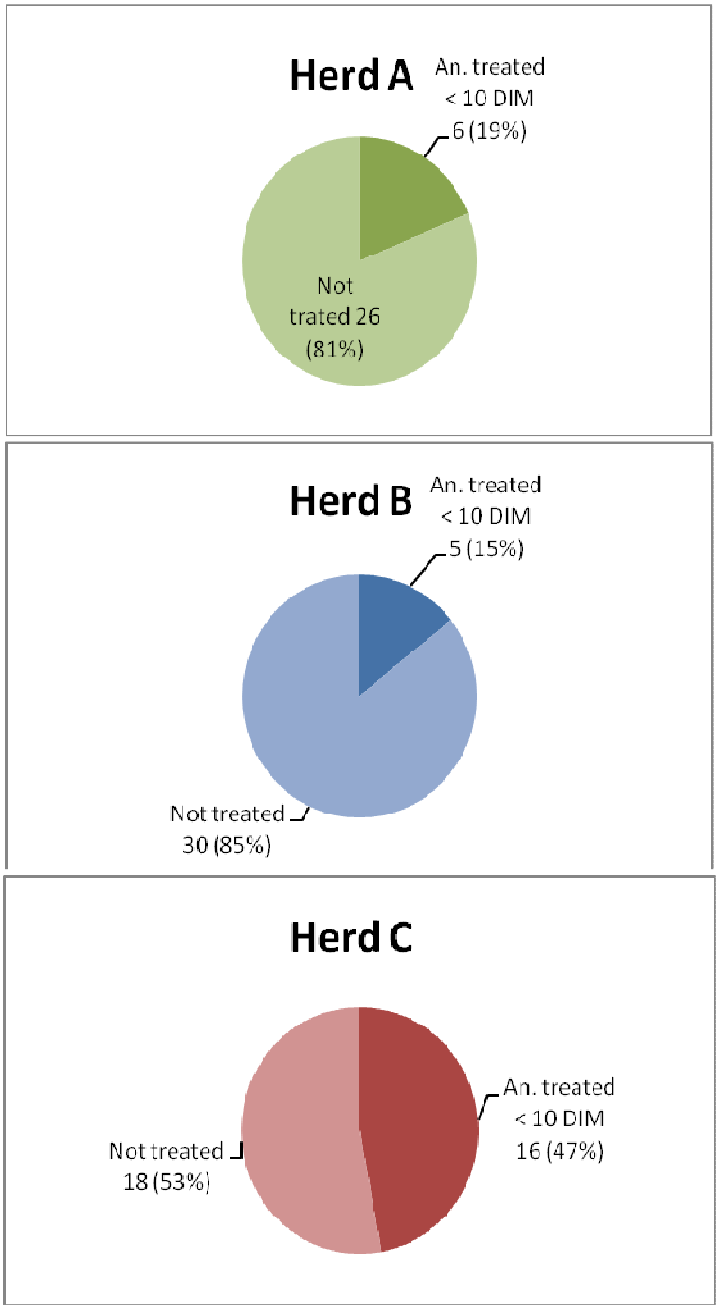


Fig. 3: N° of animals treated with any pharmacological and anti-ketosic treatment within 10 DIM and respective frequency (%) for each farm.



5.2 Hematochemical results:

Hematochemical parameters determined by laboratories have been reported as gross means within specific weeks of sampling relative to calving (Week -1 = 14-1 d pre-partum, Week +1 = 3-9 d post-partum, Week +2 = 10-16 d post-partum; Table 5). Field glucose average values have been included in the same table for completeness as well.

General outcomes were in line with expected hematochemical results from normal transition cows. Having several post-partum diseased animals in our trial and being interested in studying also those ones, a certain deviation of gross means due to pathological situations is acceptable and unavoidable. Reported reference ranges are taken from healthy animals and with 68% confidence interval, therefore they may apparently look quite restrictive for our sample but the presence of unhealthy cattle in our sturdy must be weighed. Glycaemia and insulinaemia tended to decrease after calving, with particular low glucose levels in Week +1, concurrently with the most frequent cases of hyper-ketonemia and other diseases. In reverse, NEFA and BHB concentrations peaked in Week +1, as already found by other authors (Bossaert et al., 2009). Calcium concentration was quite stable despite a slight decrease in Week +1 and +2 due to physiological hypocalcemia following parturition and onset of lactation in dairy cattle. This maintenance of calcemia trough the first days of lactation was confirmed by a very low number of milk fever cows registered in our survey (less than 3%) and could have been supported by preventive therapies with calcium boli and intravenous administration immediately after calving in weak cows, as actually routinely done by all farms. RQUICKI and HOMA indexes were rather constant in the first two weeks of lactation; pre-partum RQUICKI average values were similar to those determined after calving (0,57 – 0,60) whereas HOMA was slightly higher in the pre-partum weeks (0,53) compared to post-partum (0,41-0,43). Further, the GOT-AST is easily skewed since this parameter tends to become very high in cows suffering from any aspecific diseases. Additional reasons for variations and interpretation of this parameter will be better discussed in the next chapter.

Table 5: Average values of Field and Lab Glucose at different time samples, T80/T0 glucose ratios (Field and Lab T80/T0), Insulin, hematochemical parameters, RQUICKI and HOMA with relative standard errors of the mean (SEM) at different sampling intervals.

Parameter	Unit	week -1				week +1			week +2		
		reference values 23-7d prep. (§)	reference values < 7d prep. (§)	mean	SEM	reference values < 8d postp. (§)	mean	SEM	reference values 8-25d postp. (§)	mean	SEM
Field Glucose T0	mg/dl	-	-	48,36	0,85	-	43,21	1,37	-	44,70	1,05
Field Glucose T10	mg/dl	-	-	137,53	5,99	-	131,68	5,09	-	-	-
Field Glucose T80	mg/dl	-	-	72,40	7,59	-	54,51	3,14	-	-	-
Field T80/T0	-	-	-	1,24	0,03	-	1,24	0,04	-	-	-
Lab Glucose T0	mg/dl	55 - 66	52 - 67	62,68	6,30	53 - 73	51,38	1,61	53 - 69	69,23	19,02
Lab Glucose T10	mg/dl	-	-	162,74	15,06	-	159,87	19,84	-	-	-
Lab Glucose T80	mg/dl	-	-	86,97	13,17	-	62,00	3,53	-	-	-
Lab T80/T0	-	-	-	1,19	0,03	-	1,20	0,04	-	-	-
Insulin T0	pmol/l	-	-	23,50	1,42	-	20,01	3,76	-	19,13	1,82
Insulin T10	pmol/l	-	-	351,28	32,60	-	245,60	24,43	-	-	-
Insulin T80	pmol/l	-	-	38,76	6,27	-	28,14	5,61	-	-	-
BHB	mmol/l	0,25 - 0,42	0,33 - 0,51	0,53	0,04	0,35 - 0,62	1,21	0,11	0,32 - 0,60	1,04	0,10
NEFA	meq/l	0,08 - 0,36	0,00 - 0,54	0,42	0,03	0,58 - 1,00	0,80	0,04	0,35 - 0,88	0,66	0,03
Albumin	g/l	36 - 41	34 - 41	34,65	0,34	34,0 - 42,7	33,44	0,37	34 - 43	32,58	0,37
Globulin	g/l	34 - 43	27 - 39	43,07	0,69	34 - 43	46,21	0,70	38 - 48	48,52	0,67
Total Proteins	g/l	72 - 82	64 - 76	77,73	0,74	71 - 83	79,66	0,82	75 - 90	81,11	0,74
GOT - AST	u/l	45 - 72	50 -69	80,79	2,36	68 - 100	147,62	7,42	64 - 90	136,48	7,23
GPT - ALT	u/l	11 - 23	12 - 22	11,42	0,69	13 - 23	12,05	0,48	15 - 25	12,34	0,63
Urea	mg/dl	15,7 - 25,6	17,7 - 27,0	30,74	0,95	19,2 - 32,4	30,52	1,56	19,4 - 32,2	28,23	1,36
Calcium	mg/dl	8,92 - 9,60	8,64 - 9,64	9,84	0,08	7,96 - 9,36	9,63	0,09	8,6 - 9,52	9,65	0,08
Chlorine	mmol/l	103 - 108	105 - 110	105,19	0,34	100 - 107	101,84	0,31	99 - 104	100,70	0,31
Phosphorus	mg/dl	5,76 - 7,50	4,90 - 6,90	6,64	0,15	4,19 - 6,12	6,07	0,15	4,59 - 6,67	5,90	0,16
Magnesium	mg/dl	1,99 - 2,50	1,94 - 2,43	2,46	0,05	0,79 - 1,06	2,21	0,04	2,18 - 2,77	2,41	0,05
Potassium	mmol/l	3,93 - 4,58	3,86 - 4,71	3,73	0,05	3,70 - 4,44	3,43	0,06	3,66 - 4,34	3,29	0,06
Sodium	mmol/l	140 - 145	143 - 148	142,92	0,30	137 - 145	141,87	0,32	136 - 142	140,72	0,41
RQUICKI	-	-	-	0,60	0,01	-	0,58	0,01	-	0,57	0,01
HOMA	-	-	-	0,53	0,07	-	0,41	0,09	-	0,43	0,07

§) Reference values for multiparous healthy cows from Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna "Bruno Ubertini" (IZSLER), within 68% confidence intervals and specific periods (23-7 days pre-partum; < 7 days pre-partum; < 8 days post-partum; 8-25 days post-partum).

Fig. 4: distribution of cows in NEFA class in Week-1 (charts A, C, E) and NEFA class in Week +1 (charts B, D, F) in each herd: N° of animals and respective frequency (%) in class 0 and 1 within the farm are indicated for each class.

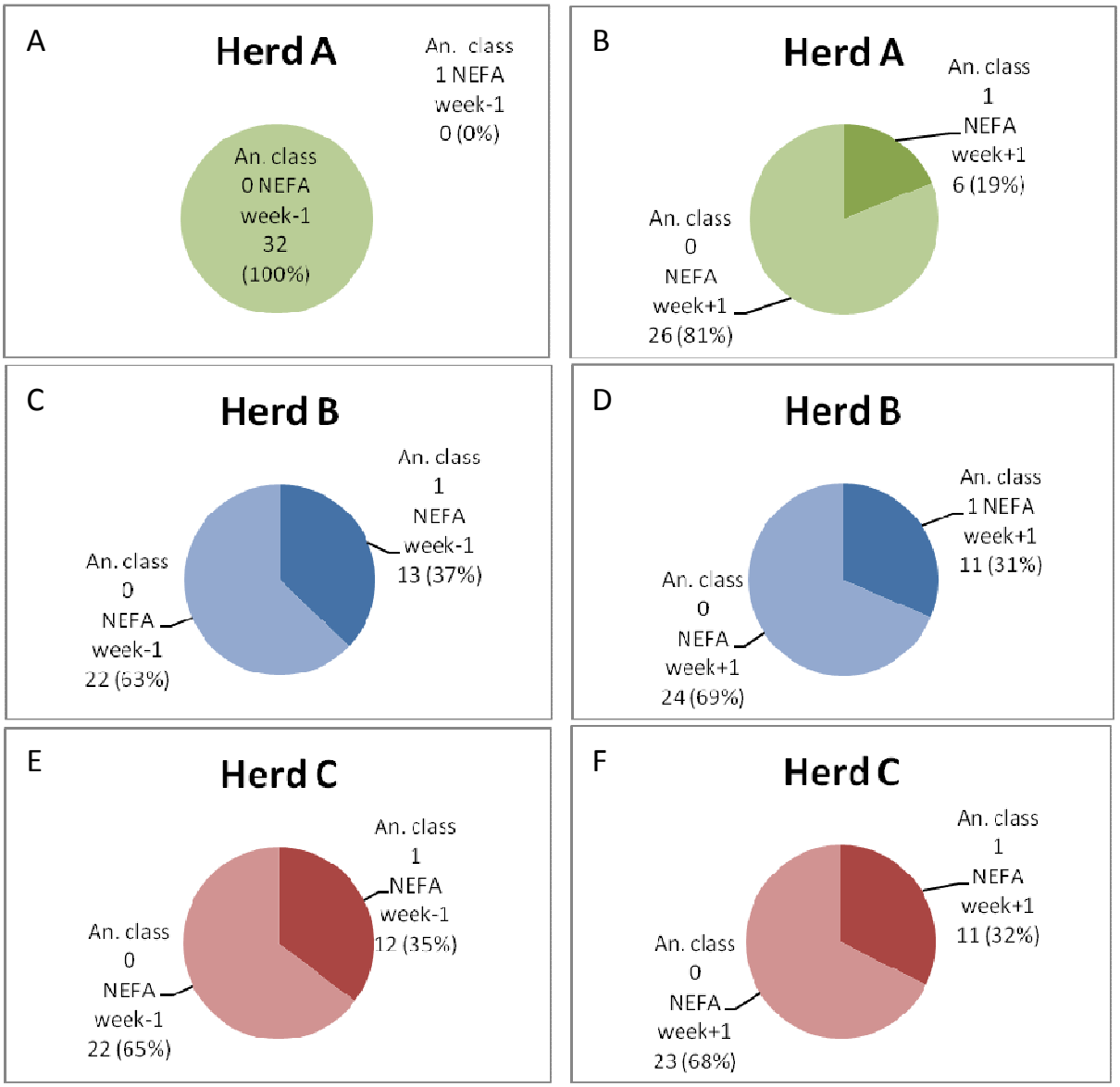


Fig. 5: distribution of cows in BHB class in Week+1 and/or +2 in each herd: N° of animals and respective frequency (%) in class 0 and 1 within the farm are indicated for each class.

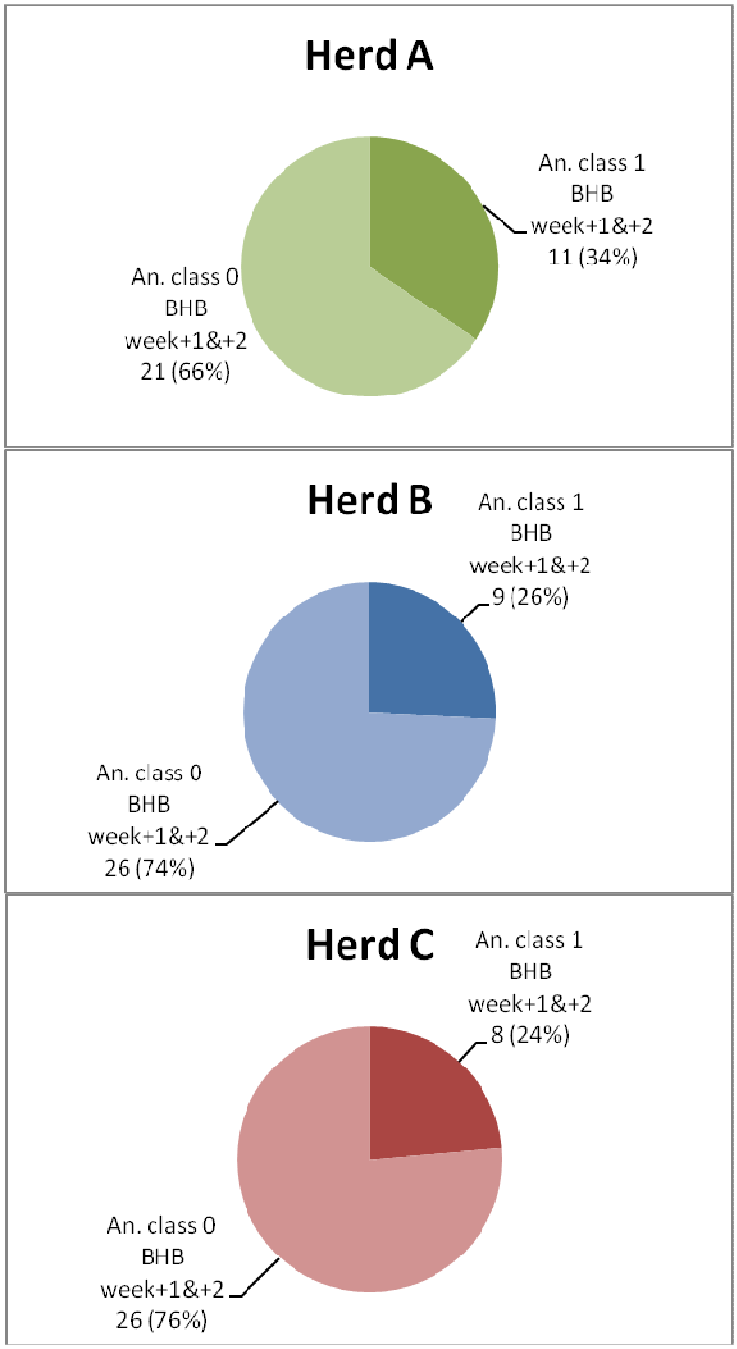
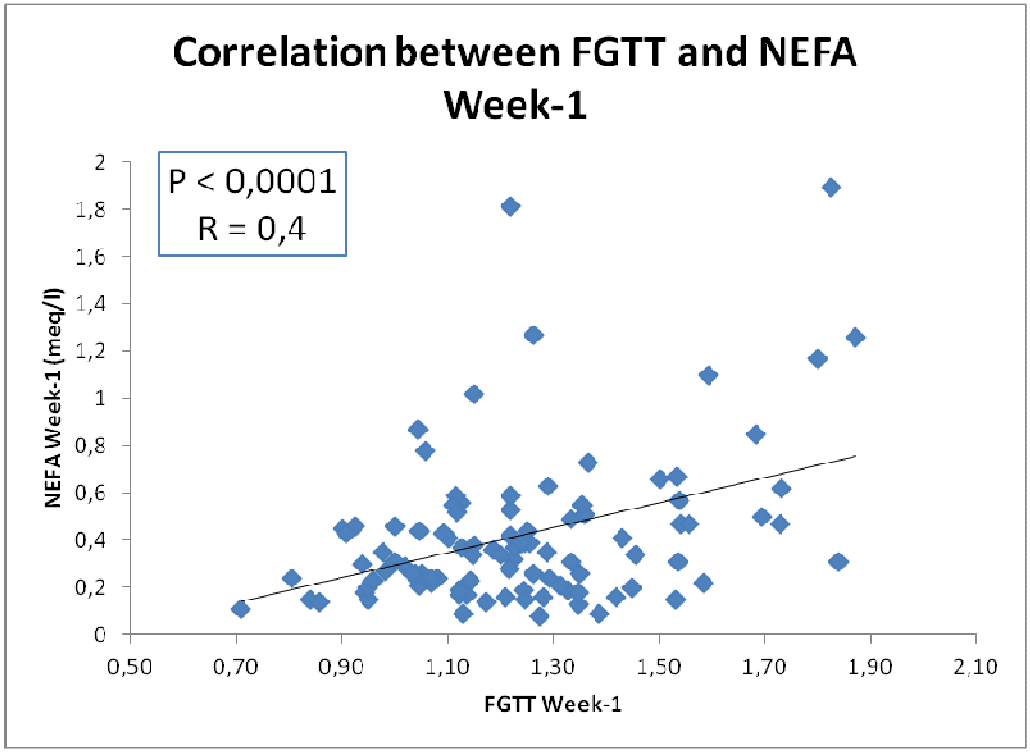


Fig. 6: Correlation between Field Glucose T80/T0 ratios (FGTT) and NEFA in Week-1.



A simple correlation between the FGTT ratios and NEFA was determined. A significant relationship was observed in Week -1 as shown in Fig. 6 ($R = 0,4$; $P < 0,0001$). Neither a significant correlation between the FGTT pre-partum and the FGTT post-partum was found, nor between the FGTT in Week -1 and NEFA or ketonemia in Week +1/+2. The FGTT in Week +1 was further compared to NEFA and BHB in the same post-partum week with no interesting results. If considering laboratories glucose measurements, the correlation coefficient R between LGTT and NEFA in Week -1 was 0,28 ($P < 0,0001$). General correlation coefficient R between LGTT and FGTT was 0,5 independently of week sample ($P < 0,0001$).

The Relative Risk (RR) of cows in FGTT class 1 pre-partum to be classified as NEFA class 1 cows pre-partum (class 1 = NEFA > 0,5 meq/l) was 2,18 (95% CI = 1,00 – 4,75; $P < 0,05$). Other risks of developing hyper-ketonemia, diseases and mastitis after calving for cows with high pre-partum NEFA were verified but no significance was observed. Only cows with NEFA higher than 1 meq/l in Week +1 had a significant risk of having high BHB in the same week sample (RR = 6,9; 95% CI = 2,70 – 17,35; $P < 0,001$).

Table 6: Relative Risk (RR) of cows in NEFA or FGTT class 1 pre-partum ("Risk cases") to be classified as Disease, Mastitis, BHB or NEFA class 1 cows post-partum ("Diseased cases"). All tested cases with respective significance levels (P) and confidence intervals (CI) are reported. In addition, the RR of cows in NEFA class 1 in Week+1 to be in BHB class 1 after calving was verified. The RR of cows in FGTT class 1 in Week-1 to be in NEFA class 1 before calving was also considered. NS = Not Significant (P < 0,05).

Risk cases	Diseased cases	P	RR	CI
NEFA week-1	Disease	NS	1,35	0,93 - 1,97
NEFA week-1	Mastitis	NS	1,22	0,74 - 1,99
NEFA week-1	BHB week+1 and +2	NS	1,77	0,98 - 3,18
NEFA week+1	BHB week+1	< 0,001	6,9	2,70 - 17,35
FGTT week-1	Disease	NS	1,32	0,88 - 1,96
FGTT week-1	BHB week+1 and +2	NS	0,73	0,41 - 1,30
FGTT week-1	BHB week+1	NS	0,64	0,28 - 1,45
FGTT week-1	NEFA week-1	0,037	2,18	1,00 - 4,75
FGTT week-1	NEFA week+1	NS	1,02	0,54 - 1,89

A preliminary analysis of Week -1 hematochemical parameters revealed a similar pattern for insulin and NEFA in herds B and C, with average higher NEFA and lower insulin compared to herd A, as shown in Fig.1. Lower Urea, GOT-AST and GPT-ALT were also found both in herd B and C compared to herd A (P<0,001). Albumin and Total proteins were lower in herd C compared to herds A and B (P<0,001) (Table 7 and Fig. 7, 8, 9).

Only means for herd class have been shown in the table because, as initially mentioned, other significant effects of pre-partum week, parity, evm and diseases classes were not evident. Nevertheless, glycaemia at T80 in Week -1 was higher in cows enrolled less than 7 d pre-partum (least squared means are 59,45 ± 14,14 in cows enrolled 14-7 d pre-partum and 90,04 ± 12,47 in cows enrolled ≤ 7 d pre-partum; P = 0,1065). Otherwise, glycaemia at T0 in the two pre-partum weeks sub-classes was rather similar (least square means are 48,33 ± 1,49 at 14-7 d pre-partum and 49,06 ± 1,32 ≤ 7 d pre-partum, P = 0,7161). Also, cows enrolled ≤ 7 d pre-partum showed lower insulin at T0 (22,33 ± 1,89 compared to 25,62 ± 2,17 at 14-7 d pre-partum, P = 0,2635) and T10 (307,68 ± 50,02 compared to 453,62 ± 56,72 at 14-7 d pre-partum, P = 0,0557).

Significantly higher plasma NEFA appeared in the same cows (least squared means are 0,49 ± 0,04 compared to 0,34 ± 0,05 at 14-7 d pre-partum, P = 0,0289).

Table 7: Least squared means (lsmean) and pooled standard errors (PSE) for basal pre-partum Field Glucose, Insulin and hematochemical parameters in different Herds, and statistical significance observed. Only significantly different lsmeans have been reported (P < 0,05).

Parameters	Unit	FValue	P	Herd			PSE
				A	B	C	
Field Glucose T0	mg/dl	3,61	0,032	50,10	44,98^{b}	51,02^{a}	1,73
Insulin T0	pmol/l	5,47	0,006	30,87^{e}	17,69^{f}	19,68^{f}	2,56
NEFA	meq/l	7,35	0,001	0,25^{f}	0,55^{e}	0,47^{e}	0,06
Albumin	g/l	14,82	< 0,0001	34,76^{e}	36,66^{e}	32,31^{f}	0,56
Total proteins	g/l	6,48	0,003	80,14^{c}	80,25^{c}	73,68^{d}	1,45
GOT-AST	u/l	8,91	0,0003	89,48^{e}	71,81^{f}	70,18^{f}	3,42
GPT-ALT	u/l	11,62	< 0,0001	16,34^{e}	9,56^{f}	9,69^{f}	1,14
Urea	mg/dl	17,27	< 0,0001	38,68^{e}	27,66^{f}	26,54^{f}	1,61
Chlorine	mmol/l	12,69	< 0,0001	105,85^{e}	107,15^{e}	102,95^{f}	0,60
Sodium	mmol/l	12,53	< 0,0001	144,29^{e}	143,33^{e}	140,48^{f}	0,54

ab, means with different superscripts on the same line differ with P < 0,05; cd P < 0,01; ef P < 0,001.

Fig. 7: Least squared means for basal Field Glucose, Insulin and NEFA in Week-1 in Herd class. Means with different letters between herds differ as indicated by superscripts in table 7.

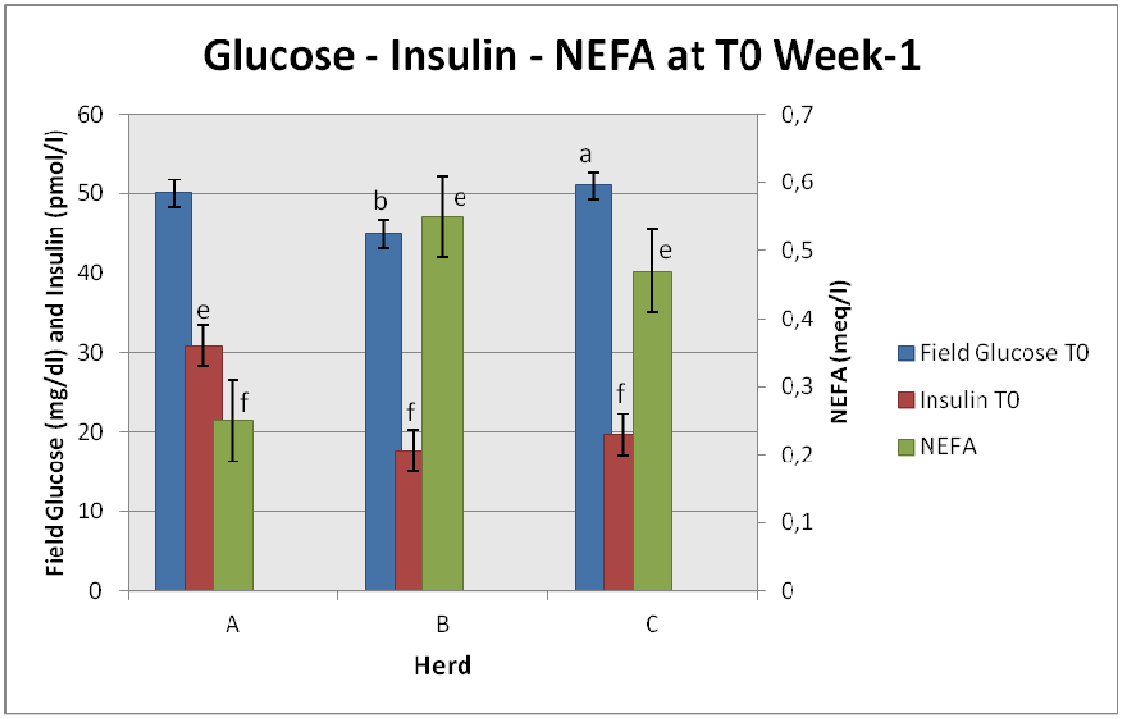


Fig. 7: Least squared means for basal Albumin, Urea and Total Proteins in Week-1 in Herd class. Means with different letters between herds differ as indicated by superscripts in table 7.

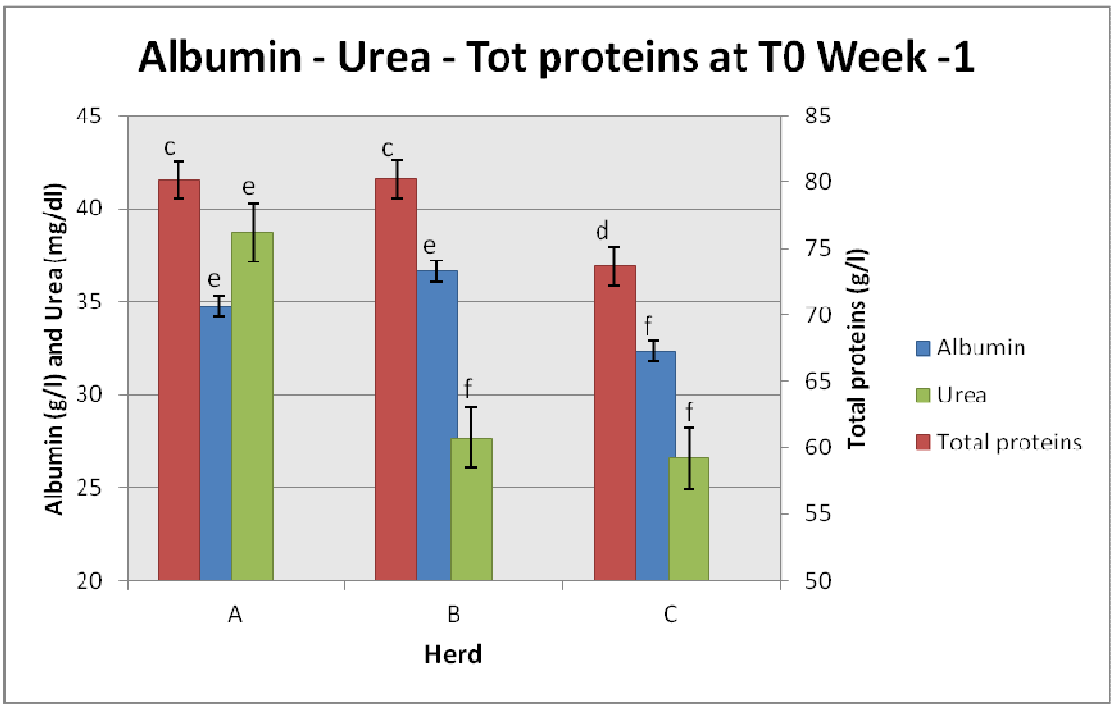


Fig. 8: Least squared means for basal GOT-AST and GPT-ALT in Week-1 in Herd class. Means with different letters between herds differ as indicated by superscripts in table 7.

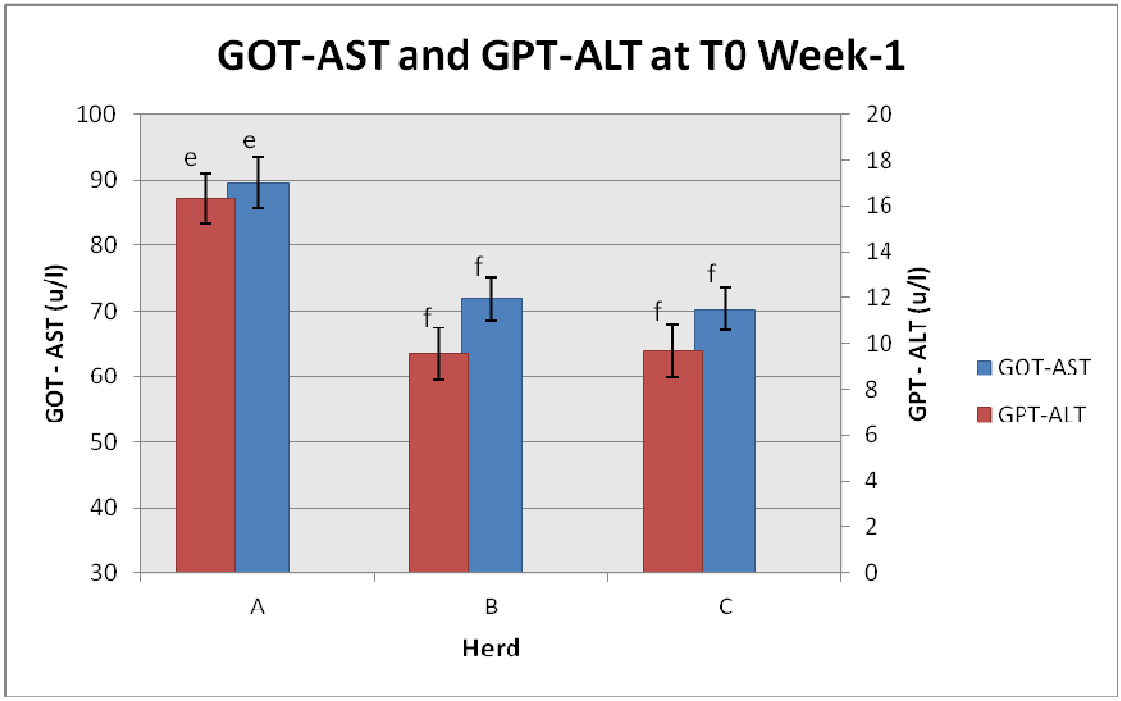


Table 8: Statistical significances observed for pre-partum Field and Lab Glucose determinations and Insulin at different time samples, for RQUICKI and HOMA, and for post-partum Field and Lab Glucose and Insulin at T10 and T80, within Herd, EVM, FGTT Week-1, Mastitis, Parity and Disease Classes. NS = Not Significant (P < 0,05).

	Parameter	Herd Class		EVM Class		FGTT Week-1 Class		Mastitis Class		Parity Class		Disease Class	
		FValue	P	FValue	P	FValue	P	FValue	P	FValue	P	FValue	P
Week-1	Field Glucose T0	3,082	NS	0,066	NS	5,719	0,019	0,766	NS	0,384	NS	0,444	NS
	Field Glucose T10	1,419	NS	0,216	NS	1,018	NS	1,296	NS	3,300	NS	0,585	NS
	Field Glucose T80	4,987	0,009	0,115	NS	31,468	< 0,0001	1,572	NS	3,378	NS	0,669	NS
	Lab Glucose T0	0,188	NS	0,311	NS	0,228	NS	0,586	NS	1,480	NS	0,025	NS
	Lab Glucose T10	0,591	NS	1,007	NS	0,302	NS	1,068	NS	0,706	NS	0,007	NS
	Lab Glucose T80	0,0067	NS	0,328	NS	1,723	NS	0,841	NS	0,437	NS	0,915	NS
	Insulin T0	7,105	0,001	2,915	NS	0,101	NS	0,017	NS	0,191	NS	0,012	NS
	Insulin T10	0,89	NS	0,327	NS	9,007	0,004	0,000	NS	0,011	NS	0,000	NS
	Insulin T80	0,255	NS	1,262	NS	0,003	NS	0,840	NS	0,663	NS	0,002	NS
	RQUICKI	2,743	NS	0,848	NS	1,113	NS	0,041	NS	1,961	NS	0,249	NS
	HOMA	5,82	0,004	1,595	NS	0,059	NS	0,082	NS	0,151	NS	0,313	NS
Week+1	Field Glucose T10	1,507	NS	0,848	NS	0,034	NS	3,125	NS	0,424	NS	0,024	NS
	Field Glucose T80	0,009	NS	0,228	NS	0,001	NS	0,049	NS	0,072	NS	0,819	NS
	Lab Glucose T10	0,368	NS	0,855	NS	0,011	NS	0,978	NS	0,416	NS	0,777	NS
	Lab Glucose T80	0,816	NS	0,428	NS	0,07	NS	0,007	NS	0,003	NS	0,298	NS
	Insulin T10	0,173	NS	2,905	NS	0,028	NS	0,66	NS	2,326	NS	0,269	NS
	Insulin T80	1,702	NS	4,266	0,018	0,839	NS	0,029	NS	0,981	NS	0,001	NS

Table 9: Statistical significances observed and cow effect (%) over post-calving repeated T0 Field and Lab Glucose determinations, Insulin, hematochemical parameters, RQUICKI and HOMA within Herd, EVM, FGTT Week-1, Mastitis, Parity, Disease and Postpartum week Classes. NS = Not Significant (P < 0,05).

Parameter	Herd Class		EVM Class		FGTT Week-1 Class		Mastitis Class		Parity Class		Disease Class		Postpartum week Class		Cow effect (%)
	FValue	P	FValue	P	FValue	P	FValue	P	FValue	P	FValue	P	FValue	P	
Field Glucose T0	3,237	0,045	3,933	0,024	1,333	NS	0,049	NS	0,334	NS	0,000	NS	0,143	NS	22
Lab Glucose T0	5,829	0,004	4,926	0,010	3,289	NS	0,018	NS	4,538	NS	0,020	NS	0,446	NS	17
Insulin T0	0,860	NS	1,382	NS	2,933	NS	2,054	NS	0,425	NS	0,836	NS	0,003	NS	3
BHB	1,765	NS	0,600	NS	0,873	NS	0,323	NS	4,747	0,032	1,216	NS	5,986	0,017	44
NEFA	2,259	NS	0,737	NS	0,497	NS	0,017	NS	8,178	0,005	0,000	NS	12,295	0,0007	37
Albumin	25,324	<0,0001	2,105	NS	5,368	0,023	0,153	NS	0,497	NS	2,086	NS	8,101	0,006	22
Globulin	1,261	NS	10,007	0,0001	0,217	NS	2,221	NS	2,099	NS	1,449	NS	13,904	0,0003	44
Total Proteins	9,357	0,0002	5,531	0,006	0,330	NS	1,517	NS	2,796	NS	0,251	NS	1,896	NS	14
GOT - AST	0,114	NS	1,214	NS	0,003	NS	0,000	NS	0,036	NS	1,066	NS	1,406	NS	49
GPT - ALT	4,478	0,015	1,177	NS	0,485	NS	3,208	NS	0,789	NS	1,354	NS	0,433	NS	42
Urea	0,443	NS	0,995	NS	0,276	NS	2,677	NS	1,228	NS	1,225	NS	4,668	0,034	57
Calcium	8,641	0,0004	1,804	NS	2,372	NS	0,084	NS	0,342	NS	0,496	NS	0,018	NS	13
Chlorine	9,423	0,0002	0,144	NS	1,419	NS	1,059	NS	0,045	NS	6,367	0,014	6,181	0,015	<0.01
Phosphorus	14,213	<0,0001	1,458	NS	0,024	NS	1,910	NS	2,489	NS	1,527	NS	2,394	NS	31
Magnesium	6,593	0,002	0,458	NS	0,591	NS	0,427	NS	0,304	NS	0,682	NS	8,945	0,004	35
Potassium	1,620	NS	0,762	NS	0,364	NS	0,027	NS	0,000	NS	1,532	NS	4,615	0,035	36
Sodium	2,229	NS	1,653	NS	0,401	NS	2,346	NS	0,528	NS	1,979	NS	5,195	0,025	22
RQUICKI	6,116	0,003	1,318	NS	6,280	0,014	0,363	NS	0,969	NS	0,006	NS	1,620	NS	20
HOMA	1,421	NS	2,358	NS	6,465	0,013	2,557	NS	0,134	NS	1,669	NS	0,038	NS	<0.01

A summary of the statistics performed by inclusion of the FGTT class was proposed in table 8 and 9 above. The most physiologically interesting and significant effects due to FGTT and herd classes have been pointed out in table 10. Differences in T80/T0 ratios between class 0 and 1 of FGTT Week-1 were given by both lower T0 and higher T80 glucose concentrations (Fig. 9). In the pre-partum weeks a deficiency in FGTT response was accompanied by lowered levels of plasma insulin at T10 (class 0 FGTT = 350,72, class 1 FGTT = 180,73), whereas the same difference was not noted at T10 in Week +1 (class 0 FGTT = 261,07 ± 38,57, class 1 FGTT = 270,27 ± 36,37; P > 0,05) (Fig. 12). Cows with pre-partum T80/T0 ratios > 1.2 interestingly had higher RQUICKI and lower HOMA indexes for the first couple of weeks after calving as displayed in Fig. 15. Herds effects over FGTT parameters and IR indexes were confirmed both pre and post-partum. Herd B and C could be qualified as more "insulin resistant" if compared to herd A on the basis of their post-partum RQUICKI indexes (Fig. 13). In particular, herd C also showed higher glucose concentrations at T0 in Week +1 against the other two farms. On the contrary the same herds B and C exhibited a higher insulin sensitivity pre-partum according to their HOMA indexes (Fig 11), which were lower than herd A, and were both characterized by lower basal insulin levels. A comment about this point will be given in the discussion.

Table 10: Least squared means (lsmean) and pooled standard errors (PSE) of Field Glucose, Insulin, Albumin, HOMA and RQUICKI for most physiologically interesting pre and post-partum effects due to FGTT Week-1 class and Herd class, and statistical significance observed. Only significantly different lsmeans have been reported; NS = Not Significant (P < 0,05). FGTT class 0 = cows with field T80/T0 ≤ 1.2; FGTT class 1 = cows with field T80/T0 > 1.2.

Parameter		Unit	FGTT Week-1 Class		Herd			PSE
			0	1	A	B	C	
Week -1	Field Glucose T0	mg/dl	51,32^{c}	46,70^{d}	NS	NS	NS	1,32
	Field Glucose T80	mg/dl	52,30^{f}	64,78^{e}	57,05	54,75^{d}	63,24^{c}	1,73
	Insulin T0	pmol/l	NS	NS	31,76^{e}	21,16^{f}	19,14^{f}	2,45
	Insulin T10	pmol/l	350,72^{c}	180,73^{d}	NS	NS	NS	40,53
	HOMA	-	NS	NS	0,53^{c}	0,31^{d}	0,30^{d}	0,04
Week +1/+2	Field Glucose T0	mg/dl	NS	NS	42,40^{b}	43,60^{b}	48,40^{a}	1,77
	Albumin	g/l	32,20^{b}	33,40^{a}	33,90^{e}	34,40^{e}	30,20^{f}	0,43
	RQUICKI	-	0,59^{a}	0,55^{b}	0,61^{c}	0,54^{d}	0,55^{d}	0,02
	HOMA	-	0,29^{d}	0,62^{c}	NS	NS	NS	0,10

ab, means with different superscripts on the same line, within FGTT or Herd class, differ with P < 0,05; cd P < 0,01; ef P < 0,001.

Fig. 9: Least squared means for Field Glucose at T0 and T80 in Week-1 within FGTT Week-1 class. Means with different letters between class 0 and class 1 differ as indicated by superscripts in table 10.

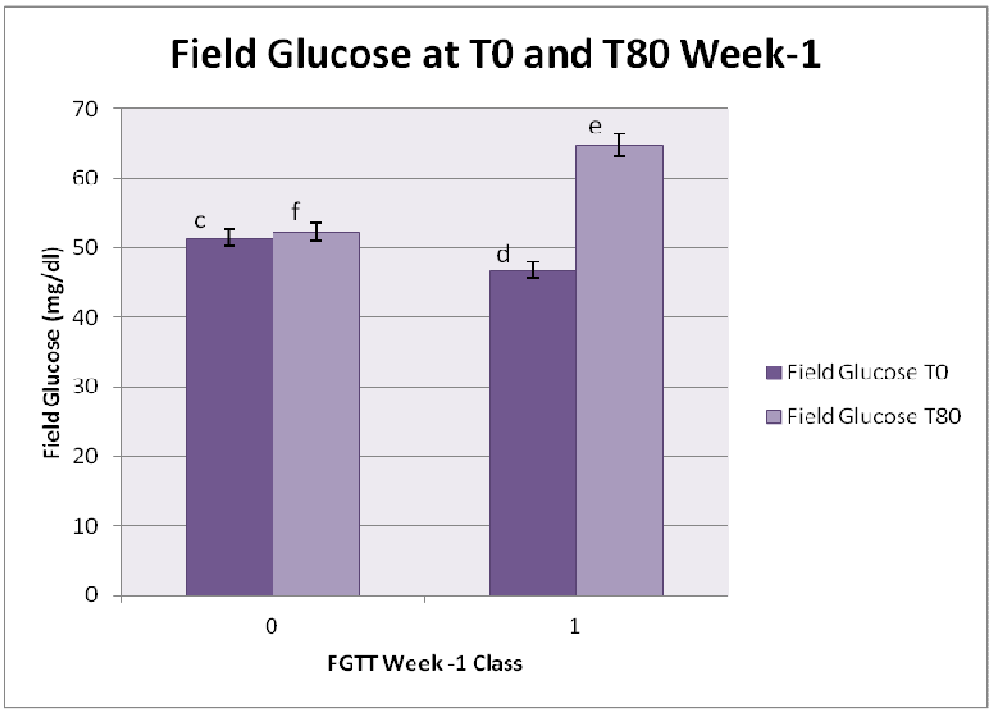


Fig. 10: Least squared means for Insulin at T0 in Week-1 within Herd class. Different letters between herds indicate a significant difference as indicated by superscripts in table 10.

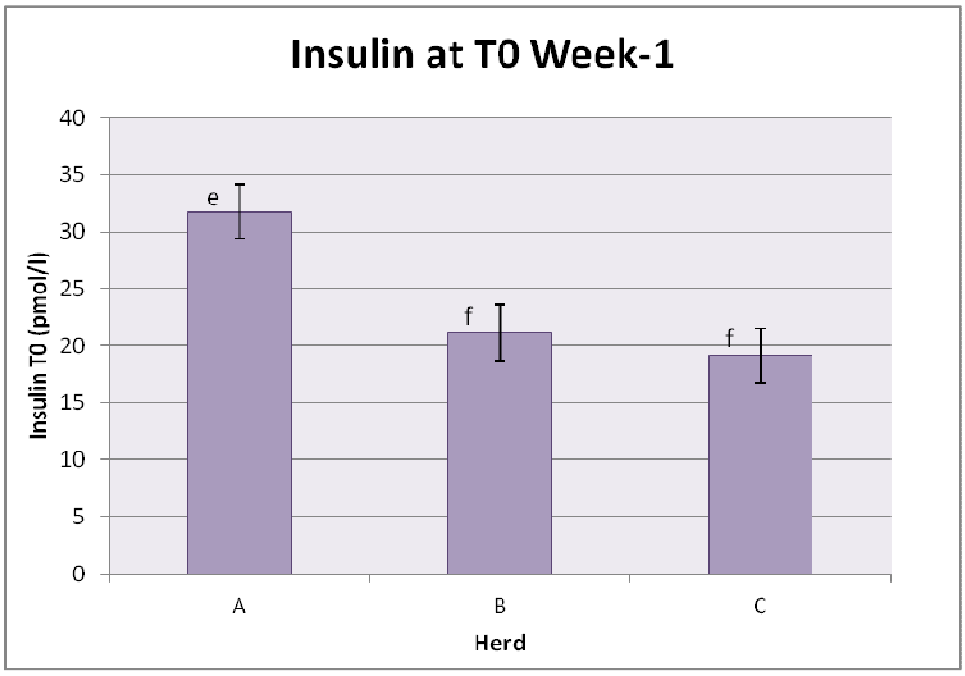


Fig. 11: Least squared means for HOMA in Week-1 within Herd class. Different letters between herds indicate a significant difference as indicated by superscripts in table 10.

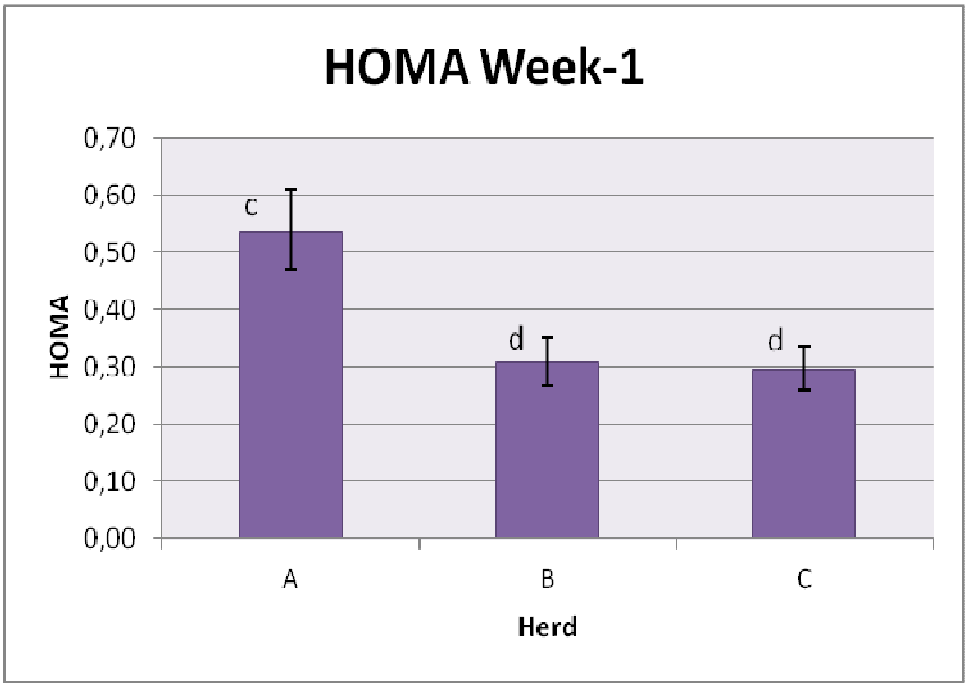


Fig. 12: Least squared means for Insulin at T10 in Week-1and Week+1 within FGTT Week-1 class. Means with different letters between class 0 and class 1 differ as indicated by superscripts in table 10.

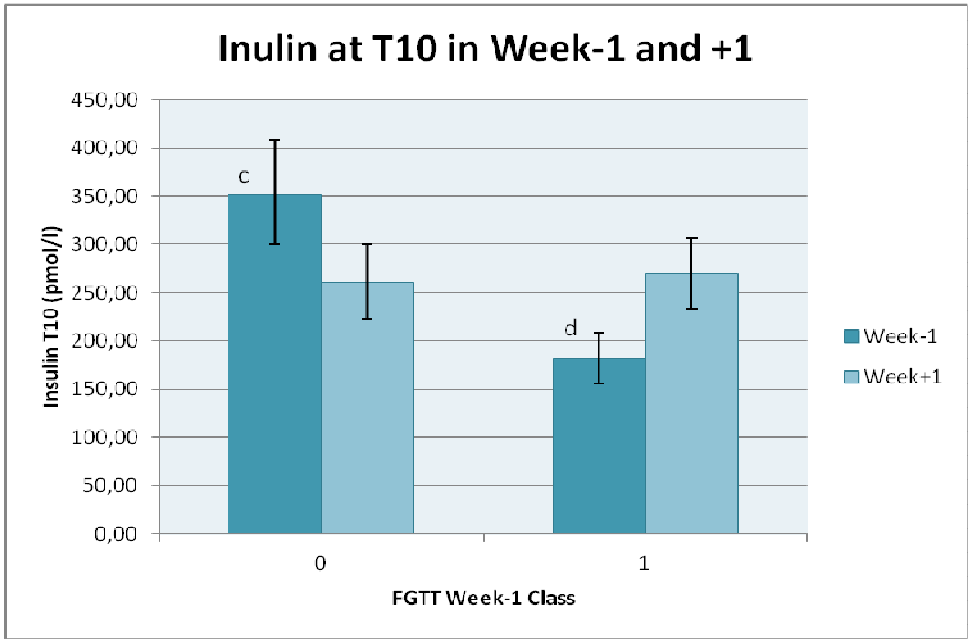


Fig. 13: Least squared means for post-partum RQUICKI and Field Glucose at T0 in different herds. Means with different letters between herds differ as indicated by superscripts in table 10.

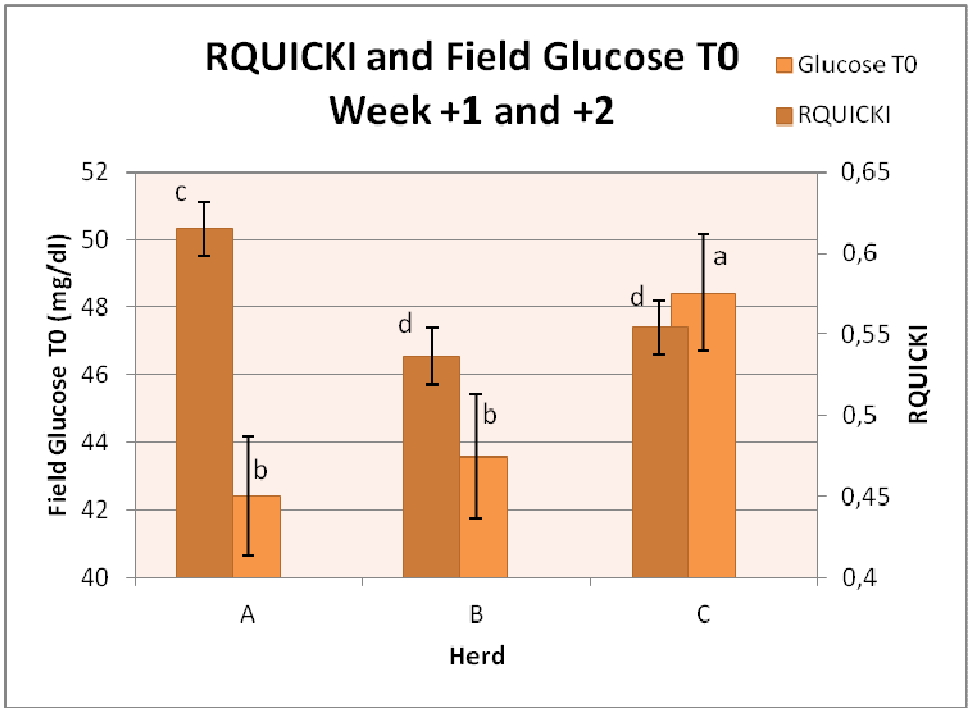


Fig. 14: Least squared means for post-partum Albumin concentration at T0 in different herds. Means with different letters between herds differ as indicated by superscripts in table 10.

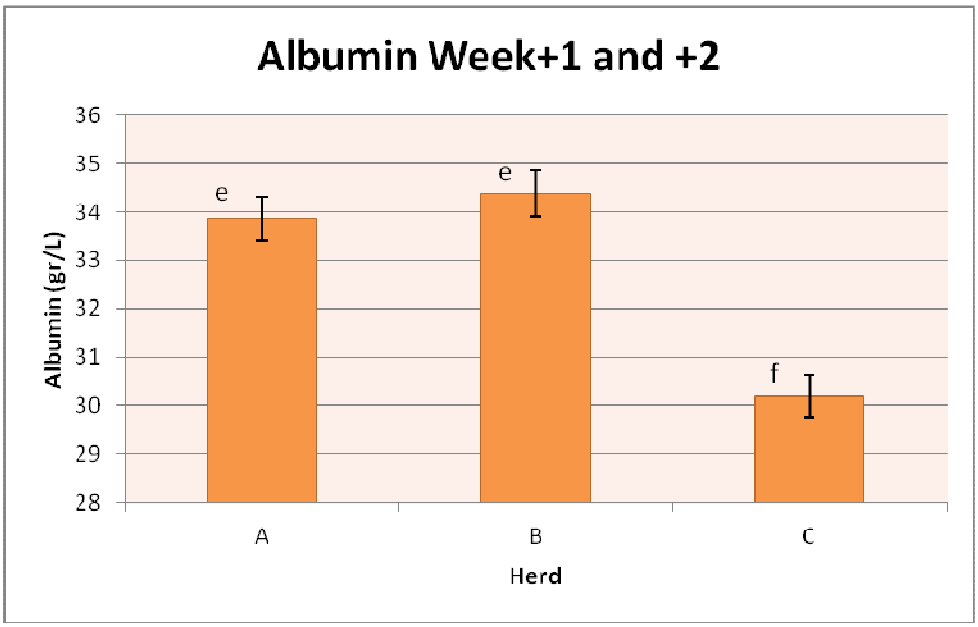
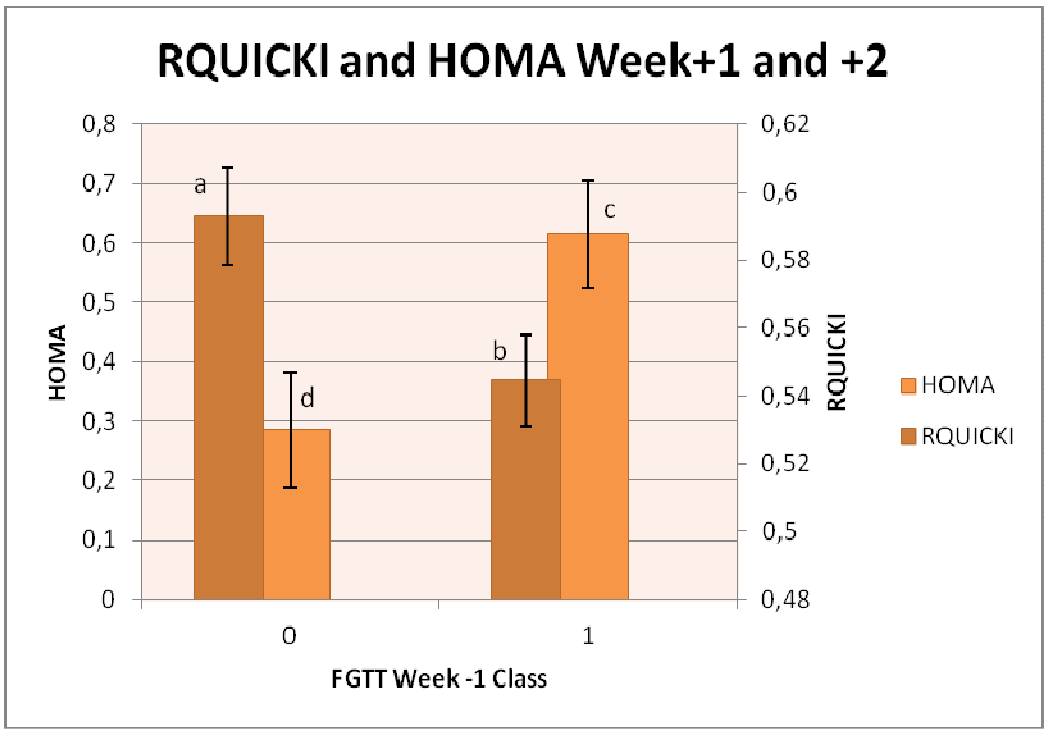


Fig. 15: Least squared means for post-partum RQUICKI and HOMA within FGTT Week-1 class. Means with different letters between class 0 and class 1 differ as indicated by superscripts in table 10.



In order to assess the accuracy of our FGTT and the applicability of RQUICKI and HOMA as marker of insulin sensitivity in dairy cattle, we applied the calculation of R.O.C. curves by comparing tests between each others and using FGTT classes (0 and 1), performed in Week -1, as classification variable. We interpreted the values of area under the curves (AUC) following Bottarelli's schema: both RQUICKI and HOMA produced an AUC of about 0,6 which would classifie the tests as poorly accurate. The value of Z is similar and higher than a critic value of 1.96 for both indexes, which means that these indexes are significantly different between class 0 and 1 of FGTT. The Z of RQUICKI is slightly higher than HOMA so it is possible to state that its performance is superior in discriminating cows above or below the T80/T0 ratio of 1.2 (with $P < 0,05$). The associated criterion gives the cut-off values of tests for identifing "insulin resistant" cows: if RQUICKI is $\leq 0,558$, than the respective T80/T0 is > 1.2 ($P < 0,01$); again if HOMA is $> 0,246$, than the T80/T0 is > 1.2 ($P < 0,05$).

Table 11. Area Under the R.O.C. Curves (AUC), Z test, and cut-off values (Association criterion) for determination of best Sensitivity and Specificity as diagnostic test of RQUICKI and HOMA used post calving (Week +1 and +2) as indexes of IR and compared to FGTT performed in Week-1 as classification variable. All statistics are followed by respective levels of significance (Standard Errors, SE; 95% Confidence Interval, 95% CI; P values, P).

Tested variables	RQUICKI Week+1/+2	HOMA Week +1/+2
AUC	0,619	0,602
SE	0,0413	0,0415
95% CI	0,545 - 0,689	0,528 - 0,673
Z statistic	2,873	2,459
P	0,0041	0,0139
Associated criterion	≤ 0,558	> 0,246
95% CI	0,476 - 0,582	0,124 - 0,326
Sensitivity (%)	63,00	55,00
95% CI	52,8 - 72,4	44,7 - 65,0
Specificity (%)	65,12	67,44
95% CI	54,1 - 75,1	56,5 - 77,2

Graphically looking at the curves, a test A is considered better than a test B when its curve is completely above the curve of the second one. Sensitivity and Specificity of tests compared to a reference variable are graphically showed by the curve trend. In this case, post-partum RQUICKI and HOMA curves are almost identical: sensitivity is 63% and 55% for RQUICKI and HOMA, respectively; specificity is inverted and slightly higher for HOMA (67%) than for RQUICKI (65%).

Fig. 16: R.O.C. curve of RQUICKI calculated during Week +1 and +2 and tested as an indicator of insulin resistance in dairy cows in association with FGTT performed in Week -1.

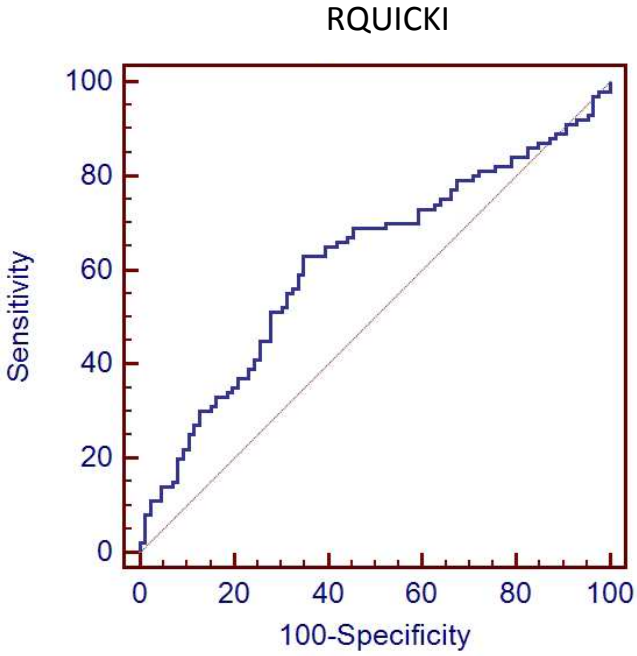
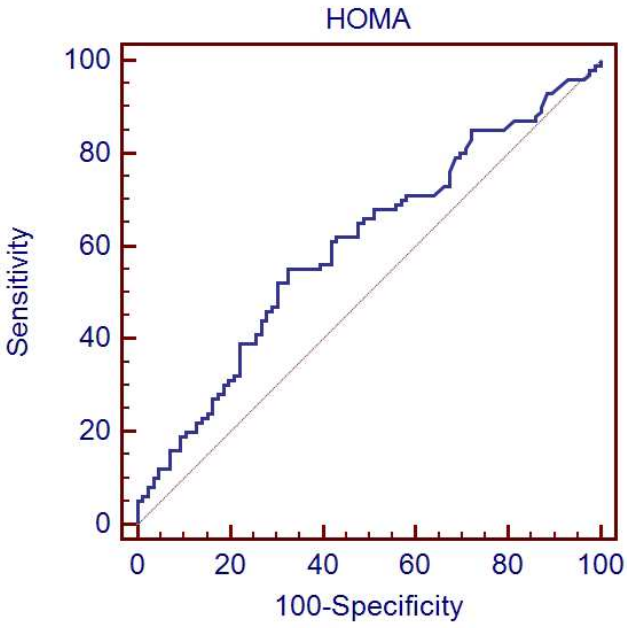


Fig. 17: R.O.C. curve of HOMA calculated during Week +1 and +2 and tested as an indicator of insulin resistance in dairy cows in association with FGTT performed in Week -1.



For concluding results, cows with lower milk production had higher basal glycaemia (EVM class 1 = 50,04 ± 2,32; class 2 = 43,07 ± 1,39; class 3 = 41,30 ± 2,06) as presented in Fig. 18. Moreover, older animals suffered from post-calving more severe negative energy balance (NEFA in parity 2 class = 0,64 ± 0,05; NEFA in parity 3 class = 0,82 ± 0,04; P < 0,01) and ketosis (BHB in parity 2 class = 0,75 ± 0,08; BHB in parity 3 class = 0,99 ±0,08; P < 0,05) (Fig. 19).

Fig. 18: Least squared means for post-partum Field Glucose concentrations at T0 within EVM class. EVM class 1: EVM305 < 9.500 kg; class 2: 9.500 kg ≤ EVM305 ≤ 11.123 kg; class 3: EVM305 > 11.123 kg. Means with different letters between classes differ (P = 0,01 – 0,05).

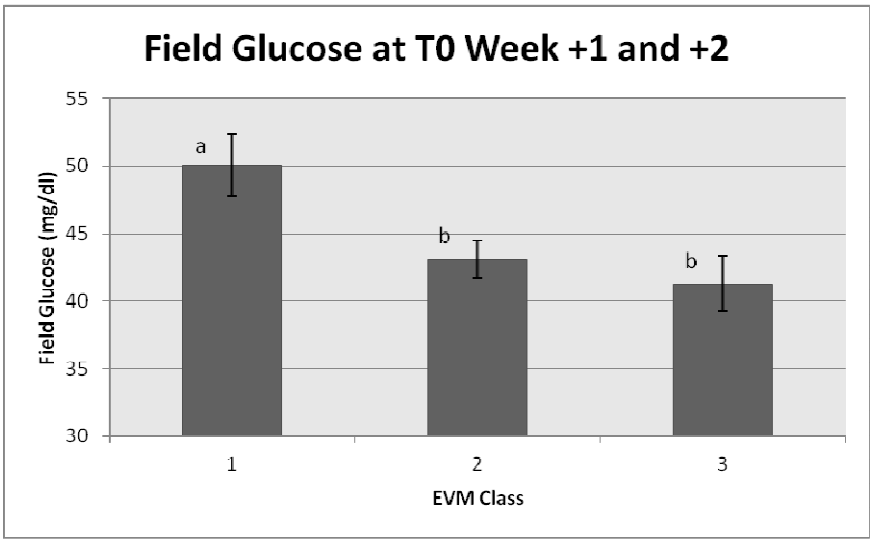
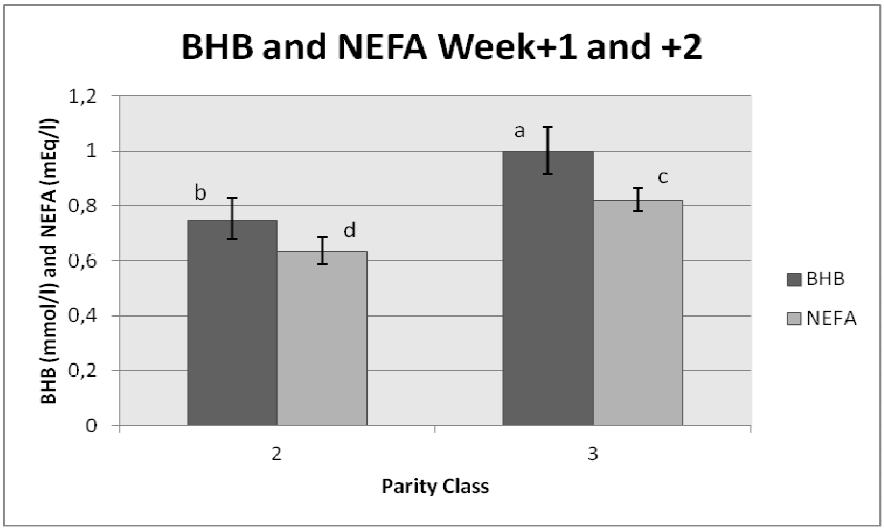


Fig. 19: Least squared means for post-partum BHB and NEFA concentrations within Parity class. Parity class 2: cows at their second lactation; parity class 3: cows ≥ 3 lactations. Means with different letters between class 2 and 3 differ (ab, P < 0,05; cd, P = 0,001 – 0,01).



6. Discussion

6.1 The FGTT

The central aim of this study was to assess the insulin response to a glucose load at different time points relative to calving in cows with different glucose clearance capacity and health status. Patterns of plasma glucose and insulin responses to GTT in this trial were comparable to those obtained elsewhere: our decision to measure glucose at T80 was derived by the expectation that it should be returned to basal levels within 80 minutes from the end of infusion under physiological conditions, in line with other authors findings during GTT carried out in the transition cow (Roche et al., 2008; Terao et al., 2010; Grunberg et al., 2011). Samples were also collected 10 minutes later with particular interest for insulin determination because T10 could likely be the moment of insulin serum peak concentration as it was found in prepartum GTT (Hayirli, 2001) and postpartum GTT (Balogh et al., 2009) characterized by similar glucose dosage administration (0.15 g/kg of BW). We verified these assumptions by performing our GTT as described above in a small number of cows and measuring both glucose and insulin concentrations at close intervals over time (every 10 minutes over 2 hours), before the beginning of our survey. In our results, a fair concordance (R = 0,5) was found between glucose ratios measured by Optium Exceed and laboratory methods, as previously affirmed by other authors: both Oetzel and McGuirk (2008) reported a correlation with R2 = 0,56, and Voyvoda and Erdogan (2010) showed a correlation coefficient (r) of 0,63 between the same hand-held device and laboratory analysis from basal samples of clinically healthy Holstein cows. A notable reduction in insulin release was pointed out at T10 within Week -1 in cows with pre-partum T80/T0 ratios > 1.2: this outcome suggests that glucose uptake by peripheral tissues in late gestation cows could be compromised not only by peripheral insulin resistance but also by an excessive reduction in insulin secretion and pancreatic synthesis in specific subjects. The degree of insulin production and insulin action are known to be affected by various hormones, as GH, cortisol, progesterone and thyroxine (Hayirli, 2006; Stern et al., 1971) and free fatty acids (Bassett & Gluckman 1987). All these hormones and metabolites normally undergo important modifications in late pregnancy. Plasma NEFA concentration increases because fat is mobilized in response to decreased plasma insulin concentration, which starts declining beginning several weeks pre-partum. Insulin and NEFA exert a reciprocal negative effect even if the exact molecular mechanisms are not extensively explained yet. In humans, prolonged periods of elevated NEFA concentrations, such as obesity, increase the risk of type II diabetes mellitus. This seems to be due to exhaustion of the Langerhans islets after prolonged episodes of insulin

resistance and hyper-insulinaemia (Golay and Ybarra, 2005). Apart from this, a direct deleterious effect of fatty acids on the pancreatic β-cell insulin secretion and viability has been demonstrated by in vitro studies in man (Zhou and Grill, 1995) and rat (Maedler et al., 2001). Because dairy cows are also exposed to high-NEFA concentrations throughout periods of NEB, it could be hypothesized that they also suffer from an impaired insulin secretion.

Higher NEFA were seen in cows enrolled in our trial at less than 7 d before calving. Indeed, free fatty acids usually increase remarkably approaching calving and they reach peak concentration within the first week of lactation (Bossaert et al., 2008). Simultaneously, these animals had lower insulin detectable before glucose infusion and 10 minutes later and their glycaemia at T80 was slightly higher as opposed to cows involved in the trial between 14 and 7 d pre-partum. A possible interference of NEFA and peri-parturient endocrinal changes with insulin secretion and glucose clearance can be assumed. Herd B and C were characterized by an elevated number of animals selected in the week period closest to delivery and were also suffering from higher NEFA and lower insulin concentrations. Other basal biochemical parameters as urea, albumin, total proteins, GOT-AST and GPT-ALT in Week -1 in these herds suggest a risk of lower dry matter intake (DMI) on the whole. In general DMI is gradually decreasing in the few weeks before parturition. Therefore, the exact time of performance of glucose challenge in terms of days relative to calving must be considered before any in-depth analysis of other factors responsible for IR problems on farms. Otherwise, the observation of a strong herd effect over glucose, insulin, energetic metabolites and IR indexes is indicative of a potential responsibility of nutritional and managerial elements in causing metabolic disorders and compromised glucose tolerance. Looking at diets composition, herd C can be theoretically differentiated by the other two herds in particular for its reduced NEL, starch and crude protein percents in close-up stage. Indeed, these elements would predispose to IR syndrome in transition cows according to many researchers. Besides this, DMI could play a crucial role: social stressors as bunk space availability and overcrowding could further affect DMI despite similar diets composition. However, we did not measure DMI and the extension of close-up periods for single cows: investigating IR causes was not our purpose. To elucidate the causal factors of this phenomenon, further experimental studies are required with particular attention to energy and protein density of close-up diets and length of close-up stage. Furthermore, hepatic and body fat content and their effects on liver processes and fat storage seem to be highly variable among cows. This indicates that individual cow factors beyond environment and feeding management affect energetic metabolism and consequently performance of high-yielding dairy cows (McNamara, 2000; Ingvartsen, 2006; Hammon et al., 2009) and should be better investigated. In addition, usually plasma glucose concentration drops precipitously at calving and partially recovers over the course of the next several weeks. Low plasma glucose likely limits milk yield because glucose is required by the mammary gland for the production of milk

lactose, the primary determinant of milk volume. Unexpectedly, we observed higher glucose levels in cows with limited milk yield at first milk test as already seen by Balogh et al. (2008): a rapid insulin-independent uptake of glucose by mammary gland following intravenous infusion could be supposed in higher producers, with concurrent reduced glycaemia. The herds, B and C, with the higher glycaemia in Week +1 and +2, had also lower insulineamia and productivity. Thus, the level of glycaemia could also be a direct consequence of actual impairment of insulin release. Reduced feed consumption may be related to insulin availability and contribute to limited milk yield in herds with lowered insulinemia and apparently elevated glucose concentrations.

In humane medicine, a form of Gestational diabetes mellitus (GDM) exists as a "slight and temporary glucose intolerance that occurs or is recognized during pregnancy for the first time and usually ceases after calving" (American Diabetes Association, Diabetes Care, Volume 27, Supplement 1, 2004). The GDM is the result of an altered adjustment of metabolism to insulin resistance produced by hormonal changes during gestation (Catalano et al., 1991). Pregnant women with no GDM reply to a reduced peripheral tissue sensitivity to insulin by increasing insulin release. In the subjects who cannot raise insulin secretion, hyper-glycaemia appears and stimulates the fetal pancreas with consequent increase of fetus growth and other metabolic disturbances (Phillipps and Jeffries, 2006). In the post-partum the decline of peripheral insulin resistance reduces the insulin demand and therefore its secretion, so that β-cells activity is sufficient regardless of persistency of a certain degree of deficit in insulin production. Anyway, controversial findings have been reported on the levels of insulin secretion in GDM patients. Peripheral insulin concentrations likely do not adequately reflect insulin secretion. A high percent of secreted insulin is metabolized by the liver at a different rate between GDM and non-GDM women (Xiang et al., 1999). Moreover, in the past many analytical assays cross-reacted with insulin precursors, which could be elevated in GDM patients (Kautzky-Willer et al., 1997). Nowadays, the best method for assessment of pre-hepatic insulin secretion is considered the detection of C peptide concentration in the venous peripheral blood and thus its use in the bovine species could be evaluated. Similarly, the development path of GDM syndrome in humans may be comparable to the dynamics of insulin resistance complex in dairy cows during pregnancy and after calving.

6.2 The Negative Energy Balance

In our study we also aimed at relating cows' response to glucose load to their metabolic status. For evaluation of possible associations between responses to GTT and ketosis syndrome, we considered BHB and NEFA concentrations. Serum concentrations of the ketone body BHB are commonly used to diagnose ketosis in dairy cows. By use of a case definition of serum BHBA concentration > 1.4 mmol/l, Geishauser and colleagues (2000) estimated an incidence rate of 12% for subclinical ketosis among postpartum cows in their first lactation; another study (Seifi et al., 2007) reported that 16% of 1.162 cows tested in the first 8 days of lactation had ketosis. Recently, McArt et al. (2012), found that 43% of 1.717 cows in 4 dairy herds had at least one BHB test of 1.2 to 2.9 mmol/l, which they defined as subclinical ketosis, when tested 6 times between 3 and 16 DIM. Ketosis incidence may vary with different case definitions but we can state that our sample was in agreement with latest epidemiological surveys on subclinical ketosis since we concluded that between 24 and 34% of recruited cows within each herd and 16 DIM had plasma BHB > 1.4 mmol/l. A greater severity of ketosis in older cows as in our sample is a consequence of a more severe refusing of food intake during transition and a rapid fat mobilization of more obese animals compared to heifers (Hayirli et al., 2002b). Cows with ketosis were expected to show low tissue responsiveness to insulin and low insulin concentrations (Holtenius, 1993; Sakai et al., 1993; Steen et al., 1997).

NEFA concentrations are a marker of fat mobilization in states of negative energy balance (Mulligan et al., 2006). Ketosis and energy balance in transition cows should be monitored by measuring the proportion of animals above a certain biological cut-point of test results, specifically NEFA in close-up and BHB in post-calving cows, within the subsample (Oetzel, 2006). Low NEFA and BHB concentrations are of little to no significance to the cow and the cows are affected only when these parameters are elevated above alarm levels. Therefore we were less concerned with the mean value of the tested groups and we aimed at interpreting herd results on a proportional basis through risk analysis. Levels of approximately 0.3 mEq/l of pre-partum NEFA and 0.7 mEq/l post-partum have recently been identified as critical limit for predicting diseases risk after calving (Ospina et al., 2010). The same author demonstrated that although both elevated NEFA, pre and post-partum, and BHB post-partum are significantly associated with development of clinical ketosis, metritis, displaced abomasum and retained placenta within 30 DIM, postpartum serum NEFA is most associated with this risk. We used 0.5 mEq/L of NEFA Week -1 and 1.0 mEq/l of NEFA Week +1 as thresholds for classification of animals. A significant relative risk was found only pre-partum by comparing NEFA class and GTT class; absence of RR of GTT class 1 cows to experience ketosis, high NEFA or any other disease after calving can be due to treatments interference: although we tried to avoid blood collections within 24 h from any treatments, a certain influence from pharmacological therapies cannot be

excluded in a field trial such this. Other researchers have encountered difficulties in analyzing these kinds of data. Drackley (1999) affirmed that: "The transition period presents several challenges to the conduct of research. Perhaps the biggest challenge is that events happen quickly and physiological state changes rapidly, with most of the adaptations probably completed within about a 4-wk period from 2 wk before to 2 wk after calving. Measurements during this time are fraught with a high degree of variability, reflecting differences among individual cows in the success of adaptation to lactation. […] The high incidence of health problems during this time contributes to the variation in DMI, milk yield, and responses to imposed treatments. Lack of suitable covariate measurements makes analysis more difficult and requires larger numbers of cows to detect differences statistically. [...] Finally, treatments may be confounded by the changes in facilities and environments that the cows may be moved through during the transition period."

6.3 The surrogate markers of insulin sensitivity: RQUICKI and HOMA

An agreement between pre-partum GTT results and surrogate markers of insulin sensitivity, RQUICKI and HOMA, was stressed after calving. In recent research, Kerestes et al. (2009) did not obtain any relevant correlation between GTT parameters and the RQUICKI, whereas Balogh et al. (2008) have already identified a significant relationship between some variables derived by GTT and the RQUICKI. A low RQUICKI index value indicates decreased insulin sensitivity. On the other side, the higher HOMA-IR, the higher is IR and the lower is insulin sensitivity. Both a lower RQUICKI and higher HOMA in class 1 cows for pre-partum GTT confirmed our expectations of an overall reduced insulin sensitivity in animals not responding to GTT within 80 minutes from infusion. As regards HOMA calculation, other researchers (Kusenda, 2010) have used a different equation, that is HOMA = G T0 x I T0 according to Matthews et al., (1985), whereas the denominator used in our formula would be specific for humans (Singh and Saxena, 2010). The denominator of 22.5 in our formula is a normalizing factor and derives from the product of normal fasting blood glucose (4.5 mmol/L) and insulin (5 μU/mL) in typical "healthy humans" (Muniyappa et al., 2008). However, it was hard to determine "normal" reference glucose and insulin levels in the first weeks of lactation in dairy cows to estimate a specific constant for HOMA in this species. Overall both HOMA calculation methods used by these authors have led to statistically significant differences and similar patterns in their trends and IR identification before and after calving in line with other studies (Sano et al., 1993; Hayirli et al., 2006). It can be noted that herds B and C had a lower HOMA in Week -1 compared to herd A, which is expression of a greater insulin sensitivity of these 2 herds pre-partum. This outcome is conflicting with their lower RQUICKI after calving, which on the contrary reveals minor insulin sensitivity. Again, a more pronounced lowered insulin production in numerous cows at less than 7 days pre-partum in these two herds could be responsible for this finding. Perhaps the use of HOMA to test IR in the proximity of calving should be avoided. The R.O.C. curves have ultimately declared RQUICKI and HOMA as poorly accurate diagnostic test for classification of cows recovering from glucose tolerance test. In effect, despite a rather clear distinction between herds when considering patterns of plasma metabolites, such as NEFA, urea, GOT-AST and GPT-ALT, in pre-partum weeks, the distribution of cows on the basis of FGTT was almost the same within each herd and the test had difficulties in underlining differences of metabolic status at the herd level. However, it must be admitted that the same differences between herds were not as easily recognizable by hematochemical parameters, diseases incidence and treatments frequency after calving as in pre-partum scenario. Although pre-analytical handling of samples, any kind of stress and feed consumption can affect baseline glucose, NEFA and insulin concentrations (Quiroz-Rocha et al. 2010; Leroy et al. 2010) and should be carefully considered before interpretation of any results, RQUICKI and HOMA would be likely best

indicators of insulin sensitivity per se than a single GTT. A further limitation of GTT could be the loss of glucose by urinary excretion after intravenous infusion or a rapid consumption by the mammary gland for colostrum synthesis and by placenta in late gestation since the absorption of glucose through these organs is insulin-independent. Cows enrolled within one single day before parturition were excluded from the trial. Anyway, these animals reported evident higher glucose levels at T80 at first sight. This observation could be associated to a considerable glucose intolerance at the time of calving and its physiological mechanisms should be better understood and fluid therapy with glucose solutions carefully assessed in clinical practice during this very delicate phase.

6.5 Conclusions

In conclusion, our study demonstrates a promising opportunity for application of surrogate indices of insulin sensitivity and GTT in field trials to detect insulin resistance syndrome in dairy cows in the future. The T80/T0 ratio derived by GTT seemed to be useful in detecting a low insulin secretion as a likely complication of altered glucose uptake following glucose load in transition cows. Withal, it positively correlates with negative energy balance represented by NEFA concentrations in the 2 weeks before calving. These findings deserve further research about the epidemiology of IR syndrome at the herd level and among herds; its correlation with several pathological conditions commonly found in the transition cows should also be investigated. However, establishment of standardized tests protocols, strategies to minimize stress and feed delivery interferences under field conditions, and accurate analytical procedure, in particular for insulin and glucose, is worthwhile before further experimentation on this topic.

7. References

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