UNIVERSITÁ DEGLI STUDI DI MILANO
Facoltà di Medicina Veterinaria
Dipartimento di Scienze veterinarie e Sanità pubblica

PhD COURSE OF
VETERINARY HYGIENE AND ANIMAL PATHOLOGY
XXVIII cycle

CLINICAL PATHOLOGICAL MONITORING OF DAIRY COWS IN THE TRANSITION PERIOD

PhD Student
Dr. Pierangelo Moretti
Matr. R10040

PhD Supervisor
Dr. Alessia Giordano

PhD Coordinator
Prof. Giuseppe Sironi

Academic Year
2014-2015

To Cá and Pé,

with love

“Pe’ fa’ ‘e cose bone ce vo’ tiemp”

Popular saying from Benevento

Table of content

CHAPTER 1:

The health monitoring of transition cows

CHAPTER 1: THE HEALTH MONITORING OF TRANSITION COWS

1.1 – Introduction

As a matter of fact, milk yield and reproductive performances are the standard economic barometers of dairy production (Esposito, 2014). Intensive genetic selection and improvements in dairy cow nutrition, housing and management, have led to significant increases in average milk yield with no signs that the limits have been reached (Barkema et al., 2015; Opsomer, 2015). The transition period for dairy cows, extending from approximately 3 weeks pre-partum until 3 weeks post partum is subject to enormous metabolic, hormonal, inflammatory and oxidative stress changes (Sordillo and Raphael, 2013). When, in early lactation, modern high yielding dairy cows are compromised by the nutritional and hormonal environment, started during the last weeks of gestation, they are able to direct higher priority on maintaining lactation rather than on maintaining their body energy reserves (Esposito, 2014; Opsomer, 2015). The increased capacity of adaptation to a lower energy availability implicates that modern cows have to deal with increased metabolic instability compared to low producing cows. If this challenge fails, and the compensatory processes are not sufficient to sustain energy requirements, subclinical and clinical diseases may develop. As all the efforts are going in the direction of milk production, also other processes that require energy, as immunity and reproduction, are at greater risk to fall (Opsomer, 2015). For these reasons, all the conditions predisposing to an inadequate adaptation to the energy requirement (subclinical diseases, environmental, nutritional errors) are recognized as openers to other metabolic and reproductive disturbances such as clinical ketosis, milk fever, displaced abomasum, endometritis, metritis, mastitis (Mulligan et al., 2008). These diseases are the major responsible for culling thus causing productive and consequently economic losses in dairy farming (Esposito, 2014; Barkema et al., 2015; Opsomer, 2015).

Adaptative processes, subclinical and clinical conditions, and infectious diseases occurring during the transition period may be all reflected by a broad range of changes in the concentration of plasma

metabolites and hematological parameters, that can be evaluated with laboratory techniques (e.g. clinical chemistry and hematology) (Stockham and Scott, 2008a). The goal from this perspective is to investigate which are the biochemical and hematological changes associated with a proper adaptative process rather than to a failure of this adaptation, to identify pathological states and possibly to find early signals of these abnormalities. In this context, the veterinary clinical pathology, a discipline of the veterinary medicine that deals with the use of laboratory methods for the diagnosis and treatment of diseases, has the primary role to assist clinicians for an adequate application and interpretation of laboratory data (Stockham and Scott, 2008a). Maintaining health and productivity in the transition period is one of the most difficult challenges that dairy herds face. Most health-promotion strategies including nutrition and other aspects of management are logically and practically implemented at the herd level rather than at individual level (Chapinal et al., 2012). The main reasons to perform laboratory analyses at a herd level are to detect an unidentified pathological states, to rule out a possible cause of illness, but especially to evaluate the nutritional and metabolic status of cows in order to detect subclinical conditions that may affect productive and reproductive performances of the herd. Finally, laboratory data may also be used for research purposes in order to contribute to the definition of the pathophysiological or pathogenetic processes underlying the diseases affecting transition cows.

1.2 – Laboratory tests in veterinary clinical pathology

Laboratory tests in veterinary clinical pathology may involve the analysis of blood, tissues and body fluids; numerous laboratory methods and techniques are available. The complete description of all the methods is beyond the scope of this thesis; therefore, in the following section, the assays commonly employed for determination of the hematological and clinical chemistry profile of cattle are only briefly described.

1.2.1 Hematology

Hematology assays are commonly performed with automated cell counters using whole blood samples, with ethylenediaminetetraacetic acid (EDTA) as preferred additive to prevent clotting of the sample. The objective of this technique is the quantification of concentration, size and cellular content (e.g. haemoglobin and peroxidase) of leukocytes, erythrocytes (RBCs), and platelets (PLTs). As many haematological instruments, included those used in veterinary laboratories are firstly designed for human patients, in veterinary species a differential leukocyte count, which is the proportion of the different classes of white cells in the blood, performed by microscopy is always recommended, since morphology, size and cellular complexity of leukocytes are not accurately identified by the instrument in many cases. Hematological analysis is not only relevant for diagnosing disorders of the hemopoietic system but also helpful in the monitoring of different organs and for the identification of systemic diseases. Specifically, obtained information may be related to the inflammatory status of the animal (mainly leukocyte related parameters) or may be an indirect evaluation of metabolic and nutritional performances (mainly erythroid related parameters) (Stockham and Scott, 2008a; Tornquist and Rigas, 2010).

Concerning abnormalities in the number of circulating white blood cells, total leukocyte number and the proportions of the different leukocyte subpopulations are influenced by stress and excitement, pathological or physiological conditions occurring around parturition (Tornquist and Rigas, 2010). For example neutrophilia, an increased concentration of neutrophils in peripheral blood, with or without the presence of immature forms (the so called left shift) may be found in association with inflammatory conditions related to bacterial, viral, fungal, protozoal, and parasitic infections, but also with neoplasia, abomasal displacement, toxicosis, immune-mediated anemia, stress and excitement, but with a lower magnitude compared to other mammals (Tornquist and Rigas, 2010). The pre-partum period is accompanied by increased leukocytes mainly due to neutrophils then followed by a decrease during the first week post-partum (Kehrli et al., 1989; Cai

et al., 1994; Zerbe et al., 2000). In contrast, neutropenia, the decreased concentration on neutrophils in peripheral blood, lasting 48 hours may be found associated with hyperacute inflammatory conditions mainly due to gram-negative infection, mastitis, peritonitis, metritis, pneumonia, and gastrointestinal disease, but also to some viral infections such as bovine viral diarrhoea (BVDV), and bluetongue virus (Tornquist and Rigas, 2010). Inflammatory neutropenia is common in adult cattle, since they have a relatively small storage neutrophil pool (Stockham and Scott, 2008b). Lymphocytosis (an increased blood concentration of lymphocytes) is not common in ruminants, though it may occur in chronic viral infections, chronic trypanosomiasis, and in bovine leukemia virus (BLV) infection. On the other hand, lymphopenia is most commonly seen as a consequence of a corticosteroid-stress response(Tornquist and Rigas, 2010). Monocytosis can be observed during acute and especially chronic inflammatory diseases, and is of rare occurrence in stress response in cattle, whereas eosinophilia and basophilia have been reported in a variety of ecto and endoparasitic infections (Tornquist and Rigas, 2010). Monocytopenia, eosinopenia and basopenia are infrequently reported during hematological examination of cattle as different techniques are needed to find this anomaly (bone marrow evaluation) (Tornquist and Rigas, 2010). Moreover is plausible that the very high imprecision of the method does not allow to reach the minimum requirement for clinical purposes as observed in other species (Kjelgaard-Hansen and Jensen, 2006)

As regards erythroid changes, common indications for RBC analysis are clinical or suspected anemia or hemorrhage (Roland et al., 2014). As typical features of anemia, RBC count, haemoglobin concentration, and/or hematocrit are decreased. A reduction of the hematocrit value (which is the percentage of blood volume that consists of red blood cells) is also caused by an increased plasma volume (e.g., during pregnancy or after fluid therapy) (Roland et al., 2014). Anemia can be categorized into regenerative and nonregenerative anemia according to the bone marrow response, and it can be also classified with regard to the cell size (normocytic, macrocytic, and microcytic, indicating normal, increased, and decreased mean cellular volume or MCV, respectively) and haemoglobin concentration (normochromic, hypochromic, and hyperchromic,

indicating normal, decreased, and increased haemoglobin, respectively) (Roland et al., 2014). Common causes for regenerative anemia are hemorrhages or hemolysis. Causes for haemorrhage in cattle include trauma, abomasal ulcers, hemorrhagic enteritis, vena cava syndrome, blood-sucking parasites, hemostasis defects, and vessel erosion or rupture (Roland et al., 2014). Nonregenerative anemia, which is due to a defective or reduced erythropoiesis, may occur during chronic inflammation, due to chronic renal disease (with erythropoietin deficiency), endocrine disorders, bone marrow depression, some drugs (e.g., estrogens), toxins (e.g., lead poisoning), abscesses, neoplasia and for nutritional causes (e.g. vitamins or iron deficiency) (Roland et al., 2014).

A platelet count might be indicated in case of severe hemorrhage or increased bleeding tendency. Thrombocytosis (increased concentration of platelets in peripheral blood) may occur physiologically as a consequence of epinephrine-induced splenic contraction (Roland et al., 2014). Essential or primary thrombocytosis is an uncommon myeloproliferative condition. Reactive or secondary thrombocytosis is commonly associated with inflammatory conditions but can be also observed with chronic blood loss, neoplasia, or iron deficiency. Thrombocytopenia is found in excessive consumption (e.g., with blood loss, disseminated intravascular coagulation), decreased platelet production (e.g., in myelophthisis or bone marrow hypoplasia due to toxins), destruction (e.g., due to infections, toxins, drugs, neoplasia, or immune-mediated), or distribution disorders (e.g., splenomegaly) (Roland et al., 2014).

1.2.2 Clinical chemistry

Quantitative, semi-quantitative and qualitative analysis of metabolites may be performed on serum and plasma in order to detect or quantify the concentration or the activity of serum or plasma chemical substances. There are many parameters that can be evaluated in plasma of animals, but they can be grouped based on their relevance in reflecting specific organ or system functionality. Concerning dairy cows, the most important analytes, included also as part of the investigation described in the present thesis, are listed here below.

Nonesterified fatty acids (NEFA) and β-hydroxybutyrate (BOHB) are energy metabolites that can be used as markers of energy balance in dairy cows (Stockham and Scott, 2008a). NEFAs form from hydrolysis of triacylglycerol in adipose tissue, in liver and mammary gland. Its increases occur with increased fat mobilization in response to negative energy balance from any cause (Kaneko, 2008). BOHB is a ketone body, derived from the β-oxidation of lipids that accumulates when an increased lipolysis is associated with increased gluconeogenesis as occurs during a negative energy balance (Bruss, 2008). Increased NEFA and BOHB are thus considered reliable markers of an excessive negative energy balance in dairy cows during the transition period when there are high energy demands for the foetus and milk production (Stockham and Scott, 2008a). When sampled in the appropriate time frame, pre-partum and post-partum NEFA and BOHB concentrations above certain threshold levels are associated with negative downstream outcomes such as increased risk of disease, and decreased milking and reproductive performance (Stockham and Scott, 2008c). BOHB concentrations can be measured with several tests of different sensitivities and specificities both cow-side and in laboratories. At present, NEFA concentrations can be measured only in laboratories (Oetzel, 2004).

Serum urea and creatinine concentration are used in the assessment of urinary system disorders but urea concentration is also a reflection of ruminal function and available dietary protein at the herd level (Stockham and Scott, 2008a). Urea is synthesized in the liver as a byproduct of dietary and muscle proteins catabolism. It is completely and freely filtrated by the renal glomerulus, so it is a reliable indicator of the glomerula filtration rate. Creatinine is a breakdown product of muscle creatine and phosphocreatine which are in turn molecules involved in the energy metabolism. As a consequence, creatinine serum concentration may be slightly affected by muscle mass but remains the test of choice in the evaluation of kidney function in cattle, since it the best indicator of the glomerular filtration rate (Russell and Roussel, 2007).

Serum calcium, magnesium and phosphorous concentrations reflect the dynamics and hormonal control of these metabolites in different organs (mobilization from bone, absorption from intestine, renal excretion, shift between intracellular and extracellular fluid, and protein concentration) (Rosol and Capen, 1997). Low serum levels of calcium are commonly adopted to identify animals with clinical and subclinical hypocalcemia which is one of the most common and relevant problem in the transition cow, due to the increased demand of calcium for foetus skeletal formation and milk production (Stockham and Scott, 2008a; Martinez et al., 2012).

Serum electrolytes typically included in a chemistry profile are sodium, potassium and chloride. Sodium is the major extracellular cation and is responsible for most of the osmotic force that maintains the size of the extracellular fluid (ECF) compartment. The concentration of serum sodium is thus related to the animal's hydration status. Potassium is the major intracellular cation. Hyperkalemia or hypokalemia cause muscle weakness affecting skeletal, cardiac, and smooth muscle. Chloride is the major anion in ECF. It is an important component in many secretions (sweat, saliva, and gastric secretions) as sodium chloride (NaCl), potassium chloride (KCl), or hydrogen chloride (HCl). Even slight changes in serum electrolytes concentration may be life-threatening, since they are paramount for the acid-base balance as well as for plasma osmolarity which are of primary relevance for the homeostasis (Russell and Roussel, 2007).
Serum electrolytes are useful in the evaluation of several body systems and for formulating and monitoring of fluid and electrolyte therapy.

The serum measurement of liver enzyme activities is pivotal in detecting possible hepatocyte damage. The increased activity of aspartate transaminase (AST), alkaline phosphatase (ALP), and γ-glutamyltransferase (GGT) may be indicative of hepatic or muscular cell damage (AST) or gall bladder and bile ducts disorders (ALP and GGT). In the latter case, GGT is known to have a better

diagnostic sensitivity. Mild increases in GGT activity have been found in association with hepatic lipidosis (Stockham and Scott, 2008d), a frequent condition in transition cows due to the fat mobilization associated with the negative energy balance. Increased ALP activity is also present in younger cows probably due to increased osteoblastic activity (Russell and Roussel, 2007).

Glucose is a tightly regulated metabolite, influenced by several hormones, thus its concentration in serum is not a useful indicator of energy metabolism in cattle. Hyperglycemia may occurs in different condition as stress, bovine milk fever, hyperammonemia, displaced abomasum, abomasal volvulus, or proximal duodenal obstruction, or associated to administration of different drugs (xylazine, corticosteroids, solutions containing dextrose) (Russell and Roussel, 2007). Hypoglicemia in contrast, is sometimes associated with ketosis in dairy cattle, during septicemia, or malnutrition.. Hypoglycemia may be also seen with pregnancy toxemia in cows as in other species.
Healthy cows present lower concentration of bilirubin compared to other species. Increased total bilirubin concentration may be indicative of colestatic liver disease, even if the increase may be only moderate (Russell and Roussel, 2007). Sick cattle with anorexia and rumen stasis may also have hyperbilirubinemia due to a lower uptake from hepatocytes, not associated with hemolysis or hepatic diseases (Stockham and Scott, 2008e).

Serum proteins concentration is related to both nutritional causes as well as the functionality of organs and systems involved in their metabolism (mainly liver, kidney, gut and immune system). Changes in the concentration of total proteins (increases and decreases) may be thus a reflection of different causes. Total proteins are composed by albumin and globulins which in turn are mainly composed by inflammatory proteins and immunoglobulins (antibodies). Concentration of serum total proteins is better measured with automated spectrophotometers, using the biuret method, whereas albumin and globulins fractions are better evaluated with electrophoretic techniques. Albumin, synthetized in the liver, is the main contributor to the oncotic pressure in plasma and it is

responsible of the transport of many substances into the blood (calcium and magnesium ions, fatty acids and hormones). It is also considered a negative acute phase protein due to decreased production by hepatocytes during inflammation. Due to the prolonged half-life of albumin in cows, hypoalbuminemia is detected only in chronic inflammatory states or during chronic hepatic failure. Many different proteins compose the globulin fraction. A large proportion of globulins consists of immunoglobulins, which are synthesized by plasmacells. Many other globulins are synthesized by the liver, with a small amount synthesized by other tissues. Hyperglobulinemia may be an indicator of an inflammatory response common to a variety of conditions (traumatic reticuloperitonitis, liver abscesses, chronic pneumonia) or a reflection of an immune stimulation due to the presence of infectious agents. Hyperproteinemia with hyperalbuminemia and hyperglobulinemia is commonly due to dehydration (Russell and Roussel, 2007).

Acute phase proteins (APPs) are a group of molecules whose liver production and release is influenced by inflammatory mediators (IL-1, IL-6, TNFα) which lead to a huge variation of their concentration during the acute phase response. APPs can be classified according to the magnitude of their increase (positive acute phase proteins) or decrease (negative acute phase proteins) in serum concentrations during an acute phase response. Examples of positive APP in cattle are haptoglobin (Hp), ceruloplasmin (Cp), and serum amyloid A (SAA), whereas negative APP are considered the paraoxonase (PON-1) and albumin (Petersen et al., 2004; Giordano et al., 2013). Evaluation of plasma concentration of APPs in cattle was found as an excellent tool to determine the presence of an inflammatory condition in cattle and to predict possible transition diseases (Petersen et al., 2004; Trevisi, 2009; Dubuc et al. 2010; Huzzey et al., 2011; Giordano et al., 2013). PON-1 is supposed to hydrolyses lipid hydroperoxides generated on low density lipoproteins (LDL) during oxidative events (Turk et al., 2004) thus determination of PON-1 concentration and the estimation of serum hydroperoxides trought the dROM test may be used to evaluate the oxidative status in cattle (Bionaz et al., 2007). Increased concentrations of hydroperoxides, and of other derivatives of

reactive oxygen metabolites are important risk factor for disorders occurring in the transition period as ketosis, fatty liver, mastitis, and retained placenta (Sordillo and Raphael, 2013).

Vitamins are divided in fat-soluble (A, D, E and K) and water-soluble (B and C) vitamins. In cows, Vitamin K, and the water-soluble vitamins B and C, are synthesised by ruminal and intestinal bacteria, whereas vitamin D may be of dietary origin and or synthesised starting from cholesterol and through conversion by the ultraviolet light (National Research Council, 2001). All vitamins are important for animal health; vitamins A and E are considered to play an important role for the immune response. Deficiencies in β-carotene and vitamin A around calving have been associated with lower reproductive performance and higher incidence of intramammary infections (Johnston & Chew, 1984), whereas vitamin E deficiency at the same period may lead to increased incidence of reproductive diseases such as retained placenta, metritis and mastitis (Smith et al., 1997; Hemingway, 2003). Vitamin D, in turn, is crucial for calcium metabolism, since it promotes the intestinal uptake of calcium and phosphates and in general it promotes hypercalcemia (Rosol and Capen, 1997).

1.3 - Determination of reference intervals

The detection of pathological and paraphysiological conditions, achievable by their reflection on alterated biochemical and hematological data, first requires the definition of which results are expected to be found in a healthy animals, the improperly defined ‘normal values’. This is based on the use of reference intervals. Reference intervals are statistically derived intervals between and including the two reference limits in which results from the 95% of the healthy population are expect to be found (Geffré et al., 2009). The determination of reference intervals is a critical aspect of veterinary clinical pathology and was recently the subject of guidelines, based on the 2008 Clinical Laboratory and Standards Institute (CLSI) recommendations (CLSI, 2008), reported by the

American Society for Veterinary Clinical Pathology (ASVCP) (Friedrichs et al., 2012). Due to the variability among different assays used to measure a single analyte and the intrinsic variability of different population of animals, each laboratory should create its own reference intervals. This procedure requires an economical effort, since a minimum number of healthy animals on which performs a complete set of analysis is required to produce a proper reference interval, so alternative options are available, such as the use of reference intervals obtained from literature. This is possible only when the methods used by the 'donating' and 'receiving' laboratories are overlapping, when all the information related to the procedures employed in their determination are available and when the reference intervals from literature are suitable for the population of the receiving laboratory through the application of specific procedures. The difficulty to perform the transference is primarily due to the unavailability of all these information. As reference intervals may vary according to age, breed, level of production, distance from parturition, number of lactations and management, the presence of published data concerning updated well established reference intervals in cows for a wide panel of laboratory data is extremely important to unravel the possible effect of all these factors, as showed in recently published studies (Quiroz-Rocha et al., 2009; Cozzi et al., 2011; Brscic et al., 2015)

1.4 - Interpretation of herd-based laboratory data

Laboratory assays in dairy cows are used on individuals to detect a pathological state, or on groups of animals in order to evaluate the nutritional and metabolic status of the group. In the first case both hematological and biochemical analyses are performed in clinically ill animals to reveal pathological states that may be linked to the reproductive apparatus (endometritis, metritis) or infectious diseases that require punctual therapeutic intervention or, as commonly happen in the dairy industry, a culling decision to avoid further costs. When laboratory assays are performed on a groups of cow, laboratory profiles are commonly performed on a representative number of animals

of the herd (that need to be clinically healthy) which belong to defined groups of similar age and lactation stage. Sampling multiple animals from a defined group allow to reduce the physiologic variation. (Stockham and Scott, 2008a). Commonly, a sample size of 7-12, regardless the herd size, is considered the minimum number of animals to obtain sufficient information at the field level for most of the parameters. Adding more individuals to the test group will allow obtaining more representative results, but often for economic reasons, smaller test groups are encouraged by the breeders. On the other hand pooling of samples is strongly discouraged since the pool often showed biased results compared to the mean values obtained from individuals. The goal of herd-based samplings is to detect metabolic abnormalities that may be corrected through interventions on nutrition or management. In this case animals with evident signs of disease are excluded because variations of the mean values of the groups may be influenced by an individual problem that may not reflect the problem of the herd. For the same reason, most of the results from a herd-based sampling will fall within reference intervals. To achieve this goal, different approaches were suggested by some authors based on their experience and researches (Herdt 2000; Oetzel, 2004). For example, mean values of the examined herd were compared with mean values from reference herds or with expected mean results. In other cases, acceptable, unacceptable and borderline percentages of sampled animals with values above or below a threshold level have been established. Both these strategies are widely adopted in field and may provide useful information; however it is questionable how thresholds that may be valid for a herd in a specific geographical area could be adopted worldwide, due to inter-laboratory variability in assays results and to other sources of variation (Stockham and Scott, 2008a).

CHAPTER 2:

Physiological changes during the transition period

CHAPTER 2: PHYSIOLOGICAL CHANGES DURING THE TRANSITION PERIOD

2.1 - Metabolic changes during transition

The transition period for dairy cows extends from approximately three weeks before parturition until three weeks after parturition (Sordillo and Raphael, 2013). During this phase, physiological, nutritional, metabolic, and immunologic changes occur as the cow leaves a gestational non-lactating state, passes through the onset of milk synthesis and secretion, and finally reaches a more stable lactation phase near peak lactation. In the weeks before parturition, the metabolism of the pregnant cow has to face with the highest nutrient requirement of the foetus in a time where the dry matter intake (DMI) decreases of 10-30% (Bell, 1995). In this phase, the suppression of cow appetite is compounded by additional factors such as stress and management, which further reduce DMI (Esposito et al., 2014). Leptin, a hormone that influence voluntary feed intake in ruminants, was found decreased in late pregnancy of dairy cows (Wathes et al., 2007). At the same time the cow experiences a physiologic increase of serum triglyceride and decreased cholesterol concentrations likely due to the reduction in the udder uptake of triglyceride and to the increased use of cholesterol for foetus development and steroid hormone synthesis (Turk et al., 2005). Moreover, an impairment of the whole antioxidative system has been found during the pre-partum as shown by the reduced paraoxonase-1 activity during this period (Turk et al., 2005). All these factors participate to the negative energy balance. The main substrate for the synthesis of glucose in a moment of normal energy demands in cow is represented by the propionate originating from rumen microbial fermentation of feed carbohydrates. As a consequence of the reduced DMI intake during late pregnancy, the energy requirement are partially met by an increased state of insulin resistance in adipose tissue and muscle, coupled with an increased sensitivity to lipolytic agents (Bell, 1995); these events reduce peripheral glucose uptake and facilitate the use of endogenous substrates,

mainly glucogenic amino acids (glutamine, alanine) from degraded endogenous protein sources, and glycerol from adipose tissue mobilization (Sordillo and Mavangira, 2014).

The inflammatory and oxidative stress experienced during parturition (Turk et al. 2004), together with the start of milk production, drive to another dramatic increase in energy requirements. At this time the drop of plasma progesterone level, together with the transient estrogen and glucocorticoid increase, contribute to the further decline in DMI (Drackley et al., 2005; Ingvartsen, 2006). The imbalance between energy consumption and energy demand for production induces a physiologic state of negative energy balance (NEB) (Grummer et al., 2004; Ingvartsen, 2006). To cope with the decreased energy availability, further sources of energy must be mobilized from tissue stores to support energy-dependent needs of the body, and adipose tissue is a major source for this. The decrease in blood glucose level reflecting both high demands for the lactation and decreased DMI results in lower insulin levels that, coupled with the elevation in growth hormone (GH) concentration, further increases the lipolytic activity started before calving (Lucy et al., 2001; Drackley et al., 2005; Ingvartsen, 2006). During lipolysis, non-esterified fatty acids (NEFA) are cleaved from triglyceride molecules within adipocytes through the action of various hormone-sensitive lipases. NEFA is then transported by albumin into the blood, where it can be used as an energy source also initiating negative feedback loops to regulate the rate of lipolysis. When blood NEFA concentration increases, NEFA are gradually taken up by the liver and can undergo complete oxidation to carbon dioxide, incomplete oxidation to ketone bodies, or can be re-esterified and stored as triacylglyceroles (Herdt, 2000; Bobe et al., 2004; Drackley et al., 2010). The overall cumulative effect should result in relatively constant blood glucose concentration, which is needed for milk synthesis without excessive NEFA accumulation in the blood. The determination of blood concentration of NEFA and beta-hydroxybutyric acid (BOHB) is widely used in the monitoring of the proper adaptation to NEB in dairy cows. Substantially, NEFA concentration reflects the use of lipid sources whereas the peripheral concentration of ketone bodies, mainly BOHB, reflects the ability of the liver to cope with the NEFA overload (Opsomer, 2015).

Finally, the process of metabolic adaptation to the increased energy requirements depends on individual factors, environmental and management conditions (Jorritsma et al., 2003; Hammon et al., 2009; van Dorland et al., 2009). Due to the genetic selection, modern cows are considered to have a more adaptable metabolism that allows them to cope with NEB, giving at the same time the possibility to reach a higher production target (Opsomer, 2015).

2.2 - Relationship between metabolism, oxidative stress and inflammation in transition cows

In recent years, researches concerning the transition period have been mainly focused on the statement that metabolic and inflammatory pathways are strictly correlated and contribute to the pathogenesis of both metabolic and infectious diseases occurring in transition cows (Sordillo and Raphael, 2013). Different studies proved that both parturition and the onset of lactation are responsible for the altered immune system of transition cows. It has been observed that in mastectomized cows, compared to cows with intact mammary glands during the peri-parturient period, only moderate increases in NEFA, no hypocalcemia, slight compromission of lymphocyte and neutrophil function and a little impact on peripheral blood leukocytes number were found (Kimura et al., 1999; Goff et al. 2002; Kimura et al., 2002a; Nonnecke et al., 2003). Changes in glucose availability during the transition period may also have adverse effects on some bovine immune responses. Leukocytes require glucose to support antimicrobial functions, thus the dramatic decrease in blood glucose concentration during intense lipid mobilisation may limit the availability for immune cells to work optimally (Sordillo and Mavangira, 2014). At the same time, the increased requirement of glucose to support the inflammatory response in diseased cow may compete with the energy requirement for production.

Also hyperketonemia can adversely affect several important immune functions leading to a possible increased disease susceptibility in transition cows. BOHB has detrimental effect on leukocyte

antimicrobial mechanisms (Hammon et al., 2006) and may exacerbate injury to bovine hepatocytes inducing oxidative stress that can in turn activate an inflammatory response (Shi et al., 2014).

Even if the clear effect on signalling pathways in bovine immune cells during transition is still not known, some authors hypothesized that the progressive increase of NEFA concentration and the changes in NEFA composition may affect the immune response of transition cows (Sordillo and Mavangira, 2014). This is supported by the finding that the exposure to a composition of NEFA similar to that present in the plasma of transition cows, resulted in a greater expression of pro-inflammatory and adhesion molecules such as cytokines (IL-6, IL-8), I-CAM and V-CAM), ROS (reactive oxygen species) production by endothelial cells (Contreras et al., 2012) and decreased antibacterial activities of cultured neutrophils (Ster et al., 2012). Previous studies in dairy cows also demonstrated that elevated NEFA during the peri-parturient period decrease lymphocytes' secretion of IFN-γ and IgM, molecules with anti-inflammatory properties (Lacetera et al., 2004); in contrast, an activation of neutrophil phagocytosis, was found by Scalia et al. (2006).

The altered regulation of the inflammatory response experienced during the transition period is also reflected by the changes in acute phase proteins (APP), molecules whose liver production and release is influenced by inflammatory mediators (IL-1, IL-6, TNFα) which lead to a huge variation of their concentration during the acute phase response. Evaluation of plasma concentration of APPs in animals is thus an excellent tool to determine the presence of an inflammatory status, and it is gaining increasing interest also due to the low cost and availability of laboratory tests. According to their changes during the acute phase of inflammation, APPs in cattle may be defined as positive if their concentration increases, for example serum amyloid A (SAA), haptoglobin (Hb) and ceruloplasmin (Cp), and negative, for example paraoxonase (PON-1) and albumin, (Petersen et al., 2004, Giordano et al., 2013). Haptoglobin is produced by the liver under stimulation of IL-6 or TNF-α. Increased concentration of serum Hp has been found to be associated with increased risk of metritis and endometritis (Dubuc et al. 2010) and concomitant with the occurrence of retained placenta (Huzzey et al., 2011). Lower concentrations of PON-1 and albumin and higher

concentration of Hp were found in association with transition disorders in a retrospective study on dairy herds (Bertoni et al., 2008). Treatment with low oral dose of IFN-α, supposed to stimulate the innate immune system, was shown to increase the plasma Hp and Cp concentration in a study (Trevisi, 2009).

The oxidative stress in dairy cattle is also considered a contributing factor of dysfunctional inflammatory response during the transition period (Sordillo and Mavangira, 2014). Reactive oxygen species (ROS) such as oxide anion (O-2), hydrogen peroxide (H2O2), and hydroxyl radical (OH-), are normal end products of the cellular metabolism responsible for the conversion of nutrients in energy and the cellular oxygen-dependent defences against bacteria (Sordillo and Mavangira, 2014). The generation of ROS has a critical role in the regulation of normal cellular processes, including the regulation of inflammation, but excessive ROS production can cause damage to lipids, DNA, proteins, and other macromolecules. The regulation of ROS concentration requires the action of enzymatic (cytosolic glutathione peroxidase) and non-enzymatic antioxidants (e.g. glutathione and vitamin E) that directly catalyse or indirectly favour, respectively, the reduction of ROS to less reactive molecules (Sordillo and Aitken, 2009). The increased oxygen requirements during increased metabolic demands, as occurs in the transition period, result in increased production of ROS in a time when the total antioxidant potential of sera is reduced (Sordillo et al., 2007). Immune cells are particularly sensitive to oxidative stress because their membranes contain high concentration of polyunsaturated fatty acids that are very susceptible to peroxidation (Sordillo and Mavangira, 2014). Increased concentrations of hydroperoxides, and of other derivatives of reactive oxygen metabolites (dROMs) are important risk factor for disorders occurring in the transition period as ketosis, fatty liver, mastitis, and retained placenta (Sordillo and Raphael, 2013). The role of PON-1 as an antioxidant was suggested by Turk et al. (2004). Increased concentrations of d-ROMs were found in cows with the lowest levels of PON-1 during the first two weeks of lactation (Bionaz et al., 2007) when concentrations of HDL-cholesterol, the carrier of PON-1, is reduced (Turk et al., 2004).

CHAPTER 3:

Diseases of the transition period

CHAPTER 3: DISEASES OF THE TRANSITION PERIOD

3.1 – Introduction

Due to the strict relationship between metabolism, immunity and oxidative status in the transition period, as described before, the term ‘production disease’, traditionally regarding the metabolic disorders of dairy cows (namely hypocalcaemia and ketosis) has been now broadened to infectious diseases and other diseases such as retained placenta, displacement of the abomasum and the laminitis (Mulligan and Doherty, 2008). All these conditions have the highest incidence in the period from calving until 10 days after calving (Ingvartsen et al., 2003). These diseases, especially ketosis and retained placenta are opener to further uterine and udder diseases. A description of the main diseases occurring during transition are reported in the following paragraphs.

3.2 - Clinical and subclinical hypocalcaemia

Conditions frequently observed in dairy farm are subclinical and clinical hypocalcemia (also known as milk fever). Subclinical conditions may have an incidence of 33% in dairy farms (increasing to about 50% if considering only pluriparous cows) whereas the clinical form has usually a lower incidence, limited to 3 - 5% (Mulligan and Doherty, 2008). Primiparous cows seem not to be less exposed to hypocalcemia around calving than multiparous cows. The pathogenesis is supposed to be linked to a reduced response of gut and bones to parathyroid hormone (PTH) due substantially to a reduced expression of receptors for this hormone on target cells at the moment of increased calcium demand such as at beginning of lactation. This condition seems to be enhanced by an excessive calcium (or vitamin D) supplementation in the diet before parturition (Rosol and Capen, 1997). The clinical form is characterized by a different severity of clinical signs ranging from mild excitement, anorexia and weakness to more severe lateral recumbency, gastrointestinal atony, and difficulty to perceive pulse, always accompanied by hypocalcemia and hypophosphatemia, within

72 hours from calving (Kelton et al., 1998). Other clinical pathological changes vary depending on the stage of disease and may be represented by increased or decreased concentration of magnesium, increased hepatic and muscle enzymes. When hematological abnormalities are present they may consist with a neutropenia, in the first phase of disease, or with a stress leukogram in more advanced conditions. The subclinical form of hypocalcemia is characterized by a reduced concentration of serum calcium without clinical signs (Roche, 2003; Martinez et al., 2012). These latter authors suggested different cut-offs to consider a condition as subclinical (ranging from 8.02 to 8.59 mg/dL). Both subclinical and clinical hypocalcemia have been correlated with dystocia, uterine prolapse, retained placenta, endometritis, infertility, mastitis, displaced abomasum, ketosis and immunosuppression (Mulligan and Doherty, 2008).

3.3 - Clinical and subclinical ketosis

Ketosis in dairy cows is a metabolic disturbance associated with NEB and characterized by abnormally elevated concentration of the ketone bodies acetoacetic acid (AcAc), acetone (Ac), and β-hydroxybutyric acid (BOHB) in the body tissues and fluids (Opsomer, 2015). The clinical signs include reduced appetite, excessive loss of body weight, reduced milk yield, ketone odour in the breath or milk, hard or dry faeces, reduced motility and, in severe cases, a variety of neurological signs. Ketosis is classified as clinical or subclinical based on levels of ketone bodies in the blood, urine, and milk and the presence or absence of clinical signs. In literature are reported many different cut-off values for BOHB concentration in blood (Opsomer, 2015). As an example, Oetzel (2004) proposed to adopt specific intervention in order to prevent ketosis when more than 10% of cows between 5 and 50 days after parturition have BOHB higher than 1400 μmol/L, while a cut-off of 3000 μmol/L is more suggestive of a clinical condition. Ketosis may increases the risk and severity of the occurrence of other diseases during lactation. The relative risk associated with subclinical ketosis, in a recently published meta-analysis was calculated in terms of odd ratio and it

was found as 3.33 for left abomasal displacement, 5.38 for clinical ketosis, 1.92 early culling and death, 1.75 for metritis, 1.52 for placental retention, 1.61 for clinical mastitis, 2.01 for lameness, and 1.42 for doubling of the somatic cell count. In the same work, the milk losses on 305 milking days, associated with subclinical ketosis were about 251 kgs (Raboisson et al., 2014).

3.4 - Left-displaced abomasum

The shift of the abomasum to the left abdominal space, left-displaced abomasum or LDA, is a disorder frequently observed in early postpartum cows that in some circumstances may cause overt clinical signs, characterized by decreased appetite accompanied by an audible, high pitched tympanic resonance (ping) produced by percussion of the left abdominal wall between the 9th and 12th ribs (Kelton et al., 1998). The pathogenesis is multifactorial with hypocalcemia, metritis, negative energy balance, and nutritional errors being predisposing factors (Esposito et al. 2014). BOHB and aspartate aminotransferase (AST) activity in the blood, both parameters associated with NEB of post- partum cows, may be used to predict the development of LDA but with low sensitivity and specificity (Esposito et al., 2014).

3.5 - Subacute ruminal acidosis

Subacute (or sub-clinical) ruminal acidosis is a common digestive problem in high-producing dairy herds. Producers respond to the demands for increased milk production with higher grain, lower fibre diets that maximize energy intake during early lactation. The combined action of a slower adaptation of rumen to the higher supplementation of early lactation diet, and a higher concentrate intake around parturition may lead to subacute ruminal acidosis (SARA) characterized by a ruminal pH lower than 5.5, a value at which rumen function is depressed (Esposito et al., 2014). This condition has a reported prevalence of 10% to 26%, it has been implicated in the aetiology of laminitis, low BCS, reduced feed intake, abomasal displacement and inflammation (Esposito et al.,

2014). The effect on these conditions seems to be related to a reduced insulin secretion, increased cortisol secretion and reduced phagocytic and migration capacity of neutrophils (Esposito et al., 2014).

3.6 – Mastitis

Mastitis is one of the most prevalent and costly diseases in the high- producing dairy herds with losses attributable to reduced milk production, discarded milk, early culling, veterinary services, and labor costs (Moyes et al., 2009). Typically, mastitis is an inflammation of the mammary gland most often due, but not limited to, bacterial infections and is characterized by the movement of leukocytes and serum proteins from the blood to the site of inflammation. It contributes to compromised milk quality and the potential spread of antimicrobial resistance if antibiotic treatment is not astutely applied. Mastitis can be classified as subclinical or clinical depending on the presence of clinical signs. During subclinical mastitis, somatic cell counts (SCC) in milk are usually elevated, milk production is decreased, inflammation occurs with or without the presence of an intramammary pathogen and no abnormalities in the milk or gland are observed. Clinical mastitis is characterized by an elevated SCC in milk and visual signs of inflammation such as clumpy, watery, bloody, or yellowish milk, and an intramammary pathogen may be isolated. Clinical mastitis may cause a decrease in DMI, swelling of the udder and, in extreme cases, septicemia or endotoxemia that can cause death (Moyes et al., 2009).

3.7 - Uterine diseases

After parturition, uterine involution, regeneration of the endometrium, elimination of bacterial contamination of the uterus and the return of ovarian cyclical activity are necessary for the cow to conceive again. Clinical and subclinical uterine diseases are thus associated with sub-fertility and infertility such as prolonged interval from calving to first insemination or conception, and failure to

conceive. As proposed by Sheldon et al. (2008) uterine diseases may be classified as puerperal and clinical metritis, or as clinical and subclinical endometritis according to the presence of systemic illness, the presence and characteristic of the vaginal discharge and the cytological characteristics of the endometrium. Metritis is a more severe condition requiring more rapid diagnosis and systemic treatments. When an animal is found with abnormal enlarged uterus and a fetid watery red-brown to suppurative uterine discharge within 21 days after parturition, is considered to have metritis. The absence or the presence of systemic illness, such as decreased milk yield, dullness or other signs of toxaemia, fever >39.5 °C, is then used to classify the severity of the disease. Clinical endometritis is defined by purulent or mucopurulent uterine discharge detected in vagina 21 to 26 days after parturition; finally, subclinical endometritis is defined as more than about 10% of neutrophils in uterine cytological samples collected in the five weeks after parturition in an animal without uterine swelling and clinical signs of endometritis. The risk factors for uterine infection include retention of the placenta, the calving environment, twins, dystocia, and diet, whereas microbial contamination of the calving environment is less well established as a predisposing factor for infection of the uterine lumen (Sheldon et al., 2009).

Comprlessively, clinical metritis may occur in 36% to 50% of cows at the herd level, with about 20% of animals having metritis with signs of systemic illness such as pyrexia. Three weeks after parturition, about 20% of cattle have clinical persistent disease (endometritis), and about 30% have chronic inflammation of the uterus without clinical signs of uterine disease (subclinical endometritis) (Sheldon et al., 2009).

3.8 - Retained placenta

Retained placenta (RP) is defined as fetal membranes not expelled after parturition. The time interval to classify a cow as affected by RP varies with the different studies (Fourichon et al., 2000). Membranes can be retained for 7 days or more if a treatment is not administered (Paisley et al.,

1986). This condition causes huge economic losses since it predisposes to metritis and mastitis (Laven and Peters, 1996; Dubuc et al., 2010), even if no association with milk yield has been found and effects on subsequent fertility are debated (Laven and Peters, 1996). The pathogenesis of this syndrome reflects the multifactorial process leading to normal delivery of the placenta. Metabolic, immunological and endocrine alterations are, independently or together, potential gateways to RP (Beagley et al., 2010). The normal release of fetal membranes require the proper activation of an immune response since endometrial leukocytes, mainly neutrophils and macrophages, are involved in the breakdown of the collagen link at the foetus-maternal interface (Beagley et al., 2010). Negative energy balance may play a role in the occurrence of RP, as a high concentration of ketone bodies is known to interfere with leukocyte activities (Scalia et al., 2006). Even if increased NEFA and BOHB concentration 7 days after parturition were found to be associated with the occurrence of RP (Seifi et al., 2007), no differences in these metabolites were found in the days before calving, suggesting that the metabolic milieu preceding the parturition is similar for both cows that will retain and cows that will not retain the fetal membranes. Anyway, leukocytes from cows with RP show, around the time of parturition, a decreased chemotaxis (Gunnink, 1984a; Gunnink, 1984b; Gunnink 1984c; Gunnink 1984d) and a decreased phagocytic activity (Kimura et al., 2002b), confirming the role of a defective immune system in the occurrence of RP. The antioxidant capacity may also affect the proper release of the fetal membranes. This is also suggested by lower prepartum level of superoxide dismutase found in cows with RP (Beagley et al., 2010) and by the beneficial effect of vitamin E supplementation in reducing the occurrence of RP (Bourne et al., 2007). The role of hypocalcaemia as a predisposing factor to RP is still debated. Even if hypocalcaemia may predispose to dystocia and may interfere with the final stage of placental release, interfering with uterine contractions, no differences were found in RP incidence when cows were supplemented with oral calcium or not (Beagley et al., 2010). Despite the presence of an extensive literature, the pathogenesis of RP is still largely unknown (Beagley et al., 2010).

CHAPTER 4:

Aims of the study

CHAPTER 4: AIMS OF THE STUDY

The general aim of this PhD thesis is to determine a diagnostic approach, using methods and techniques usually employed in veterinary clinical pathology, to investigate and monitor the health status of cows in the transition period, paying a special attention to some of the diseases that usually affect cows in this very sensitive period. As previously stated, the transition period is a crucial period for dairy cows as both reproductive and productive challenges are in course. The management of the dry period, the hormonal changes near parturition and the following metabolic, inflammatory and oxidative challenges occurring before and after parturition drive the cow through a period of substantially low energy availability. All these factors predispose to the occurrence of different subclinical conditions that often represent the initial phases of more overt diseases frequently observed mainly in early lactation. Both subclinical and clinical diseases directly or indirectly affect the productive and reproductive outcome of the affected cows, leading to evident economic losses and increased use of drugs. The presence of a not identified subclinical condition has more detrimental effects on the herd health, as it may lead to a delay in the proper nutritional or management intervention necessary to ameliorate the health of the farm. In this context the clinical pathologist may help farmers and clinicians to develop strategies to prevent, identify, and control subclinical diseases affecting dairy cows through the use of laboratory analysis.

The identification of abnormal hematological or biochemical results first requires the knowledge of which results may be considered as ‘normal’. The **chapter 5** describes a study that was conducted to establish reference intervals for healthy high producing dairy cows around 3 and 30 days after calving. In this study the possible influence of variables such as herd, distance from parturition and parity, was also examined. The results obtained from this study were then applied to interpret data from further studies. In literature in fact, there are several publications reporting reference intervals of the most common analytes in cows, but none of them has compared results obtained at parturition and one month later for a huge panel of analytes as it has been done in the present thesis.

The retained placenta is one of the most debated diseases of the peri-partum in cows. Besides the variable occurrence of this condition (the incidence is variable depending on farm and, within a farm, also among different years), and the demonstrated predisposing role for productive and reproductive issues, its pathogenesis and the related hematological variations are still largely unknown. The identification of a specific pattern associated with the occurrence of the disease could have beneficial effects in the timely intervention and could be useful to recommend new strategic treatments. The second aim of this thesis, covered in **chapter 6**, was thus the evaluation of the possible laboratory changes that may be associated with or that may be predisposing for retention of the fetal membranes around calving. The first part of the work (**section 6.1**) concerned a retrospective analysis of data regarding hematological and biochemical parameters, acute phase proteins and markers of oxidative stress on samples obtained soon after parturition from high producing cows with and without retained placenta. In the second section of the chapter (**6.2**), a prospective study was focused on the hematological variations occurring around calving in cows with retained placenta and in cows that normally expelled the placenta, in order to explore the dynamics of these changes and the possible relationship with the pathogenesis of the disease.

As stated above, veterinary clinical pathology may also have a role in the prevention and monitoring of subclinical diseases and in the evaluation of efficacy of specific treatments. Results from these investigations are presented in **chapter 7,** where a systematic screening for sub-clinical ketosis during the transition period was performed. The measurement of the blood levels of BHOB and NEFA, eventually followed by ad hoc treatment with propylene glycol, calcium propionate, betaine, niacin and molasses and by selective treatment with calcium, was adopted to monitor the morbidity and incidence of milk fever, retained placenta, metritis and displaced abomasum during the post-partum period.

CHAPTER 5:

Reference intervals for hematological and biochemical parameters, acute phase proteins, and markers of oxidative stress in holstein dairy cows at 3 and 30 days post-partum

CHAPTER 5: REFERENCE INTERVALS FOR HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS, ACUTE PHASE PROTEINS, AND MARKERS OF OXIDATIVE STRESS IN HOLSTEIN DAIRY COWS AT 3 AND 30 DAYS POST-PARTUM

5.1. Introduction

The first step in determining the health status of an animal through the evaluation of blood analytes is the comparison of laboratory results with a set of reference values representing healthy animals. Population-based reference intervals (RIs) have become one of the most commonly used tools employed in the clinical decision-making process (Horn and Pesce, 2005).

Recently, the American Society for Veterinary Clinical Pathology (ASVCP) has established the guidelines for the determination of de novo reference intervals in veterinary species (Friedrichs et al., 2012). The establishment of specific RIs is advisable when different metabolic status, habits, gender, ages and breeds in the patient population are present, or when existing RIs are not suitable for the laboratory's patient population (Lefebvre, 2011; Friedrichs et al., 2012;).

As known, soon after parturition, the lactation in cows is characterized by the start of homeostatic mechanisms that have to maintain proper blood nutrients concentration in order to sustain the increased needs for synthesis of colostrum and milk (Goff et al., 2008; Bertoni et al., 2009; Bertoni and Trevisi, 2013). These increased energy requirements, often associated with a decline of food intake during late gestation, generally result in a state of negative energy and protein balance that persists for the first weeks of lactation (Bell, 1995; Grummer et al., 2004; Ingvartsen, 2006). Moreover, this period is characterized by an altered immune system function that predisposes the cows to different pathological conditions (Goff, 2006; Kehrli et al., 2006). As a matter of fact, most metabolic diseases, including milk fever, ketosis, abomasal displacement, and retained placenta primarily affect cows within the first two weeks of lactation (Fronk et al., 1980; Erb et al., 1984;

Drackley 1999, Heuer et al., 1999) while metritis, mastitis and lameness are diagnosed mostly within the first month of lactation (Erb et al., 1984; Kinsel et al., 1998). Four weeks after calving, even if the homeostatic processes are not yet terminated, healthy dairy cows show a more balanced metabolic condition that will allow them to reach the lactation peak (Opsomer, 2015). It is well known that lactation stage and the presence of subclinical problems are factors that may determine variations in metabolic and inflammatory profiles (Bionaz et al., 2007; Bertoni et al., 2008; Cozzi et al., 2011;). Consequently, laboratory results obtained from a heterogenic population of lactating cows, regerdless the lactation stage, will probably generate wider RIs leading to misdiagnosis when these RIs are adopted to identify abnormalities in a specific lactation moment. The introduction of new technologies makes problematic the use of reference values generated in the past (Lumsden et al., 1980; Roussel et al., 1982; Dubreil et al., 1997). Moreover, only few recent works were focused on the establishment of biochemical and haematological reference limits for transition cows (Quiroz-Rocha et al., 2009; Cozzi et al., 2011; Brscic et al., 2015). The present study was realized in order to define specific reference intervals for hematological and biochemical variables in a population of clinically healthy lactating Holstein cows sampled around 3 and 30 days in milk (DIM) selecting results from a database concerning a three years period.

5.2 - Material and methods

5.2.1 Retrospective analysis of the database

This study started with a retrospective search of data recorded in the database of the ProZoo project, a research project aimed to investigate the relationship between genomic traits and bovine health and production. The ProZoo database lists information about production, reproduction, and health status, including results of blood samplings recorded over a 3 year period (from June 2010 to February 2013) from 4 intensive farms in the area of Lodi (Lombardy region, Italy). The evaluated herds had 265, 415, 423 and 206 milking cows respectively; a mean (±SD) days in milk (DIM)

duration of 304 (±37), 321 (±35), 311 (±37) and 318 (41); a mean (±SD) normalized production at 305 days of 9577 (±1794), 10404 (±1953), 10278 (±1965), 9319 (±1851) kilograms of milk respectively for herd A, B, C, and D. All the cows were fed with a TMR (total mixed ration). Milking was performed twice a day, at 12 h intervals.

The database was searched in order to select data corresponding to cows that fulfilled the following inclusion criteria.

- availability of data about the complete blood count (CBC), biochemical profile, serum protein electrophoresis, and markers of inflammation/oxidative stress at 3±1 DIM and 30±3 DIM;
- negative history for any clinical disease or laboratory abnormality during the gestation period and normal parturition course;
- no clinical events or abnormal laboratory results during the first month of lactation;
- no anti-inflammatory or antibiotic treatments administered in the days before collection of blood samples;
- average production adjusted for 305 days during the lactation period included in this study: ≥7.000 kg;
- lactation period of at least 200 days.

Exclusion criteria were:

- the absence of at least one of the above mentioned inclusion criteria
- samples characterized by evidence of lipemia, hemolysis or icterus or by the presence of visible clots in samples collected in EDTA for routine hematology.

A complete clinical examination, included the record of body condition score (BCS), was performed in the first days after calving and at the time of the gynecological visits performed at the end of the first month of lactation.

5.2.2 Blood sampling

In all the animals included in this study, peripheral blood samples were collected at 3±1 DIM and at 30±3 DIM. Specifically, 30 mLs of venous blood were collected from the jugular vein: 10 mLs of blood were placed in plain tubes (Venosafe plastic tubes for serum, Terumo, Europe) to perform routine biochemistry, 10 mLs were placed in tubes with EDTA (Venosafe plastic tubes for hematology, Terumo, Europe) to perform routine hematology, and 10 mLs were placed in tubes with lithium heparin (Venosafe plastic tubes for plasma, Terumo, Europe) for the measurement of acute phase proteins (APPs) and of analytes associated with oxidative stress metabolism.

All the samples were immediately placed at 4 °C and submitted to the Central Laboratory of the Veterinary Teaching Hospital of the University of Milan where routine hematology was performed as described below. Plain tubes and tubes with lithium heparin were immediately centrifuged at 2,200 x g for 10 minutes upon arrival at the lab. Harvested heparinized plasma and sera were then frozen at -80°C for a maximum of 3 months before biochemical tests were performed.

5.2.3 Routine hematology

Routine hematology was performed using an automated laser hematology analyzer (ADVIA 120 with multispecies software for veterinary use, Siemens Healthcare Diagnostics, Milan, Italy). The leukocyte differential provided by the instrument was checked microscopically on blood smears prepared upon arrival of the sample at the laboratory and stained with a modified Romanowsky stain (Dif-stain kit, Titolchimica S.P.A., Rovigo, Italy). Only samples without visible clots were processed.

5.2.4 Clinical chemistry and serum protein electrophoresis

Routine biochemical analyses were run on serum or plasma with automated spectrophotometers (ILAB300 plus and ILAB600, Instrumentation Laboratory S.p.a., Milan, Italy) using reagents provided by the manufacturer of the instruments, except when otherwise specified. The following analytes were measured: alkaline phosphatase (ALP, kinetic IFCC method), aspartate aminotransferase (AST, kinetic IFCC method), calcium (orthocresoftaleine method), creatinine (Jaffè method), total proteins (biuret method), albumin (bromochresol green method), total bilirubin (diazo reactive with sulphanilic acid), glucose (GOD-POD method), total cholesterol (cholesterol oxidase method), urea (urease method), phosphate (phosphomolibdate method), γ-glutamyl transferase (GGT, kinetic IFCC method), zinc (colorimetric with Nitro-PAPS), sodium, potassium and chloride (ion selective electrodes method), non-esterified fatty acid (NEFA, ACS-ACOD method, Wako Chemicals GmbH, Neuss, Germany), β-hydroxybutyrate (BOHB, D-3-Hydroxybutyrate dehydrogenase method, Randox Laboratories Ltd., Crumlin, Co. Antrium, UK), fructosamine (Randox Laboratories Ltd., Crumlin, Co. Antrium, UK).

Serum protein electrophoresis was performed on agarose gel using the automated analyzer Hydrasis (Sebia Italia Srl, Bagno a Ripoli, Florence, Italy) and the specific manufacturer's reagents (Hydragel 15 β1-β2, Sebia Italia Srl) following the procedure described in another study (Paltrinieri et al., 2014). Absolute concentrations (g/L) for each electrophoretic fraction were calculated based on total serum protein and on the percentage of the area under each peak.

5.2.5 Acute phase proteins (APPs) and other markers of inflammation and oxidative stress

Heparinized plasma was periodically sent to the Institute of Zootechnics (Università Cattolica del Sacro Cuore, Piacenza), to measure the markers of inflammation and oxidative stress: ceruloplasmin (Cp) with the method described by Sunderman and Nomoto (1970); haptoglobin (Hp), using the method described by Skinner et al. (1991); paraoxonase-1 (PON-1) with the method described by Ferré et al. (2002); derivates of reactive oxygen metabolites (d-ROMs) using the Kit

"d-ROMs Test" from Diacron International S.r.l. (Grosseto, Italy); thiol groups (SHp) measured using a specific colorimetric kit (Diacron International S.r.l.); myeloperoxidase (MPO) determined through a colorimetric method described by Bradley et al. (1982). These methods were run on plasma with an automated spectrophotometer (ILAB 600, Instrumentation Laboratory S.p.a., Milan, Italy).

Moreover, plasma retinol, tocopherol and β-carotene were detected in accordance to the procedure described by Trevisi et al., 2013. Briefly, vitamins were extracted from plasma with hexane and analyzed by reverse-phase HPLC using Spherisorb ODS-2.3 μm, in a 150 × 4.6 mm column (Alltech, Deerfield, IL); a UV detector set at 325 nm (for retinol) or 290 nm (for tocopherol) or 460 nm (for β-carotene); and 80:20 methanol: tetrahydrofurane as the mobile phase.

5.2.6 Establishment of new RIs

Specific RIs were generated for the two sampling times (3±1 DIM and 30±3 DIM) using an Excel spreadsheet with the Reference Value Advisor (v 2.1) set of macroinstructions (Geffré et al., 2011). The software performs the following computations recommended by the International Federation of Clinical Chemistry-Clinical and Laboratory Standards Institute (CLSI, 2008): descriptive statistics (e.g. mean, median, SD, minimum and maximum values); tests of normality (Anderson–Darling with histograms and Q–Q plots and Box–Cox transformation); outlier analysis. Both Dixon–Reed and Tukey tests were used, and outliers classified as 'suspected' were retained, as recommended by the ASVCP guidelines (Friedrichs et al., 2012), while far outliers were removed from the analysis. RIs were calculated using standard and robust methods on both non-transformed and transformed data. The software indicates the best method to define the RI based on data distribution. A non-parametric bootstrap method was used to calculate the 90% confidence interval.

5.2.7 Statistical analysis and partitioning of RIs

For all the variables, the results at the two sampling times (3±1 DIM and 30±3 DIM) were compared with a paired T-test or a Wilcoxon signed-rank test according to normality and homogeneity of variance of the parameter. Within each sampling time (3±1 and 28±3 DIM), the effect of herd (n=4) was evaluated by a Kruskal-Wallis test followed by a Bonferroni post-test procedure whereas the effects of day of sampling (3 at 3±1 DIM and 7 at 30±3 DIM) and parity (1 vs 2 vs >2) were evaluated by regression analysis followed by Kruskal-Wallis test with Bonferroni post-test procedure on significant results. For all the calculation the p-value was set at 0.05.

When statistically significant differences were found between the two sampling times, the Harris and Boyd z-test (Harris and Boyd, 1990) was applied to verify the possible need to adopt separated reference intervals.

Table 5.1: detail of the numbers of animals for herd and day of sampling finally included in the reference interval study

	Herd A	Herd B	Herd C	Herd D	TOTAL
	n	n	n	n	n
3±1 DIM	**37**	**41**	**39**	**28**	**145**
2 DIM	14	20	13	13	60
3 DIM	14	10	15	5	44
4 DIM	9	11	11	10	41
30±3 DIM	**37**	**41**	**39**	**28**	**145**
27 DIM	4	8	2	3	17
28 DIM	6	6	8	5	25
29 DIM	4	6	6	4	20
30 DIM	12	7	9	4	32
31 DIM	5	8	9	5	27
32 DIM	5	5	4	4	18
33 DIM	1	1	1	3	6

5.3. Results

5.3.1 Characteristics of the reference sample group and new RIs

After adoption of inclusion and exclusion criteria, laboratory results from 145 Holstein dairy cows from four different herds were included. Details of the number of animals sampled in each herd and at each sampling time are reported in table 5.1. A lower number of samples was available for routine hematology, due to the need to immediately process whole blood samples: samples in EDTA that cannot be processed in the day of sampling were not included in the study. Similarly, serum protein electrophoresis was performed on a lower number of samples, due to the insufficient volume of serum available in the other cases. The median (min-max) BCS at 3±1 DIM were 3.25 (3-3.75), 3.5 (3-3.75), 3.5 (3-4), 3.25 (3-4) for herd A, B, C and D respectively. The median BCS at 30±1 DIM was 3.00 in all the herds (minimum and maximum values were 2.75-3.25 in herd A, 2.75-3.50 in herd B and C and 2.50-3.25 in herd D). No statistically significant differences between herds were observed for BCS at both times whereas, as expected, in all the herds a significant decrease in the BCS was observed according to time (P=0.000 for the four herds). The mean milk production normalized at 305 days was significantly lower (P=0.000) in herd D (mean±SD 8831±516 kgs) compared to the other herds (10028 ±806, 10948±1176, 9845±903 kgs, and P values of 0.017, 0.000, and 0.001 for herd A, B, and C respectively). A significantly lower (P=0.002) mean DIM was found in herd B (mean±SD 298±29 days) compared to herd C (355±88 days with P=0.011), and D (308±43 days with P=0.029) whereas herd A had a mean DIM of 312±40 days. Finally, no differences were found in the means of the interval from partum to conception in the four herds (mean±SD were 101±50, 81±25, 129±87, and 90±38 days for herd A, B, C and D respectively).

5.3.2 Differences between sampling times

The reference intervals determined at the two sampling times are reported in tables 5.2 (hematology and serum protein electrophoresis), 5.3 (clinical chemistry), and 5.4 (markers of inflammation/oxidative stress). In these tables the results of the statistical analysis are also reported. Details on the distribution of data for each reference interval, as well as on the final number of data used for each calculation after exclusion of outliers are reported in tables 5.5 to 5.8.

Data of 14/49 analytes (WBC, eosinophils, MCH, potassium, albumin, total globulin, A/G ratio, α1-globulin, α2-globulin, β1-gloubulin, γ-globulin, SHp and MPO) were not significantly different at the two sampling times and according to the recommendation from Harris and Boyd (1990) common reference interval can be used for these analytes, however due to the nature of data (the two sets of data belong to same animals sampled at two different times) data may not be used together to generate common RI and the use of the lowest lower reference limit (LRL) and higher upper reference limit (URL) might be used for cows at 3±1 and 30 ±3 DIM. On the contrary, RIs for 35/49 variables were significantly different between the two sampling times. Concerning hematological and electrophoretical results, compared with the early post-partum (3±1 DIM), the RIs recorded at 30±3 DIM had the following differences: RBC, Hb, Ht and MCV had a lower LRL, neutrophils and lymphocytes had both a lower LRL and a lower URL, monocytes had a lower URL, MCHC, and PLT, had both a higher LRL and a higher URL, and finally total globulin and β2-globulin had a significantly higher URL.

Concerning biochemical analytes and markers of inflammation/oxidation, compared with the early post-partum (3±1 DIM), the RIs recorded at 30±3 DIM had the following differences: ALP, AST, creatinine, sodium, total bilirubin, NEFA, BOHB, fructosamine, Hp and dROMs had both lower LRL and URL, calcium, GGT, magnesium, phosphorous, total protein, urea, glucose, cholesterol, zinc, PON-1, vitamin A, vitamin E, β-carotene had both higher LRL and URL, Cp and chloride had a lower LRL but an higher URL.

5.3.3 Differences between herds, days of sampling, and parity group

Regression analysis revealed a few day-related differences at day 3±1 (eosinophils, AST, and fructosamine) and 30±3 (lymphocytes, monocytes, and cholesterol) that, however, were not supported by significant results at Kruskal-Wallis test. According to these findings, the adoption of separated reference intervals for the two days of sampling was considered not necessary.

Conversely, the comparison of results between herds revealed, both at 3±1 DIM and at 30±3 DIM, several differences that were significant according to both regression analysis and Kruskal-Wallis test and all required a herd-specific RIs, according to the Harris and Boyd z-test. These include, at 3±1 DIM, RBC, WBC, AST, creatinine, potassium, sodium, NEFA, fructosamine, PON-1, d-ROMs, SHp, vitamin A and β-carotene, and at day 30±3, HCT, RBC, Hb, MCH, MCHC, RDW, lymphocytes, eosinophils, calcium, creatinine, potassium, magnesium, phosphorous, total proteins, urea, zinc, NEFA, BOHB, fructosamine, Cp, Hp, d-ROMs, vitamin E and β-carotene. However, in most cases the differences between single samplings were not univocal and/or not depending on a specific difference between a single herd and all the others. This occurred only for phosphorous of herd B at 30 ±3 DIM , for creatinine at 3±1 DIM and for chloride and potassium at 30 ±3 DIM in herd C, for MCHC, total proteins, urea, zinc, and Cp of herd D at 30 ±3 DIM, that were different compared with other herds.

Finally, significant effects according to parity were found only for eosinophils at 3±1 DIM and for HCT, RBC, Hb, MCV, MCH, WBC, neutrophils, lymphocytes, eosinophils, ALP, calcium, creatinine, potassium, phosphorous, total proteins, glucose, total bilirubin, PON-1 and vitamin A at 30±3 DIM. Except for eosinophils at 3±1 DIM and neutrophils, eosinophils, and total proteins at day 30±3 DIM, all these variables had significant differences at Kruskal-Wallis comparison as shown in tables 5.2 and 5.3 and in figure 5.1. Again, the differences between parity groups were not univocal and/or not depending on a specific difference between one group and to all the others. This

occurred, at 30±3 DIM, only for WBC, ALP, calcium and creatinine of cows at first lactation and for total bilirubin, PON-1, and vitamin A of cows with more than 3 lactations that, compared to the other groups, were significantly higher and lower, respectively.

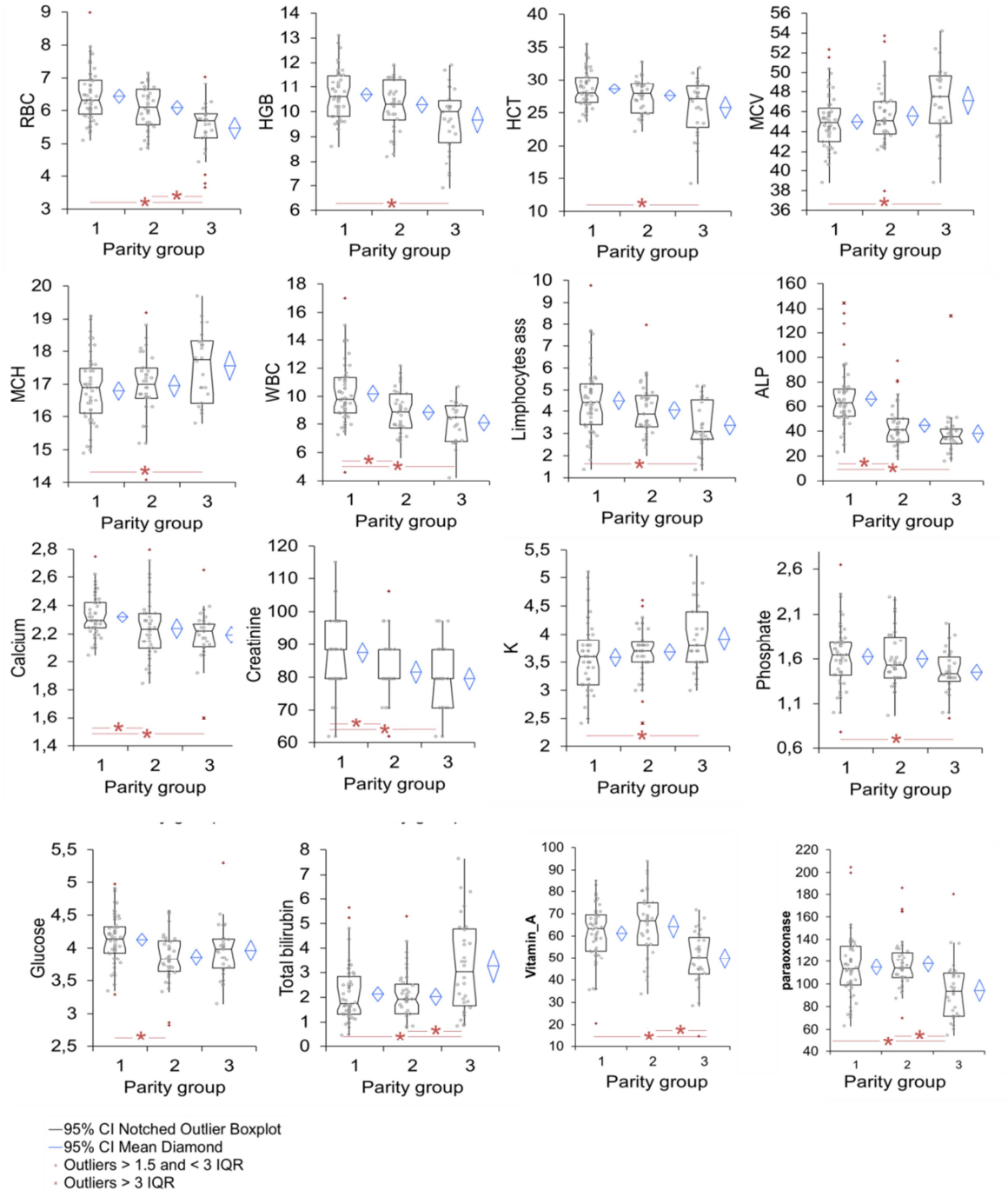

Figure 5.1: Laboratory parameters significantly different between parity groups. 1 = cows at first lactation; 2 = cows at second lactation; 3 = cows with three or more lactations. *P value <0.05

Table 5.2: Hematological and serum protein electrophoresis reference intervals (RIs) specific for cow at 3±1 and 30±3 DIM and results of the statistical analysis. The RIs previously in use in the laboratory are shown in the column on the right. RIs in bold means that significant differences were found between results used to generate the RI of cows sampled at 3±1 and 30±3 DIM.

Analyte	RI Day 3±1	P days 3±1 DIM[a]	P herd 3±1 DIM[b]	P lactation 3±1 DIM[a]	RI Day 30±3	P days 30±3 DIM[a]	P herd 30±3 DIM[b]	P lactation 30±3 DIM[a]	P Day 3±1 vs 30±3[c]	Previous RI in use[d]
HCT (L/L)	**0.25-0.38**	ns	*	ns	**0.20-0.34**	ns	0.003 A,B<C; C>D	0.000 1>3	**0.000**	0.26-0.40
RBC count (10^{12}/L)	**5.3-8.3**	ns	0.011 B<D	ns	**4.1-7.8**	ns	0.022 A<C	0.000 1,2>3	**0.000**	7.4-11.6
Hemoglobin (g/L)	**93.3-137.8**	ns	*	ns	**75.2-127.9**	ns	0.001 A,B<C; C>D	0.000 1>3	**0.000**	98-153
MCV (μm^3)	**40.1-53.2**	ns	*	ns	**38.9-53.1**	ns	*	0.005 1<3	**0.0018**	29-40.4
MCH (pg/cell)	13.7-19.5	ns	*	ns	15.0-19.1	ns	0.036 A>D	0.005 1<3	ns	11.1-15.3
MCHC (g/L)	**32.8-37.4**	ns	*	ns	**33.5-37.9**	ns	0.000 A,B,C<D; C,A>B	ns	**0.0005**	34-38
RDW (%)	14.1-19.7	ns	*	ns	14.4-19.0	ns	0.022 B<C	ns	ns	23.3-33.6
WBC (x 10^6/L)	4.7-13.9	ns	0.011 B<D	ns	5.4-14.0	ns	*	0.000 1>2,3	ns	6.2-13.6
Neutrophils (x 10^9/L)	**2.6-12.7**	ns	*	ns	**1.5-9.0**	ns	*	0.014*	**0.000**	1.8-6.3
Lymphocytes (x 10^9/L)	**3.9-13.8**	ns	*	ns	**1.6-7.7**	0.021*	0.017 C>D	0.001 1>3	**0.000**	1.6-5.6
Monocytes (x 10^9/L)	**0.0-2.8**	ns	*	ns	**0.0-1.1**	ns	*	ns	**0.000**	0-0.8
Eosinophils (x 10^9/L)	**0.0-1.6**	0.000*	*	0.000*	**0.0-1.3**	0.000*	0.006 B<C,D	0.000*	ns	0-0.9
Platelets (x 10^9/L)	**189-510**	ns	*	ns	**285-752**	ns	*	ns	**0.000**	412-1003
Albumin (g/L)	15.3-52.3	ns	*	ns	14.1-61.7	ns	*	ns	ns	27-43
Globulin (g/L)	**19.3-57.6**	ns	*	ns	**20.6-75.7**	ns	*	ns	**0.0443**	
A/G ratio	0.31-2.29	ns	*	ns	0.19-2.26	ns	*	ns	ns	0.7-1.5
α1-globulin (g/L)	1.8-5.4	ns	*	ns	1.7-5.7	ns	*	ns	ns	1.3-2.8
α2-globulin (g/L)	3.6-11.3	ns	*	ns	3.9-10.2	ns	*	ns	ns	6.0-13
β1-globulin (g/L)	2.2-11.2	ns	*	ns	3.1-9.9	ns	*	ns	ns	1.8-6.6
β2-globulin (g/L)	**3.4-11.5**	ns	*	ns	**3.0-21.0**	ns	*	ns	**0.0155**	5.1-13
γ-globulin (g/L)	4.0-23.5	ns	*	ns	5.6-34.6	ns	*	ns	ns	3.5-9.4

[a] P values according to results of regression analysis and differences between means of the subgroups according to Kruskal-Wallis test (P<0.05). *ns* not significant at regression analysis; * not significant at Kruskal-Wallis test; 1: cows at first lactation; 2: cows at second lactation; 3: cows with three or more lactations.

[b] P values according to results of Kruskall-Wallis test performed on results from four different herds (A to D). * not significant at Kruskal-Wallis test.

[c] P values of the paired test performed on results from cows sampled at 3±1 and 30±3 DIM. *ns* not significant.

[d] unknown population and methods

Table 5.3: Reference intervals for biochemical parameters specific for cow at 3±1 and 30±3 DIM and results of the statistical analysis. The RIs previously in use in the laboratory are shown in the column on the right. RIs in bold means that significant differences were found between results used to generate the RI of cows sampled at 3±1 and 30±3 DIM.

Analyte	RI Day 3±1	P days 3±1 DIM[a]	P herd 3±1 DIM[b]	P lactation 3±1 DIM[a]	RI Day 30±3	P days 30±3 DIM[a]	P herd 30±3 DIM[b]	P lactation 30±3 DIM[a]	P Day 3±1 vs 30±3[c]	Previous RI in use[d]
ALP (U/L)	**26-145**	ns	*	ns	**16-132**	ns	*	0.000 1>2,3	**0.000**	29-99
AST (U/L)	**58.8-150.5**	0.029*	0.022 A>C	ns	**52.3-111.8**	ns	*	ns	**0.000**	48-100
Ca (mmol/L)	**1.60-2.54**	ns	*	ns	**1.92-2.70**	ns	0.003 A<B,C	0.001 1>2,3	**0.000**	1.97-2.49
Creatinine (µmol/L)	**70.7-130.2**	ns	0.002 A,B,D>C	ns	**61.9-106.1**	ns	*	0.001 1>2,3	**0.000**	0-141
GGT (U/L)	**12.0-36.8**	ns	*	ns	**13.2-48.0**	ns	*	ns	**0.000**	28-48
Cl (mmol/L)	**87.0-102.8**	ns	*	ns	**81-103**	ns	0.000 A,B,D>C	ns	**0.000**	90-110
K (mmol/L)	2.7-4.7	ns	0.019 A<B	ns	2.5-5.0	ns	0.000 A,B,D>C	0.008 1<3	ns	4-5.3
Na (mmol/L)	**126.0-144.0**	ns	0.002 A>C,D; B>D	ns	**123.3-143.0**	ns	*	ns	**0.000**	135-155
Mg (mmol/L)	**0.62-1.25**	ns	*	ns	**0.71-1.35**	ns	0.018 C>D	ns	**0.000**	0.57-0.95
Phosphorous (mmol/L)	**0.82-2.02**	ns	*	ns	**0.98-2.29**	ns	0.002 B>A,C,D	0.017 1>3	**0.000**	1.48-2.9
Total proteins (g/L)	**48.3-83.4**	ns	*	ns	**59.8-95.7**	ns	0.000 A,B,C>D	0.002*	**0.000**	59-77
Urea (mmol/L)	**1.67-4.45**	ns	*	ns	**2.21-6.95**	ns	0.000 C>A,B,D; A>D	ns	**0.000**	2.27-7.5
Glucose (mmol/L)	**2.85-4.81**	ns	*	ns	**3.18-4.90**	ns	*	0.020 1>2	**0.0018**	2.77-3.88
Cholesterol (mmol/L)	**1.35-3.34**	ns	*	ns	**2.67-6.90**	0.019*	*	ns	**0.000**	2.25-6.57
Zinc (µmol/L)	**2.80-17.3**	ns	*	ns	**10.71-19.89**	ns	0.003 A,B,C>D	ns	**0.0037**	70-130
Total bilirubin (µmol/L)	**1.03-19.45**	ns	*	ns	**0.51-6.10**	ns	*	0.001 1,2>3	**0.000**	1.71-5.13
NEFA (mmol/L)	**0.16-1.63**	ns	0.001 A>B,C	ns	**0.09-0.89**	ns	0.005 C>B,D	ns	**0.000**	0.4-0.8
BOHB (mmol/L)	**0.38-1.71**	ns	*	ns	**0.26-1.12**	ns	0.003 A,B<C	ns	**0.000**	0.03-0.3
Fructosamine (µmol/L)	**135-228**	0.016*	0.001	ns	**113-210**	ns	0.000 D>A,B; B<C	ns	**0.000**	na

[a] P values according to results of regression analysis and differences between means of the subgroups according to Kruskal-Wallis test (P<0.05). *ns* not significant at regression analysis; * not significant at Kruskal-Wallis test; 1: cows at first lactation; 2: cows at second lactation; 3: cows with three or more lactations.
[b] P values according to results of Kruskall-Wallis test performed on results from four different herds (A to D). * not significant at Kruskal-Wallis test.
[c] P values of the paired test performed on results from cows sampled at 3±1 and 30±3 DIM. *ns* not significant.
[d] unknown population and methods

Table 5.4: Reference intervals for some markers of inflammation/oxidation specific for cow at 3±1 and 30±3 DIM and results of the statistical analysis. The RIs previously in use in the laboratory are shown in the column on the right. RIs in bold means that significant differences were found between results used to generate the RI of cows sampled at 3±1 and 30±3 DIM.

Analyte	RI Day 3±1	P days 3±1 DIM[a]	P herd 3±1 DIM[b]	P lactation 3±1 DIM[a]	RI Day 30±3	P days 30±3 DIM[a]	P herd 30±3 DIM[b]	P lactation 30±3 DIM[a]	P Day 3±1 vs 30±3[c]	Previous RI in use[d]
Ceruloplasmin (µmol/L)	**2.35-4.98**	ns	*	ns	**1.35-5.23**	ns	0.000 A<B,C,D	ns	**0.000**	na
Haptoglobin (g/L)	**0.08-2.18**	ns	*	ns	**0.01-0.44**	ns	0.000 A,D<B	ns	**0.000**	na
Paraoxonase-1 (U/mL)	**46.3-135.0**	ns	0.000 A,B<C,D	ns	**63.2-183.9**	ns	*	0.002 1,2>3	**0.000**	na
dROMs (mg H_2O_2/100 mL)	**9.4-24.2**	ns	0.033 A>C	ns	**7.0-21.5**	ns	0.018 C<D	ns	**0.000**	na
Thiol groups (µmol/L)	226-522	ns	0.014 A>B,C	ns	240-517	ns	*	ns	ns	na
Myeloperoxidase (U/L)	262-742	ns	*	ns	310-634	ns	*	ns	ns	na
Vitamin A (µg/100 mL)	**16.6-68.0**	ns	0.000 A,B<C,D	ns	**22.9-87.4**	ns	*	0.002 2<3	**0.000**	na
Vitamin E (µg/mL)	**0.36-3.92**	ns	*	ns	**1.02-5.86**	ns	0.012 B<C	ns	**0.000**	na
Beta-carotene (mg/100 mL)	**0.03-0.24**	ns	0.000 A,B<C,D; A<B	ns	**0.05-0.27**	ns	0.000 A,B<C,D	ns	**0.0001**	na

[a] P values according to results of regression analysis and differences between means of the subgroups according to Kruskal-Wallis test (P<0.05). *ns* not significant at regression analysis; * not significant at Kruskal-Wallis test; 1: cows at first lactation; 2: cows at second lactation; 3: cows with three or more lactations.
[b] P values according to results of Kruskall-Wallis test performed on results from four different herds (A to D). * not significant at Kruskal-Wallis test.
[c] P values of the paired test performed on results from cows sampled at 3±1 and 30±3 DIM. *ns* not significant.
[d] unknown population and methods

Table 5.5: Descriptive statistics of hematological reference intervals for cows at 3±1 DIM. Number of reference values included, mean, standard deviation, median, minimum, maximum, RI, number of outliers, confidence intervals of the lower and upper reference limits, distribution of data and method employed to generate the reference interval.

Analyte	SI Units	N	Mean	SD	Median	Min	Max	RI	Outliers	Lower Ref Lim 90% CI	Upper Ref Lim 90% CI	Dist.	Method
HCT	L/L	126	0.31	0.03	0.31	0.24	0.39	0.25-0.38	1S	0.24-0.27	0.37-0.39	G	NP
RBC count	10^{12}/L	126	6.7	0.7	6.7	4.8	8.5	5.3-8.3	2S	4.8-5.7	7.8-8.5	G	NP
Hemoglobin	g/L	126	114.7	11.0	114.5	87.0	141.0	93.3-137.8	0	87-96	133-141	G	NP
MCV	μm^3	126	47.0	3.1	46.6	39.9	54.8	40.1-53.2	0	39.9-42.4	52.9-54.8	G	NP
MCH	pg/cell	126	17.1	1.3	17.2	12.7	19.9	13.7-19.5	3	12.7-14.7	19.3-19.9	G	NP
MCHC	g/L	126	35.2	1.2	35.0	29.6	38.2	32.8-37.4	2S	29.6-33.6	37.1-38.2	NG	NP
RDW	%	125	16.7	1.2	16.7	13.7	20.1	14.1-19.7	3S, 1R	13.7-15.1	18.9-20.1	NG	NP
WBC	10^9/L	126	9.4	2.6	9.4	3.4	17.1	4.7-13.9	0	3.4-5.4	13.2-17.1	G	NP
Neutrophils	10^9/L	126	7.3	2.7	7.3	2.0	13.3	2.6-12.7	0	2.0-3.2	11.7-13.3	G	NP
Lymphocytes	10^9/L	126	8.4	2.7	8.4	2.6	15.3	3.9-13.8	0	2.6-4.6	12.5-15.3	G	NP
Monocyte	10^9/L	125	1.0	0.7	0.9	0.0	3.4	0.0-2.8	1S, 1R	0.0-0.0	2.1-3.4	NG	NP
Eosinophils	10^9/L	125	0.3	0.4	0.2	0.0	1.8	0.0-1.6	1S, 1R	0.0-0.0	1.1-1.8	NG	NP
Platelets (optical)	10^9/L	125	339	87	337	40	550	189-510	1S, 1R	40-218	474-550	G	NP

S: suspected outliers; R: removed outliers; G: Gaussian distribution of data; NG: non-gaussian distribution of data; NP: nonparametric method.

Table 5.6: Descriptive statistics of biochemical reference intervals for cows at 3±1 DIM. Number of reference values included, mean, standard deviation, median, minimum, maximum, RI, number of outliers, confidence intervals of the lower and upper reference limits, distribution of data and method employed to generate the reference interval.

Analyte	SI Units	N	Mean	SD	Median	Min	Max	RI	Outliers	Lower Ref Lim 90% CI	Upper Ref Lim 90% CI	Dist.	Method
ALP	U/L	131	69	28	64	17	166	26-145	1S	17-35	121-166	NG	NP
AST	U/L	129	86.7	20.4	82.0	52.0	164.0	58.8-150.5	5S, 2R	52.0-62.0	127.0-164.0	NG	NP
Ca	mmol/L	131	2.09	0.23	2.10	1.20	2.84	1.60-2.54	3S, 1R	1.20-1.70	2.40-2.84	NG	NP
Creatinine	µmol/L	130	96.7	14.3	97.2	70.7	132.6	70.7-130.2	3S, 1R	70.7-70.7	123.8-132.6	NG	NP
GGT	U/L	128	20.5	6.5	19.0	7.0	47.0	12.0-36.8	3S, 3R	7.0-12.0	33.0-47.0	NG	NP
Cl	mmol/L	129	95.9	3.7	96.0	85.0	104.0	87.0-102.8	5S, 2R	85-88	102-104	NG	NP
K	mmol/L	131	3.7	0.5	3.6	2.5	5.0	2.7-4.7	0	2.5-2.9	4.6-5.0	NG	NP
Na	mmol/L	128	137.7	4.1	139.0	125.0	146.0	126.0-144.0	6S, 3R	125.0-130.0	146.0-149.0	NG	NP
Mg	mmol/L	129	0.89	0.16	0.91	0.53	1.32	0.62-1.25	1S, 2R	0.53-0.62	1.11-1.32	G	NP
Phosphate	mmol/L	131	1.37	0.33	1.32	0.71	2.39	0.82-2.02	1S	0.71-0.90	1.97-2.39	NG	NP
Total protein	g/L	131	67.4	8.4	68.0	46.0	97.0	48.3-83.4	1S	46.0-52.0	79.0-97.0	NG	NP
Urea	mmol/L	131	2.91	0.75	2.83	1.50	5.00	1.67-4.45	0	1.50-1.83	4.16-5.00	NG	NP
Glucose	mmol/L	131	3.81	0.53	3.85	1.90	5.74	2.85-4.81	0	1.90-2.97	4.68-5.74	G	NP
Cholesterol	mmol/L	130	2.16	0.51	2.14	1.25	3.95	1.35-3.34	6S, 1R	1.25-1.38	3.22-3.95	NG	NP
Zinc	µmol/L	131	10.60	3.64	10.86	1.07	19.28	2.79-17.30	0	1.07-5.20	16.52-19.28	G	NP
Total bilirubin	mmol/L	130	6.57	4.43	5.48	0.29	21.99	1.03-19.45	6S, 1R	0.29-1.86	15.57-21.99	NG	NP
NEFA	mmol/L	131	0.66	0.38	0.55	0.11	1.96	0.16-1.63	0	0.11-0.20	1.43-1.96	NG	NP
BOHB	mmol/L	129	0.75	0.31	0.71	0.31	1.76	0.38-1.71	7S, 2R	0.31-0.40	1.44-1.76	NG	NP
Fructosamine	µmol/L	131	182	22	179	119	244	135-228	3S	119-144	217-244	G	NP

S: suspected outliers; R: removed outliers; G: Gaussian distribution of data; NG: non-gaussian distribution of data; NP: nonparametric method.

Table 5.7: Descriptive statistics of reference intervals for some markers of inflammation/oxidation for cows at 3±1 DIM. Number of reference values included, mean, standard deviation, median, minimum, maximum, RI, number of outliers, confidence intervals of the lower and upper reference limits, distribution of data and method employed to generate the reference interval.

Analyte	SI Units	N	Mean	SD	Median	Min	Max	RI	Outliers	Lower Ref Lim 90% CI	Upper Ref Lim 90% CI	Dist.	Method
Ceruloplasmin	µmol/L	131	3.65	0.67	3.69	2.24	5.04	2.35-4.98	0	2.24-2.53	4.88-5.04	G	NP
Haptoglobin	g/L	131	0.85	0.54	0.78	0.01	2.36	0.08-2.18	2S	0.01-0.11	1.99-2.36	NG	NP
Paraoxonase	U/L	131	84.1	21.4	84.0	39.4	142.5	46.3-135.0	1S	39.4-53.6	128.4-142.5	G	NP
dROMs(H_2O_2)	mg/100 mL	131	15.3	3.9	14.5	7.7	25.9	9.4-24.2	1S	7.7-10.0	22.5-25.9	NG	NP
Thiol groups	µmol/L	131	354	68	352	190	563	226-522	4S	190-244	474-563	G	NP
Myeloperoxidase	U/L	131	476	99	469	237	808	262-742	3S	239-339	637-808	G	NP
Vitamin A	µmol/L	130	36.7	13.6	34.8	10.1	87.9	16.6-68.0	2S, 1R	10.1-16.9	62.3-87.9	NG	NP
Vitamin E	µg/mL	130	1.51	0.81	1.37	0.20	4.38	0.36-3.92	5S, 1R	0.20-0.46	3.09-4.38	NG	NP
Beta-carotene	mg/100 ml	130	0.10	0.06	0.09	0.02	0.27	0.03-0.24	1R	0.02-0.04	0.22-0.27	NG	NP

S: suspected outliers; R: removed outliers; G: Gaussian distribution of data; NG: non-gaussian distribution of data; NP: nonparametric method.

Table 5.8: Descriptive statistics of reference intervals for electrophoretical parameters for cows at 3±1 DIM. Number of reference values included, mean, standard deviation, median, minimum, maximum, RI, number of outliers, confidence intervals of the lower and upper reference limits, distribution of data and method employed to generate the reference interval.

Analyte	SI Units	N	Mean	SD	Median	Min	Max	RI	Outliers	Lower Ref Lim 90% CI	Upper Ref Lim 90% CI	Dist.	Method
Albumin	g/L	44	37.2	8.3	37.2	14.7	52.5	15.3-52.3	1S	14.7-25.1	48.7-52.5	G	NP
Globulin	g/L	44	33.3	10.0	32.3	19.3	58.5	19.3-57.6	0	19.3-20.8	49.6-58.5	G	NP
A/G ratio	ratio	44	1.26	0.55	1.17	0.29	2.31	0.31-2.29	0	0.29-0.49	2.14-2.31	G	NP
α1-globulin	g/L	44	3.2	0.9	3.1	1.8	5.5	1.8-5.4	1S	1.8-2.0	5.1-5.5	NG	NP
α2-globulin	g/L	44	6.7	1.7	6.7	3.6	11.5	3.6-11.3	1S	3.6-4.6	9.2-11.5	NG	NP
β1-globulin	g/L	44	5.8	1.9	5.6	2.2	11.2	2.2-11.2	2S	2.2-2.9	8.9-11.2	NG	NP
β2-globulin	g/L	44	6.7	2.2	6.3	3.4	11.6	3.4-11.5	0	3.4-3.8	10.4-11.6	NG	NP
γ-globulin	g/L	44	10.9	5.1	10.0	3.9	23.9	4.0-23.5	0	3.9-4.6	19.1-23.9	NG	NP

S: suspected outliers; R: removed outliers; G: Gaussian distribution of data; NG: non-gaussian distribution of data; NP: nonparametric method.

5.4. Discussion and conclusions

In routine practice, hematological and biochemical RIs for cows are established using the transference method, based on RIs obtained from literature (Friedrichs et al., 2012), or generated within the lab from the patient's database, which includes a wide population of animals irrespective on possible biological peculiarities associated with pregnancy or lactation. However, the ASVCP guidelines recommend that in some circumstances (e.g. differences in pre-analytical or analytical methods) transference may not be appropriate (Friedrichs et al., 2012). Due to the unavailability of information concerning their study population, the most common used biochemical RIs cited in textbooks (for example Kaneko, 2008) were not considered acceptable for the transference procedures. A recently published study on hematological RIs of Holstein cows (George et al., 2010) included only cows with at least 18 weeks in lactation. Other studies on biochemical analytes classified as "early lactation" the period ranging from 30 to 90 DIM (Cozzi et al., 2011; Bertoni and Trevisi, 2013). Studies on acute phase proteins and other analytes related with inflammation were not focused on generation of RIs (Bionaz et al., 2007, Bertoni et al., 2008, Trevisi et al., 2012). Therefore, there are no specific studies on the RIs regarding hematology, clinical chemistry and acute phase proteins/inflammatory markers in the first days post-partum compared with one month later, when the metabolic status of lactating cows is deeply different. Hence, we focused our study on these two periods. To this aim we applied recently published guidelines for veterinary species (Friedrichs et al., 2012). According to these guidelines, strict inclusion criteria were applied, in order to exclude from the study not only sick animals but also any animal potentially affected by subclinical diseases. To achieve this objective, we reviewed the health status of cows included in a larger study focused on genetic bases of bovine disease susceptibility (ProZoo Project) and we excluded from the study either the cows that had health problems during the whole lactation period subsequent to the samplings included in this study, or the animals that had problems of fertility or low production, potentially consistent with subclinical abnormalities. This allowed us to include in

the study only samples from animals that were clinically healthy for the whole lactation period and had production and fertility consistent with the normal standard of dairy herds of our region. The application of these strict inclusion criteria induced us to exclude from the study a large number of animals (the original database with the whole set of analyses, included more than 1,000 samples per each lactation period) but despite this, the final dataset remained higher than the minimum caseload recommended for establishing RIs (n= 120, Geffrè et al., 2009). To our knowledge, other studies on bovine RIs were generally based on numbers of samples largely lower than those included in this study and this further reinforce the statistical power of our results.

Our results confirm the importance to establish different RIs, appropriate for the lactation stage, since results recorded at 3±1 DIM and at 30±3 DIM were often significantly different either in terms of mean and median values or in terms of RIs. In most of the cases, the differences were justifiable with the biological events that characterize early post-partum or early lactation. In these cases, the adoption of separated RIs for these parameters was thus supported both from the statistical analysis and from a biological point of view.

In particular, the biological events occurring soon after parturition may justify some of the differences recorded between day 3±1 and 30±3: for example, the reference limits for RBC, Hb and Ht are higher at 3±1 DIM maybe due to hemoconcentration associated with parturition (Enemark et al., 2009). Moreover, at this sampling time, reference limits of MCV and MCHC were slightly higher and lower, respectively, likely due to the moderate blood loss that may occur after parturition, suggesting the release into the circulation of younger RBCs (that show higher mean cell volume). Mild blood losses may also justify the lower reference limits for PLT count (Stockham and Scott, 2008f). Hemoconcentration, stress or inflammatory stimuli associated with parturition, may also justify the higher counts of all the leukocyte populations (Tornquist and Rigas, 2010), except eosinophils, recorded at 3±1 DIM compared with 30±3 DIM. The production of colostrum and milk and the low food intake in the days around calving may explain the lower calcium,

phosphorous, magnesium, glucose, total proteins and GGT as many of these metabolites cross the hemato-mammary barrier or are used as source for milk production (Ramberg et al., 1970; Goff and Horst, 1997; Butler, 2005, Hoffman and Solter, 2008). The high creatinine concentration at 3±1 DIM may be justified by several factors such as dehydration, increased muscle catabolism associated with the decrease food intake in the peri-partum, or an increased catabolism of proteins from placental tissues or allantoic fluids (Braun and Lefabvre, 2008). The negative energy balance following the start of the lactation justify the high NEFA and BOHB level (Drackley et al., 2005; Martinez et al., 2012). The higher AST and total bilirubin may be explained by a higher degree of hepatic exploitation also due to the increased inflammatory stimuli or by an increased degradation of fetal hemoglobin and increased muscle damage around parturition (Hoffmann and Solter, 2008; Bertoni and Trevisi, 2013). Finally, dehydration may explain the higher chloride and sodium concentration found in early post-partum (Carlson and Bruss, 2008).

Parturition represents a sort of inflammatory stimulus. Therefore, is not surprising that RIs for Cp and Hp, that are positive APPs in cattle (Arthington et al., 2008; Trevisi et al., 2009; Eckersall and Bell, 2010) are higher and the RI for PON-1, that is a negative APP in cattle (Bionaz et al., 2007; Trevisi et al., 2009; Giordano et al., 2013) is lower at parturition. Moreover, oxidative stress associated with inflammation may justify the higher dROMs (Trevisi et al., 2009) and the lower zinc, vitamin A, vitamin E and β-carotene (Goff and Stabel, 1990; Wilde, 2006; Bionaz et al., 2007) recorded at parturition..

Conversely, the approaching peak of lactation, starting approximately six weeks after parturition may justify other findings. The β2-globulin fraction, is higher at 30±3 DIM than in early post-partum likely due to an increased antibody production (Stockham and Scott, 2008g), or to a smaller loss of immunoglobulins in milk than in colostrum. These changes may reflect also in the higher total globulin encountered at the same time. The more balanced energy state that occurs at 30±3 DIM may lead to a reduction in calcium resorption from bones, thus explaining the lower ALP compared with early post-partum (Rosol and Capen, 1997). The higher cholesterol is consistent

with other studies, where this analyte was found to increase with the progress of lactation (Herdt and Smith, 1996; Bionaz et al., 2007; Bertoni et al., 2008; Cozzi et al., 2011) maybe due to changes in serum lipoprotein concentration (Raphael et al., 1973).

Finally, it is more difficult to explain the lower concentration of urea that reflects an increased protein catabolism, at 3±1 DIM, when fetal and placental catabolism is active. In another study, lower serum urea concentration was found in cows in the first week after parturition (RIs are very similar to that obtained in the present study for cow at 3±1 DIM) compared to the pre-calving period (Quiroz-Rocha et al., 2009). The authors attributed the finding to the increased recycling of ruminal urea as source of proteins synthesis at the starting of lactation, when a decreased dry matter intake may reduce the endogenous availability of ammonia for urea production. In our case, data regarding urea in the two periods minimally overlap and therefore the use of different RIs is highly justified, independently on its possible pathogenic mechanism.

Hence, in all the cases above, the adoption of RIs specific for each period is highly recommendable in order to avoid that results actually depending solely on the lactation stage are erroneously interpreted as associated to pathophysiological or pathological conditions.

The guidelines for establishing reference intervals, recommend also investigating any possible pre-analytical source of variation that may justify the adoption of additional RIs. From this perspective, the herd of origin, the precise day of sampling and the parity group may represent additional variables that may induce laboratory changes so severe to require additional day-, herd-or parity-specific RIs. Regression analysis based on the day of sampling revealed only few differences according to time that however were not supported by the statistical paired test. In this case, the utility of separated RIs according to days is questionable and, moreover, in routine practice it may be difficult to use a different RI for each specific day of lactation.

Similarly, despite partitioning based on the herd of origin revealed several significant differences and the possible need of herd-specific RIs, the data distribution evidenced only in a few cases an univocal trend (i.e. a given change or RI found in a single herd and not in the other ones).

Conversely, in most cases, the significant differences seemed to be a “statistical artifact” without practical or biological significance. As regards the few parameters that were significantly different in a single herd (in our caseload, herd B, C or D, depending on the analyte), the recorded differences may be associated with different dietary or management procedures that, although very similar in the 4 herds, may have been characterized by slight differences (e.g. vitamin supplementation, management of groups, microclimate management). In addition, differences among herds could be also justified by the presence of specific subclinical conditions, which were not identified. Also in this case, herd-specific RIs have not been determined in this study, either because the number of animal per herd was often too low to allow a reliable statistical analysis, or because in routine practice it may not be applicable the use of an herd-specific RI only for some analyte and not for the others.

Finally, many significant differences according to parity were found but only in few cases there were specific differences between the group of cows at first lactation or than that of cows with more than 3 lactations and the others groups. Interestingly all these differences were found at 30±3 DIM. The higher ALP activity and creatinine concentration observed in primiparous cows may be associated with the increased osteoblastic activity that occurs in young growing cattle and with higher muscle activity in young growing cattle as observed also in similar studies at different sampling times (Brscic et al., 2015; Cozzi et al. 2011). The higher calcium concentration in cows at first lactation may be related to an age dependent effect as shown in another study (Quiroz-Rocha et al., 2009). One of the reasons suggested in this latter study is the decreased numbers of receptors for 1,25-dihydroxyvitamin D in the intestine, resulting in decreased absorption of the mineral. Finally, the higher total bilirubin and PON-1 found in older cows (3 or more lactations) may be explained with a slower return to normal condition after a period of liver exploitation and with a less reactive immune system supported also by the higher total leukocytes count found in younger cows.

In conclusion, this study confirmed the results of previous studies that evidenced different metabolic/pro-inflammatory states in different periods in lactating cows. Based on these differences

we were able to establish different RIs to be applied just after parturition (3±1 DIM) or when lactation is approaching the peak (30±3 DIM). The use of different RIs in the different periods is highly justified not only on a statistical basis but also on a biological point of view, since the analytes that were different between the two periods are involved in the metabolic processes that differ in the two lactation phases. Further differences may be found in the different herds included in this study or in each different sampling day. The application of additional day- or herd-specific RIs, however, has several practical limitations and therefore it may be recommendable, in routine practice, to use different RIs at early post-partum and one month later and, within this period, carefully consider results that in a given day or in a given herd seem to move towards one or the other reference limit since this trend may depend on day-, herd- or parity specific pre-analytical factors. These specificities may have determined the definition of wider RIs in the past. However in our opinion the RIs generated in the present study may be a useful tool to interpret hematological, biochemical and inflammatory parameters of dairy cows belonging to commercial herds, in the more critical phases of lactation as the immediate post calving and the peak of lactation.

CHAPTER 6:

Evaluation of peri-partum laboratory changes in dairy cows with and without retained placenta

CHAPTER 6: SECTION 1 - EARLY POST-PARTUM HEMATOLOGICAL CHANGES IN HOLSTEIN DAIRY COWS WITH RETAINED PLACENTA

Moretti, P., Probo, M., Morandi, N., Trevisi, E., Ferrari, A., Minuti, A., Venturini, M., Paltrinieri, S., Giordano, A.: Early post-partum hematological changes in Holstein dairy cows with retained placenta. Animal Reproduction Science. 2015, 152, 17–25

6.1.1 Introduction

Retention of fetal membranes or retained placenta (RP) occurs frequently in high-yielding dairy cows and has been proven to cause negative effects on reproductive performances (Kelton et al., 1998). A worldwide survey by Kelton et al. (1998) estimates an incidence of between 1.3% and 39.2% with a median of 8.6%. These results agree with those obtained by a preliminary study on Italian herds (Bolla and Fantini, 2003).

Retained placenta is defined as fetal membranes not expelled after parturition. The time interval to classify a cow as affected by RP varies with different studies (Fourichon et al., 2000). Membranes can be retained for 7 days or more if a treatment is not administered (Paisley et al., 1986). This condition causes huge economic losses since it predisposes to a variety of reproductive and productive problems (Laven and Peters, 1996; Trevisi et al., 2008; Dubuc et al., 2010).

Although there are many studies concerning RP in cows, its pathogenesis is still not well understood (Schlafer et al., 2000; Boos et al., 2003). Pregnancy maintenance requires suppression of the immune response in order to avoid rejection of the fetal-placental unit and RP might result from a failure in switching off these immune-protective mechanisms.

In the last few years the understanding of the role of the innate immune system in the pathogenesis of reproductive diseases which occur in the transition period has been improved (Cai et al., 1994; Kimura et al., 2002b; Hammon et al., 2006; Bertoni et al., 2008). The increasing number and activity of endometrial leucocytes appears to play an important role in placental detachment and neutrophil dysfunction may be involved in RP. It has been observed that leukocytes from cows with RP show, around the time of parturition, decreased chemotaxis (Gunnink, 1984a; Gunnink, 1984b; Gunnink 1984c; Gunnink 1984d) and decreased phagocytic activity (Kimura et al., 2002b). Moreover, leukocytes of cows with hyperketonemia, a condition frequently associated with RP, have a lower phagocytic activity, decreased cytokine production and chemotactic activity (Scalia et al., 2006).

Changes in clinical biochemistry associated with RP have also been described. Compared to healthy cows, cows with RP have higher serum concentration of non-esterified fatty acids (NEFA) and D-3-hydroxybutyrate (BOHB), lower serum concentration of vitamin E and calcium (Seifi et al., 2007). Inflammatory changes occurring after RP were described by Trevisi et al. (2008); cows with RP having lower concentrations of albumin (a negative acute phase protein) whilst the serum concentration of typical inflammatory markers (haptoglobin and ceruloplasmin) were similar to those of cows that normally expelled fetal membranes. Conversely, little is known about hematological changes occurring soon after parturition in affected cows. Therefore this retrospective study was designed to investigate the hematological changes associated with RP in the immediate post-partum period and to assess whether hematological changes are associated with an acute phase reaction, in order to provide additional insights on the pathogenesis of this condition.

6.1.2 Material and methods

6.1.2.1 Retrospective analysis of the database

This study started with a retrospective search of data recorded in the database of the ProZoo project, a research project aimed to investigate the relationship between genomic traits and bovine health and production. The ProZoo database includes information about production, reproduction, and health status, including results of blood samplings recorded over a 3 year period (2010-2013) from 5 intensive farms in the area of Lodi (Lombardy region, Italy), two of which (herds A and B) had a high prevalence of RP (20% and 29%, respectively). These two herds were composed of 187 and 360 milking cows, respectively, with a mean days in milk (DIM) of 199 for the farm A and 188 for the farm B. All the cows were fed with a TMR (total mixed ration). Milking was performed twice a day, at 12 h intervals. A thorough gynecological investigation (transrectal uterine palpation and ultrasonography) was conducted at 30 DIM on all the cows.

The database was examined in order to select data corresponding to cows from herds A and B that fulfilled the following inclusion criteria.

- availability of data from the complete blood count (CBC) and biochemical profile performed at 3±1 DIM;
- negative history for any clinical disease or laboratory abnormality during the gestation period and normal parturition course;
- average production adjusted for 305 days during the lactation period included in this study: ≥7.000 kg;
- lactation period of at least 200 days;
- no clinical events or abnormal laboratory results during the first month of lactation, except for RP and associated laboratory changes for animals included in the RP group;

- no anti-inflammatory or antibiotic treatments administered in the days before collection of blood samples.

In order to exclude from the study all the animals that did not fulfill the criteria above, data regarding clinical visits recorded in the first days after calving were examined. At each visit, the evaluation for RP was conducted visually and vaginally by the veterinarian. A cow was judged to have an RP when the placental membranes had been retained for at least 24 h. All cows from the two farms were monitored for the occurrence of vaginal discharge in the following 30 days.

6.1.2.2 Blood sampling

In all the animals included in this study, peripheral blood samples were collected at 3±1 DIM. Specifically, 30 mLs of venous blood were collected: 10 mLs of blood were placed in a tube without anticoagulant (Venosafe plastic tubes for serum, Terumo, Europe) to perform routine biochemical analyses, 10 mLs were placed in tubes with EDTA (Venosafe plastic tubes for hematology, Terumo, Europe) to perform routine hematology, and 10 mLs were placed in tubes with lithium heparin (Venosafe plastic tubes for plasma, Terumo, Europe) for the measurement of acute phase proteins (APPs).

All the samples were immediately placed at 4 °C and submitted to the Central Laboratory of the Veterinary Teaching Hospital of the University of Milan where routine hematology was performed as described below. Tubes with lithium heparin were immediately centrifuged at 2,200 x g for 10 minutes upon arrival at the Central Laboratory. Samples in tubes without anticoagulant were allowed to clot at room temperature for 30 minutes and then centrifuged at 2,200 g for 10 minutes. Harvested heparinized plasma and sera were then frozen at -80°C for a maximum of 3 months before biochemical tests were performed.

6.1.2.3 Routine hematology

Routine hematology was performed at the Central Laboratory of the Veterinary Teaching Hospital of the University of Milan using an automated laser hematology analyzer (ADVIA 120 with multispecies software for veterinary use, Siemens Healthcare Diagnostics, Milan, Italy). The following variables generated by the instrument were recorded: hemoglobin (Hb) concentration, hematocrit (HCT), erythrocyte (RBC) counts, white blood cells (WBC) counts.

The leukocyte differential provided by the instrument was checked microscopically on blood smears prepared upon arrival of the sample at the laboratory and stained with a modified Romanowsky stain (Dif-stain kit, Titolchimica S.P.A., Rovigo, Italy). The total number of the leukocyte populations was then calculated based on the total number of WBC and on the percentage of each cell population.

6.1.2.4 Clinical chemistry

Routine biochemical analyses were run on serum or plasma with automated spectrophotometers (ILAB300 plus and ILAB600, Instrumentation Laboratory S.p.a., Milan, Italy) using reagents provided by the manufacturer of the instruments, except when otherwise specified. The following analytes were measured: alkaline phosphatase (ALP, kinetic IFCC method), aspartate aminotransferase (AST, kinetic IFCC method), calcium (orthocresoftaleine method), creatinine (Jaffè method), total proteins (biuret method), albumin (bromochresol green method), total bilirubin (diazo reactive with sulphanilic acid), glucose (GOD-POD method), total cholesterol (cholesterol oxidase method), urea (urease method), phosphate (phosphomolibdate method), □-glutamyl transferase (GGT, kinetic IFCC method), zinc (colorimetric with Nitro-PAPS), sodium, potassium and chloride (ion selective electrodes method), non-esterified fatty acid (NEFA, ACS-ACOD method, Wako Chemicals GmbH, Neuss, Germany) and β-hydroxybutyrate (BOHB, D-3-Hydroxybutyrate dehydrogenase method, Randox Laboratories Ltd., Crumlin, Co. Antrium, UK).

6.1.2.5 Acute phase proteins (APPs) and other markers of inflammation

Heparinized plasma was periodically sent to the Institute of Zootechnics, Faculty of Agriculture, Università Cattolica del Sacro Cuore, Piacenza, to measure the following APPs and other markers of inflammation: ceruloplasmin (Cp) with the method described by Sunderman and Nomoto (1970); haptoglobin (Hp), using the method described by Skinner et al. (1991); paraoxonase (PON1) with the method described by Ferré et al. (2002); reactive oxygen metabolites (d-ROMs) using the Kit "d-ROMs Test" from Diacron International S.r.l. (Grosseto, Italy); thiol groups (SHp) measured using a specific colorimetric kit (Diacron International S.r.l.); myeloperoxidase (MPO) determined through a colorimetric method described by Bradley et al. (1982). All the methods were run on plasma with an automated spectrophotometer (ILAB600, Instrumentation Laboratory S.p.a., Milan, Italy).

6.1.2.6 Statistical analysis

Statistical analyses were done on an Excel (Microsoft Corp, Redmond, WA, USA) spreadsheet using the Analyse-it software (Analyse-it Software Ltd, Leeds, UK). Results recorded at day 3±1 after calving from both groups were compared with the reference intervals established in our laboratory from clinically healthy animals sampled in the same post-partum period. Results from cows affected by RP and from CTRL cows were compared to each other using a Wilcoxon signed rank test. Comparison of neutrophils was done either for the whole group of animals or for animals subgrouped according to the herd of origin. The Wilcoxon signed rank test was also used to compare the neutrophil counts obtained in the two herds either in cows affected by RP or in CTRL cows.

6.1.3. Results

6.1.3.1 Case selection and characteristics of the study population

As shown in figure 6.1.1, most of the animals sampled in the study period were finally excluded because they did not fulfill the inclusion criteria. Therefore, the study population was finally composed of 22 cows with RP (14 from herd A and 8 from herd B), sampled at 2 (n=12), 3 (n=6) and 4 (n=4) days post-partum, and of 23 cows without RP and without any other clinical symptoms in the 1st month after calving (CTRL group, 9 from herd A and 14 form herd B), sampled at 2 (n=5), 3 (n=14) and 4 (n=4) days post-partum.

The median (min-max) BCS at 3±1 DIM were 3.25 (3.00-4.00) points in the RP group and 3.50 (3.00-3.75) in the CTRL group. The median (min-max) 305-days milk production were 9647 (7099-11990) and 9954 (7268-11950) Kg for the RP and the CTRL group respectively. The median (min-max) number of lactations were 3 (1-6) in the RP group and 3 (1-5) in the CTRL group. Regarding these parameters, no statistically significant differences between the two farms were observed.

A combination of manual removal of the fetal membranes and intramuscular administration of 30,000 UI/kg of benzylpenicillin (Depomicina, Intervet Italia, Peschiera Borromeo, Milan, Italy) was performed in 15 cows with RP whereas a combination of manual removal of the fetal membranes and intrauterine administration of 1.2 g of rifaximin (Fatroximin Pessari, Fatro s.p.a., Ozzano dell'Emilia, Bologna, Italy) was performed in 5 cows with RP. All the treatments were administered on the day of blood collection.

On the basis of the 30 DIM gynecological visits all animals with RP recovered completely.

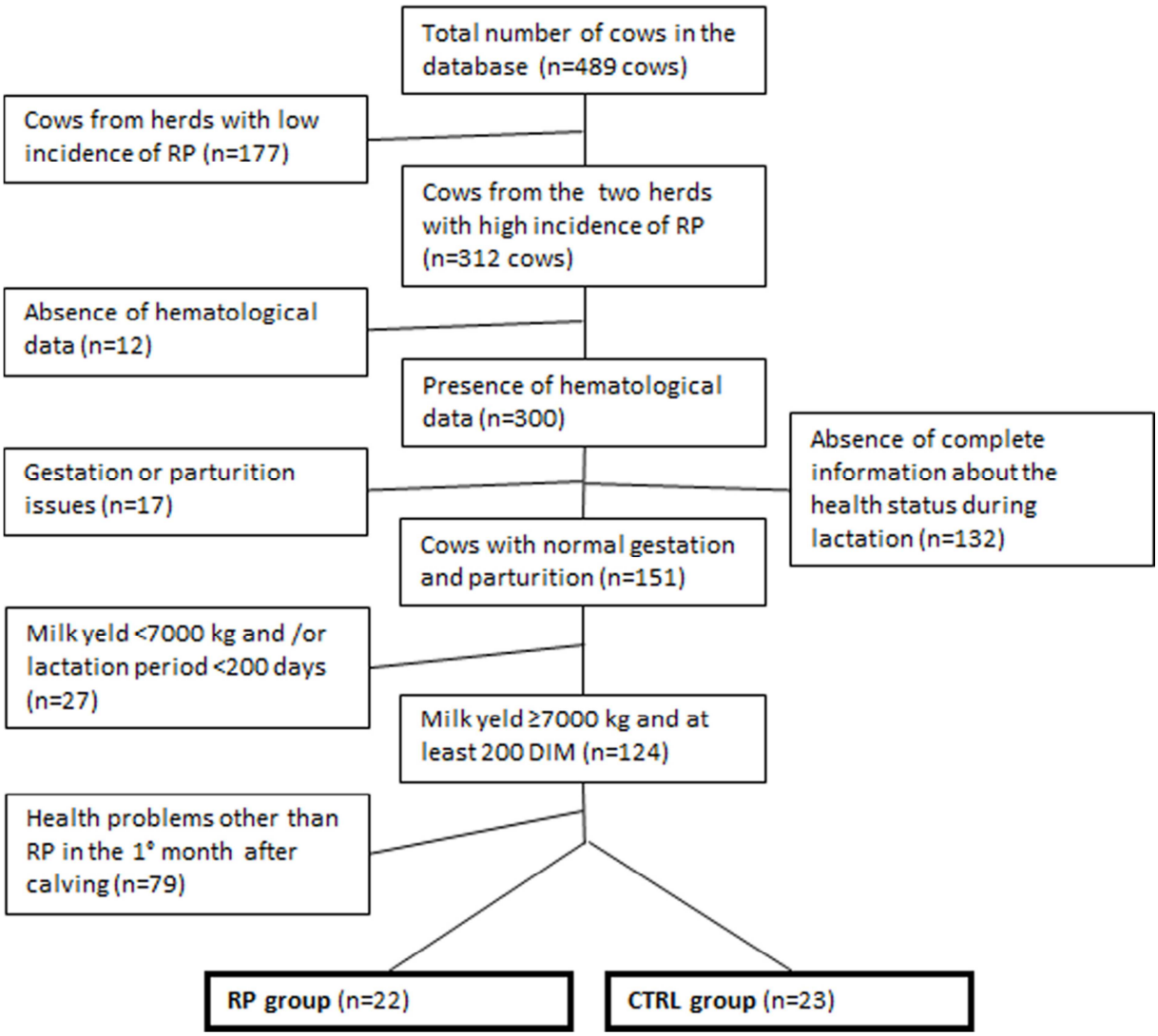

Figure 6.1.1: flowchart summarizing the results of the retrospective search in the database and the final composition of the study group.

6.1.3.2 Hematology and clinical chemistry

Results recorded at 3±1 DIM are reported in Figure 6.1.2 and 6.1.3 and shows that RBC counts were significantly higher in cows affected by RP compared with CTRL cows (P=0.034) but values were within the reference intervals in both groups. Conversely, neutrophils counts were significantly lower (P=0.015) in the RP affected group, in which about one third of the cows had values lower than the lower limit of the reference interval, compared with controls which, conversely, had values included in the reference interval, with rare exceptions. The differences between neutrophil counts of CTRL and RP groups of herd A (CTRL = mean ± S.D: 2.93 ± 1.51 x 10^3/µL; median: 2.59 x 10^3/µL; RP = 1.69 ± 1.11 x 10^3/µL; 1.35 x 10^3/µL) and B (CTRL = 3.76 ± 1.90 x 10^3/µL; 3.57 x 10^3/µL; RP = 2.26 ± 2.35 x 10^3/µL; 1.45 x 10^3/µL) were statistically significant (P=0.038 and P=0.042, respectively). Conversely, no significant differences were found when neutrophil counts recorded in CTRL cows of herd A and B were compared to each other or when neutrophil counts recorded in RP cows of herd A and B were compared to each other.

With regard to the whole set of data (i.e. herds A and B), no significant differences were found for total WBC, lymphocytes, and monocytes. Band neutrophils, basophils and eosinophils were only occasionally seen in both groups but the total number of these cells was always within the reference interval, without differences between the two groups (data not shown). Similarly, no significant differences for the markers of inflammation and oxidation were recorded between CTRL and RP cows, except for a significant increase of d-ROMs and for a significant decrease of thiol groups although these largely remained within the reference intervals in both groups. Biochemistry was also unremarkable, with no significant differences between groups except for AST activity, which was significantly higher in the RP group, and serum zinc concentration, that was significantly lower

in the RP group. However, for both these analytes values were largely within the reference intervals in both groups. Interestingly, no significant differences between the groups were found regarding the analytes that are considered as risk factors for RP such as Calcium, NEFA and BOHB.

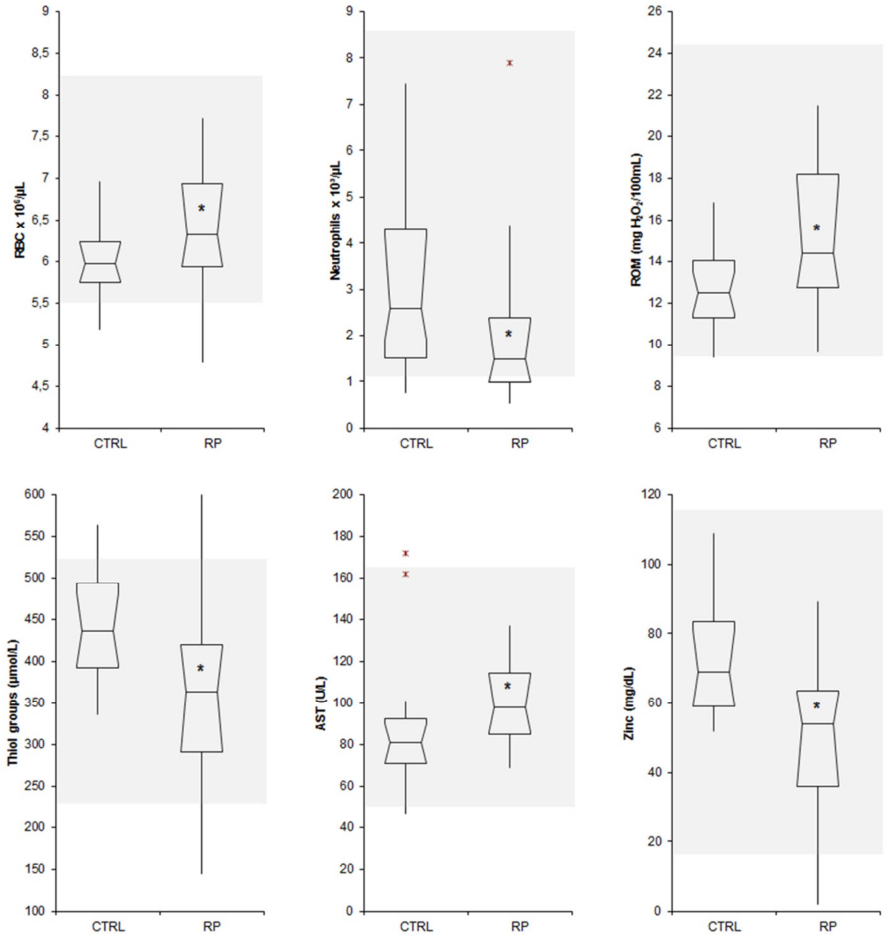

Figure 6.1.2: Results regarding hematological parameters, inflammatory markers and biochemical parameters showing significant differences between groups. The figure displays the comparison between animals with retained placenta (RP) and controls (CTRL). Boxe indicate the I-III interquartile interval, the horizontal line corresponds to the median, vertical lines are the limits of outlier distribution according to the Tukey rule. Near outliers are indicated by the symbols "x" and far outliers with asterisks outside the boxes. The black bolded asterisks within the boxes indicate significant differences between groups (* = P<0.05). When no asterisks are reported within boxes, the difference between groups are not significant. The gray areas display the reference interval of our lab referred to cows at 3±DIM

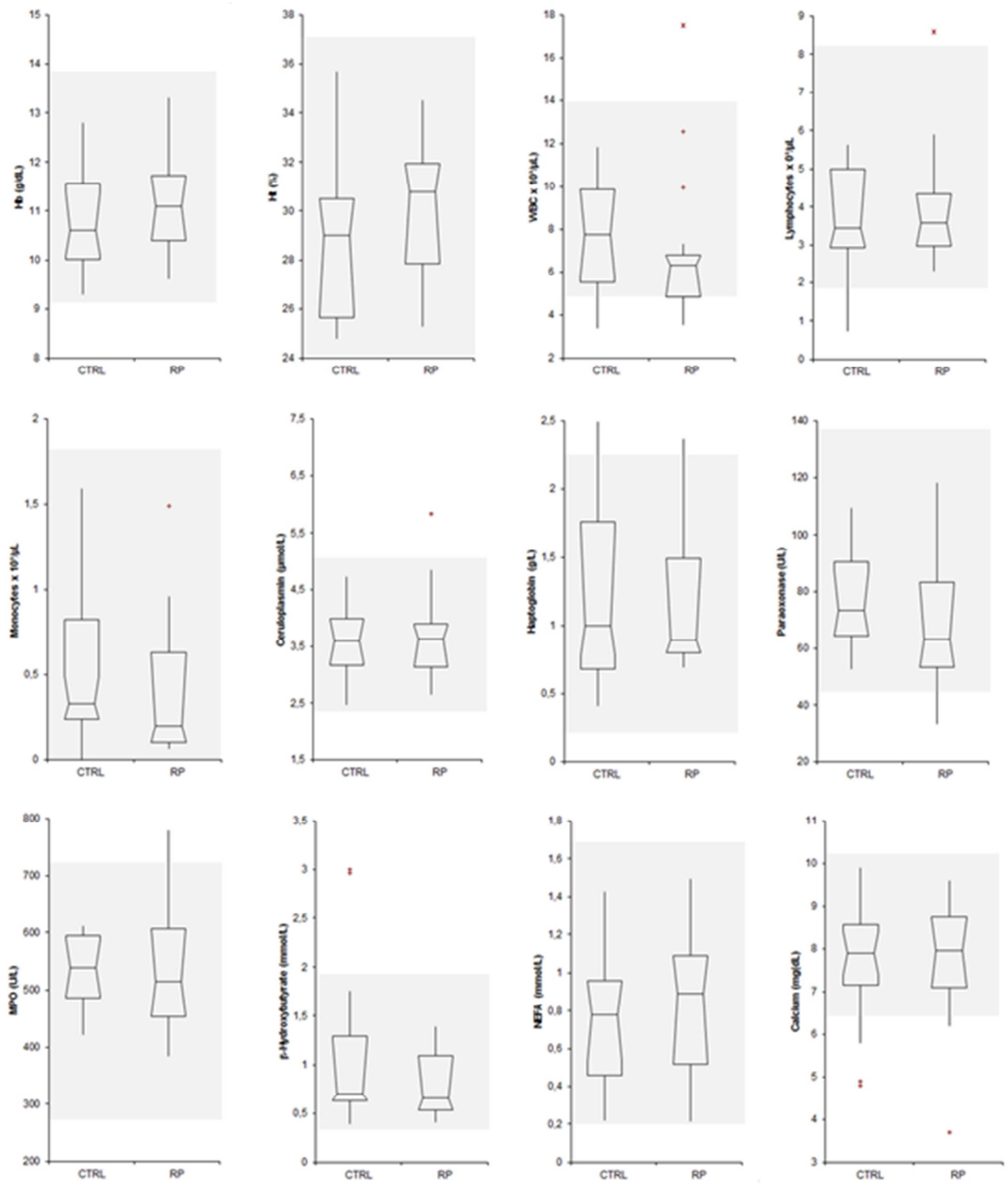

Figure 6.1.3: Results regarding hematological parameters, inflammatory markers and biochemical parameters of potential interest for placental retention that did not were significantly different between groups. The figure displays the comparison between anima with retained placenta (RP) and controls (CTRL). See figure 6.1.1 for interpretation of box and whiskers.

6.1.4. Discussion

This study is focused on the hematological profile analysis of cows affected by retained placenta, since most of the previous reports were focused on biochemical alterations (Peter and Bosu, 1987; Melendez et al., 2004; Seifi et al., 2007; Ospina et al., 2010; Huzzey et al., 2011) or in vitro leukocyte functions (Gunnink, 1984a; Gunnink, 1984b; Gunnink, 1984c; Gunnink, 1984d; Hammon et al., 2006) associated with RP while alterations of erythrogram and leukogram were not considered. These may be particularly important in the management of animals that are at risk for or are affected by RP, since the depression of neutrophil functions and the inflammatory state, and the association with RP (Gunnink, 1984b; Gunnink, 1984d; Gilbert et al., 1993; Hammon et al., 2006; Trevisi et al., 2008), may change the amount of circulating neutrophils. The most relevant findings recorded in RP affected cows compared to controls in the present study are represented by a higher RBC count and a lower neutrophil count. The high RBC count probably reflects a moderate dehydration. This condition is usually associated with increased concentration of albumin and total proteins. However, they were not increased in this study, probably because albumin usually decreases immediately post-partum (Seifi et al., 2007; Trevisi et al., 2009; Trevisi et al., 2012) especially in RP affected cows (Trevisi et al. 2008). In addition, the increase of RBCs, although statistically significant, is probably not relevant on a biological or pathological point of view, since all the values (including those of the RP group) were within the reference intervals.

The lower number of circulating neutrophils recorded in RP affected cows is potentially associated with a variety of metabolic and pathological conditions, we thus investigated the possible presence of inflammation, evaluating results concerning serum inflammatory markers and biochemical profiles in order to exclude that hematological changes were related to any generic alteration of the health status or to conditions such as hyperketonemia or hypocalcemia, that have been reported to play a role in the pathogenesis of RP in cows (LeBlanc et al., 2004; Seifi et al., 2007; Ospina et al., 2010).

The analysis of the obtained biochemical data did not keep identity with peculiar abnormalities at 3±1 DIM in animals with RP, as previously reported by Trevisi et al. (2008). In the present study the concentration of the analytes recorded in most of the animals from both RP and CTRL groups was within reference intervals although slightly higher than in previous studies (Trevisi et al., 2009; Trevisi et al., 2012). The lack of significant changes in NEFA and BOHB suggests that any possible difference in leukocyte and neutrophil counts between the two groups are probably unrelated to metabolic abnormalities, since severe changes in neutrophil number and functions may be found only when severe increases of NEFA and BOHB or severe hypocalcemia are present (Sartorelli et al., 1999; Sartorelli et al., 2000; Zerbe et al., 2000; Scalia et al., 2006; Martinez et al., 2012).

The decrease of neutrophils at 3±1 DIM is particularly severe, with values in many cases below the lower limit of the reference intervals in the RP group, suggesting that neutropenia is consistent with an actual pathological condition. Moreover, this seems to be a constant finding since it occurred in both herds. Therefore, the association between RP and neutropenia is the most important finding detected in this study. Severe neutropenia may be primary (e.g. associated to a decreased myelopoiesis) or secondary to severe inflammation (consumption due to an increased peripheral demand) (Tornquist and Rigas, 2010; Harvey, 2012). This latter condition may depend on a severe systemic inflammation, mostly of bacterial origin (e.g. septicemia), that is usually associated with severe clinical signs (fever and/or hypothermia, depression, etc.), and to the recruitment of neutrophils in organs with focal inflammatory changes, that leads to a macroscopic evidence of the neutrophils gathering in inflamed sites (e.g. purulent inflammation, abscesses, etc.). However, none of the animals with RP included in this study had evident vaginal purulent discharges or clinical signs potentially associated with a severe systemic inflammatory response. On the contrary, the persistency of fetal membranes from calving to the time of sampling may have induced a moderate inflammatory response (e.g. inflammation not associated with severe migration of neutrophils in inflamed sites). This type of inflammation, however, is usually associated with neutrophilia rather

than neutropenia (Tornquist and Rigas, 2010). Therefore, an excessive peripheral consumption of neutrophils is unlikely in our study. Results regarding inflammatory markers support once more this hypothesis since in this study the serum concentration of positive APPs (i.e. proteins whose concentration increases in blood during inflammation), such as haptoglobin or ceruloplasmin, was normal. The increases of the APPs concentration in serum is considered the most rapid event occurring in blood after inflammation (Petersen et al., 2004). In the current study, the serum concentration of APPs did not significantly differ in cattle with RP compared with CTRL cows suggesting that an acute phase reaction had not yet been mounted in cattle with RP. Reference intervals for Hp are wider in lactating than in non-lactating cows, since the metabolic events of the transition period induce changes in the serum concentration of hormones and cytokines that mimic an inflammatory reaction (Bionaz et al., 2007; Trevisi et al., 2012). Theoretically, both groups may have a reduced rate of hepatic synthesis of negative APPs, as it may occur in the peri-partum period (Bertoni et al., 2008; Trevisi et al., 2012). However, this is very unlikely since the possible indicators of liver failure (bilirubin, albumin, urea, APPs) were in normal concentration in both groups, and only AST, that ultimately is not an indicator of liver function (Stockham and Scott, 2008e), significantly increased in cattle with RP compared with CTRL cows; this increase was of small proportions and not exceeding, in most cows, the upper reference limit. As an additional support to this hypothesis, paraoxonase activity, that has been shown to decrease during inflammation due to both a decreased production and to an increased consumption associated with oxidative phenomena typical of inflammatory states (Turk et al., 2004; Bionaz et al., 2007; Giordano et al., 2013), was not significantly different between RP and CTRL cows. However, oxidation was likely present in cows with RP since reactive oxygen species and thiol groups were respectively increased and decreased in the RP group compared with CTRL. Additionally, cows with RP had a low serum concentration of zinc, that is considered to have antioxidant properties, contributes to the efficiency of immune responsiveness and has been thought to be a co-factor in the pathogenesis of RP (Laven and Peters, 1996; Wilde, 2006). However, all the changes in the serum

concentration of these molecules were moderate and did not exceed the reference intervals. More importantly, they were not associated with an increase of myeloperoxidase, whose serum concentration may increase in association with increased d-ROMs when oxidants are released from inflammatory cells (Bochsler and Slauson, 2002; Sordillo and Aitken, 2009; van der Veen et al., 2009; Mittal et al., 2014).

Neutropenia may also depend on antibiotic treatments. However, this is unlikely since the antibiotics used are not reported to induce alterations in leukocyte counts at conventional therapeutic doses (Papich and Riviere, 2009) and, additionally, the period of time between administration of antibiotics and samplings was too short (both were done in the same day) to induce changes in the leukograms.

Therefore, all these findings support the hypothesis that neutropenia in cattle affected by RP may exist independently on the presence of inflammation. If this interpretation is correct, neutropenia may be considered as an additional predisposing factor for placental retention. This may be also consistent with the presence of neutrophil dysfunction demonstrated in previous studies on animals with RP (Gunnink, 1984a; Gunnink, 1984b; Gunnink, 1984c; Gunnink, 1984d; Hammon et al., 2006).

The design of this study does not allow us to formulate any hypothesis about the mechanism responsible for such a severe neutropenia. More specifically, this was an observational study focused on hematological findings recorded at the time of occurrence of RP and the number of cases included in the study was quite low, due to the application of strict inclusion criteria to exclude any sample potentially affected by confounding factors (e.g. other infectious, metabolic, reproductive or productive disorders). Longitudinal studies, possibly on a higher number of animals, based on samples collected before parturition and at the occurrence of RP would provide more information about the temporal relationship between the appearance of neutropenia and RP.

In conclusion, although retained placenta has to be considered as a syndrome with multifactorial causes, many of which associated with parturition or with altered metabolic states leading to hyperketonemia and/or hypocalcemia (Beagley et al., 2010), the obtained results suggest that neutropenia may serve as a co-factor involved in its pathogenesis. Further studies are needed to clarify whether neutropenia acts as a contributor in the pathogenesis of RP or if it is a very early consequence of RP, preceding any other inflammatory changes in blood. Moreover it would be interesting to investigate the mechanism responsible for this hematological change, as well as the possible genetic predisposition leading to this condition.

CHAPTER 6: SECTION 2 - HEMATOLOGICAL CHANGES AROUND PARTURITION IN HOLSTEIN DAIRY COWS WITH RETAINED PLACENTA

6.2.1 Introduction

Retained placenta (RP) occurs frequently in high-yielding dairy cows and it has been proved to cause negative effects on productive and reproductive performances (Laven and Peters, 1996; Kelton et al., 1998; Trevisi et al., 2008; Dubuc et al., 2010). Its pathogenesis is still not well understood. In normal conditions, the breakdown of the collagenous fetal-maternal link is the result of endocrine and immunological pathways (Beagley et al., 2010). At parturition, the activation of a cellular immune response against the placenta mediated by the release of pro-inflammatory cytokines and chemokines, seems necessary for the proper dissolution of this link (Davies et al., 2000; Davies et al., 2004; Beagley et al., 2010). Alteration of leukocyte functions may contribute to a failure of this process. This hypothesis is supported by different studies where cows with RP have shown to have decreased neutrophil function around parturition (Gunnink, 1984a, 1984b, 1984c, 1984d; Cai et al., 1994; Kimura et al., 2002b; Hammon et al., 2006). In a previous work we found that cows with RP and without evidence of metabolic abnormalities and inflammatory conditions have lower circulating neutrophil number soon after parturition compared to normal cows (Moretti et al., 2015). After parturition, the possible mechanisms involved in RP have just occurred, thus it was not possible to deduce if neutropenia was a predisposing factor or a consequence of RP. The objective of the present study is to examine the temporal dynamics of some hematological parameters around parturition in cows with RP in order to better clarify the relationship between neutropenia and RP and to assess the possible role of this finding as a contributing factor for pathogenesis of the disease.

6.2.2 Material and methods

6.2.21 Study design, herds and groups

A prospective study was carried out on 4 Holstein intensive dairy farms located in the Po valley (Italy) from November 2013 to December 2014. The herds were composed of 270, 300, 700, and 300 animals, respectively, with 150, 130, 250 and 150 milking cows each, a mean days in milk (DIM) of 320, 180, 297 and 220 days respectively, and a normalized production of 9500, 10500, 11360 and 8900 kgs respectively. All the cows were fed with a TMR (total mixed ration). Milking was performed twice a day, at 12 h intervals.

A cow was judged to have an RP when the placental membranes had been retained for at least 24 h. All cows were monitored for the occurrence of vaginal discharge in the following 30 days. The electronic database of each farm was searched in order to retrieve information concerning health and management (clinical diseases, treatments, production, and days in milk) covering the study period.

Cows with RP and without other pathological conditions within the following 30 DIM were assigned to the RP group (n=12) whereas, 17 cows, randomly selected within the cows with a normal parturition course and without other pathological conditions in the following 30 DIM, were assigned to the control group (CTRL).

6.2.2.2 Blood sampling

Peripheral blood samples from the coccygeal vein were collected in EDTA tubes (Venosafe plastic tubes for hematology, Terumo, Europe) and in plain tubes (Venosafe plastic tubes for serum, Terumo, Europe) 2 to 7 days before parturition (T0), within 12 h from calving (T1) and between 48 and 72 hours after parturition (T2). All the samples were immediately placed at 4 °C and submitted to the Central Laboratory of the Veterinary Teaching Hospital of the University of Milan where

routine hematology was immediately performed as described below. Samples in tubes without anticoagulant were allowed to clot at room temperature for 30 minutes and then centrifuged at 2,200 g for 10 minutes. Harvested sera were then frozen at -80°C for a maximum of 3 months before biochemical tests were performed.

6.2.2.3 Hematology

Routine hematology was performed using an automated laser hematology analyzer (ADVIA 120 with multispecies software for veterinary use, Siemens Healthcare Diagnostics, Milan, Italy). The following variables generated by the instrument were recorded: hemoglobin (Hb) concentration, hematocrit (HCT), erythrocyte (RBC) counts, white blood cells (WBC) counts, mean corpuscular hemoglobin (MCH), corpuscular hemoglobin concentration mean (CHCM), mean cellular volume (MCV), and platelet (PLT) counts. The leukocyte differential provided by the instrument was checked microscopically on blood smears prepared upon arrival of the sample at the laboratory and stained with a modified Romanowsky stain (Dif-stain kit, Titolchimica S.P.A., Rovigo, Italy). The total number of each leukocyte population was then calculated based on the total number of WBC and on the percentage of each cell population.

6.2.2.4 Clinical chemistry

In order to obtain information on the metabolic status of the cows, routine biochemical analyses were run on serum or plasma with an automated spectrophotometer (ILAB300 plus, Instrumentation Laboratory S.p.a., Milan, Italy) using reagents provided by the manufacturer of the instrument, except when otherwise specified. The following analytes were measured: β-hydroxybutyrate (BOHB, D-3-Hydroxybutyrate dehydrogenase method, Randox Laboratories Ltd., Crumlin, Co. Antrium, UK), calcium (orthocresoftaleine method), creatinine (Jaffè method), glucose (GOD-POD method), non-esterified fatty acid (NEFA, ACS-ACOD method, Randox Laboratories Ltd.,

Crumlin, Co. Antrium, UK), phosphate (phosphomolibdate method), and total proteins (biuret method).

6.2.2.5 Statistical analysis

At each sampling time (T0, T1 and T2) results from cows affected by RP and from CTRL group were compared using a Mann-Whitney U test. Within the two groups results were also compared over time with a Friedman test followed by a Wilcoxon test, when a significant difference was found. Statistical analyses were done on an Excel (Microsoft Corp, Redmond, WA, USA) spreadsheet using the Analyse-it software (Analyse-it Software Ltd, Leeds, UK) with P value set at 0.05 for all calculations.

6.2.3. Results

6.2.3.1 Characteristics of the study population

A total of 111 cows were sampled during the study period (13, 46, 36 and 16 animals from herd A, B, C and D respectively). The prevalence of RP (diseased animals / (diseased animals + not diseased animals) was 0% (0/13), 13% (6/46), 5.5% (2/36), and 25% (4/16) in herd A, B, C, and D respectively. One cow with RP from herd C had a dystocic calving (assisted parturition longer than 15 minutes) and one from herd D was unable to stand after the parturition. No twins occurred.

6.2.3.2 Erythrocytes and platelets

Results (mean values ± SD) of hematological parameters recorded at T0, T1 and T2 from RP and CTRL groups are reported in table 6.2.1. Parameters significantly different between the two groups are shown graphically in figure 6.2.1. Results of the comparison over time are graphically represented in figures 6.2.2 and 6.2.3 respectively for RP and CTRL groups.

Concerning erythroid parameters, the analysis of results revealed that RP cows had significantly lower HCT at T0 and significantly higher CHCM at T0, T1, and T2 compared to CTRL cows. All

the other parameters were not statistically different between the groups at the three time points examined. A trend with increased RBC counts and HCT in CTRL cows was observed at all the time points.

Concerning the comparison over time, both in RP cows and CTRL cows RBC, Hb and HCT, significantly varied over time (respectively P=0.0052, P=0.0006 and P=0.0052 for RP cows and P=0.0004, P=0.0003 and P<0.0001 for CTRL cows). Compared to the day of parturition, all these parameters had significantly lower value in the week before parturition (T0) and 2 days after (T2) in both the groups (figures 6.2.2 and 6.2.3), but at T2, values were significantly lower compared to T0 only in CTRL group. The same trend was observed for MCV in CTRL cows (P=0.0003) but at T2 values were significantly higher compared to T0. Finally, only in CTRL group CHCM appeared significantly affected by time (P=0.0008) with T0 values significantly higher than other time points.

6.2.3.3 Peripheral leukocytes

Concerning leukocytes, WBC and absolute neutrophil counts were significantly lower in RP cows than CTRL cows at T1 whereas significantly higher absolute lymphocyte counts were found at T0 in RP cows. Finally, absolute monocytes counts were significantly lower in RP cows compared to CTRL cows at all the sampling times. All the other parameters were not statistically different between the two groups at the three time points examined. A tendency to lower absolute neutrophil counts was observed in RP cows at all the time points even if was not supported by the statistics maybe due to the low number of animals included in the study.

Concerning the differences over time, only CTRL cows showed a significant increase of WBC counts together with a significant increase of neutrophil counts at T1 followed by a significant decrease of these parameters at T2. Whereas in both RP and CTRL cows, eosinophils counts exhibit significantly higher values at T0 compared to T1 and T2.

6.2.3.4 Clinical chemistry

Results (mean values ± SD) concerning the clinical chemistry profile performed on samples from RP and CTRL cows at T0, T1, and T2 are reported in table 6.2.2. Parameters significantly different between the two groups are shown graphically in figure 6.2.1. Results of the comparison over time are graphically represented in figures 6.2.4 and 6.2.5 for RP and CTRL groups, respectively.

The majority of the investigated parameters (calcium, creatinine, glucose, NEFA, phosphorous and total proteins) were not statistically different in RP and CTRL cows at the three time points. Magnesium was found significantly higher in RP cows than CTRL cows only at T2. The analysis of results revealed also that RP cows had significantly higher BOHB both before (T0) and after (T2) parturition compared to CTRL cows. Concerning variations of biochemical parameters over time, results are represented in figures 6.2.4 and 6.2.5. Calcium, creatinine, NEFA, and BOHB had identical pattern of variation in both RP (P=0.0004, P=0.0273, P=0.0022, and P=0.0450 respectively) and CTRL cows (P=0.0125, P=0.0015, P=0.0092 and P=0.0498 respectively) with calcium significantly higher and NEFA significantly lower at T0 compared to T1 and T2, BOHB value lower at T0 than T1, and creatinine values lower at T2 compared to the other times (figures 6.2.4 and 6.2.5). Glucose concentration also varied significantly in both the groups (P=0.0033 and P=0.0111 respectively in RP and CTRL cows). Glucose concentration increased with parturition but this finding was clearly supported by the statistical analysis only in RP cows whereas in CTRL cows the P value was only slightly above the level of significance (P=0.0547 for T0 versus T1). Finally, only in CTRL cows differences over time were found for phosphorous and magnesium concentrations (P=0.0001 and P=0.0125 respectively). Phosphorous was found significantly higher before parturition compared to T1 and T2 whereas magnesium had the lowest values at T2 compared to the other two time points.

Table 6.2.1: results concerning hematological parameters expressed as means (±SD) in cows with a normal parturition course and without other pathological condition within 30 DIM (CTRL) and in cows with retained placenta (RP). T0=2 to 7 days before parturition; T1=within 12 h from calving; T2=between 48 and 72 hours after parturition. § Reference interval obtained from at least 120 healthy cows at 3±1 DIM. DIM = days in milk. * P value<0.05 versus CTRL

		RBC (10^6/µl)	Hgb (g/dL)	HCT (%)	MCV fL	MCH (pg)	CHCM (g/dL)	PLT (10^3/µl)	WBC (10^3/µl)	Neutrophils (10^3/µl)	Lymphocytes (10^3/µl)	Monocytes (10^3/µl)	Eosinophils (10^3/µl)
Reference intervals §		5.3-8.3	9.3-13.7	25-38	40-53	13.7-19.5	32.8-37.4	189-510	4.7-13.9	2.6-13.9	3.9-13.8	3.3-2.8	0-1.6
CTRL (T0)	Mean (±SD)	6.38 (± 0.62)	11.88 (±0.95)	31.44 (±2.73)	49.41 (±2.97)	18.66 (±1.11)	35.05 (±0.53)	252.7 (±90.2)	10.10 (±1.93)	5.01 (±1.35)	3.41 (±1.09)	1.17 (±0.88)	0.51 (±0.26)
RP (T0)	Mean (±SD)	5.99 (± 0.60)	11.28 (± 0.73)	28.80* (± 2.53)	48.20 (±2.60)	18.88 (±1.27)	37.21* (± 1.80)	235.6 (±105.2)	9.67 (± 2.52)	3.94 (±1.87)	4.64* (±1.67)	0.57* (±0.31)	0.50 (±0.31)
CTRL (T1)	Mean (±SD)	6.83 (±0.69)	12.69 (±1.04)	34.56 (±2.97)	50.72 (±3.15)	18.63 (±1.08)	34.49 (±1.01)	193.2 (±117.7)	13.93 (±4.38)	8.88 (±4.15)	3.65 (±1.47)	1.14 (±0.76)	0.26 (±0.33)
RP (T1)	Mean (±SD)	6.62 (±0.57)	12.74 (±0.76)	32.41 (±3.01)	49.04 (±3.04)	19.33 (±1.51)	36.28* (±1.79)	251.0 (±84.9)	9.10* (±4.60)	3.34* (±2.47)	5.06 (±3.19)	0.54* (±0.52)	0.15 (±0.20)
CTRL (T2)	Mean (±SD)	6.07 (± 0.51)	11.26 (± 0.95)	30.29 (±2.57)	50.0 (± 3.2)	18.55 (± 1.07)	34.71 (± 0.47)	203.2 (± 94.92)	9.01 (± 2.20)	4.08 (± 2.49)	3.51 (±1.60)	1.13 (± 0.47)	0.21 (± 0.18)
RP (T2)	Mean (±SD)	6.08 (±0.58)	11.69 (±0.95)	29.58 (±2.86)	48.7 (±2.6)	19.29 (±1.65)	36.54* (±1.88)	271.8 (±114)	8.23 (±2.45)	3.54 (±3.11)	3.66 (±1.23)	0.63* (±0.43)	0.09 (±0.12)

Table 6.2.2: clinical chemistry results expressed as means (±SD) in cows with a normal parturition course and without other pathological condition within 30 DIM (CTRL) and in cows with retained placenta (RP). T0=2 to 7 days before parturition; T1=within 12 h from calving; T2=between 48 and 72 hours after parturition. § Reference interval obtained from at least 120 healthy cows at 3±1 DIM. DIM = days in milk. * P value<0.05 versus CTRL

		Calcium (mg/dL)	Creatinine (mg/dL)	Glucose (mg/dL)	Magnesium (mg/dL)	NEFA (mmol/L)	Phosphorous (mg/dL)	BOHB (mmol/L)	Total proteins (g/dL)
Reference intervals at 3 ±1 DIM §		6.41-10.18	<1.47	51-87	1.51-3.04	0.16-1.63	2.54-6.26	0.38-1.71	4.8-8.3
CTRL (T0)	Mean (±SD)	9.49 (± 0.91)	1.30 (±0.20)	66.6 (±3.6)	2.11 (±0.22)	0.26 (±0.15)	5.69 (±0.94)	0.38 (±0.12)	5.78 (±0.45)
RP (T0)	Mean (±SD)	9.55 (± 0.36)	1.19 (± 0.20)	65.7 (± 5.4)	2.21 (± 0.36)	0.22 (± 0.20)	6.17 (±0.72)	0.48* (± 0.10)	5.63 (±0.55)
CTRL (T1)	Mean (±SD)	7.84 (±1.78)	1.42 (±0.16)	95.8 (±29.0)	2.23 (±0.32)	0.69 (±0.45)	3.50 (±1.46)	0.52 (±0.19)	5.75 (±0.39)
RP (T1)	Mean (±SD)	7.05 (±1.30)	1.22 (±0.27)	81.8 (±16.1)	2.52 (±0.51)	0.78 (±0.50)	4.16 (±1.49)	0.66 (±0.18)	5.89 (±0.43)
CTRL (T2)	Mean (±SD)	8.53 (± 0.79)	1.08 (± 0.15)	53.1 (± 11.7)	1.76 (± 0.39)	0.54 (± 0.25)	4.36 (± 0.86)	0.44 (± 0.11)	5.48 (± 0.55)
RP (T2)	Mean (±SD))	7.93 (±1.04)	1.08 (±0.14)	64.5 (±15.0)	2.43* (±0.70)	0.48 (±0.25)	5.40 (±1.37)	1.00* (±0.76)	5.79 (±0.59)

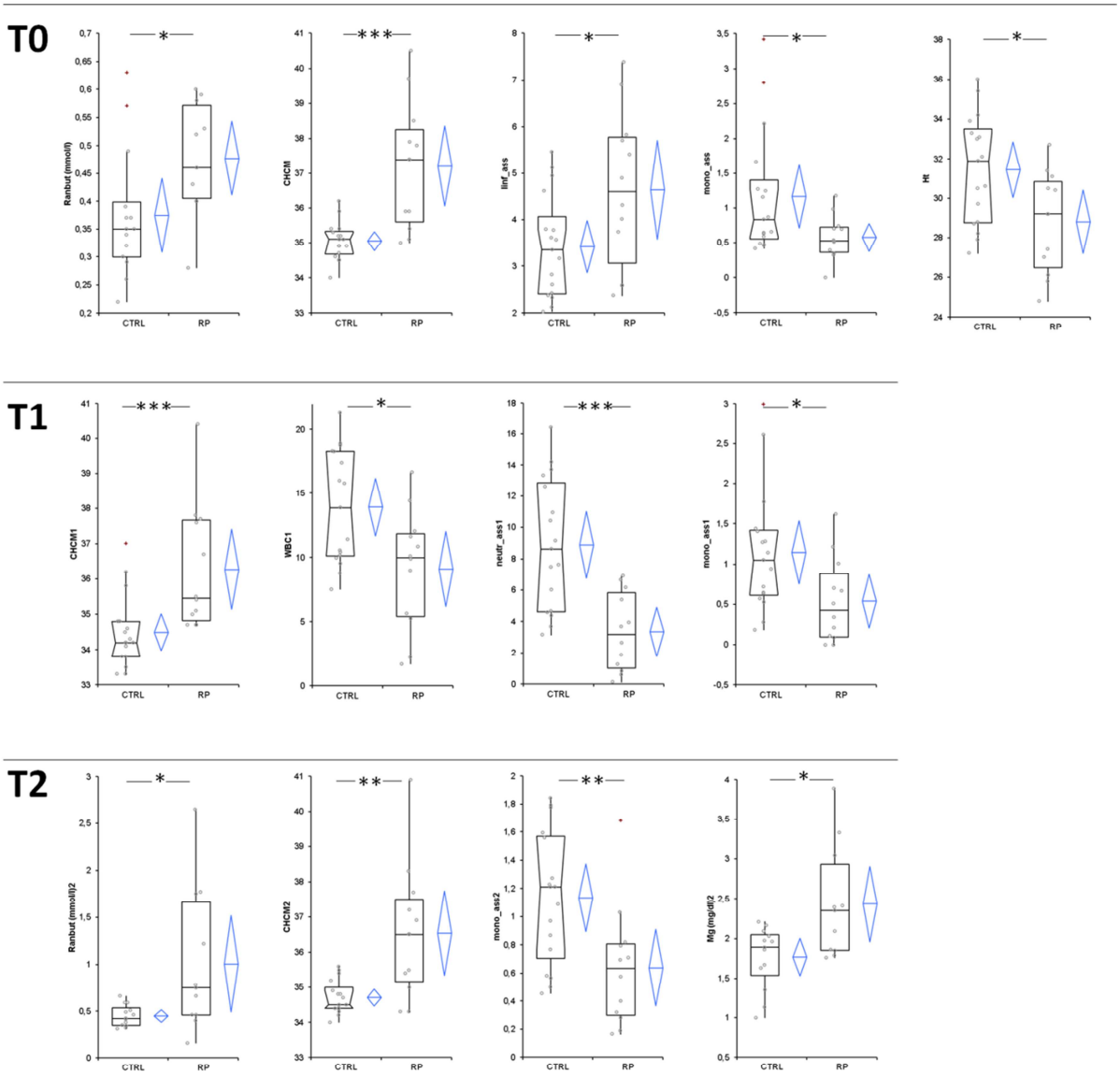

Figure 6.2.1: clinical chemistry and hematological results significantly different at comparison between cows with retained placenta (RP) and controls (CTRL) at the different sampling times. T0=2 to 7 days before parturition; T1=within 12 h from calving; T2=between 48 and 72 hours after parturition. * = P<0.05; **=P<0.01; ***=P≤0.001

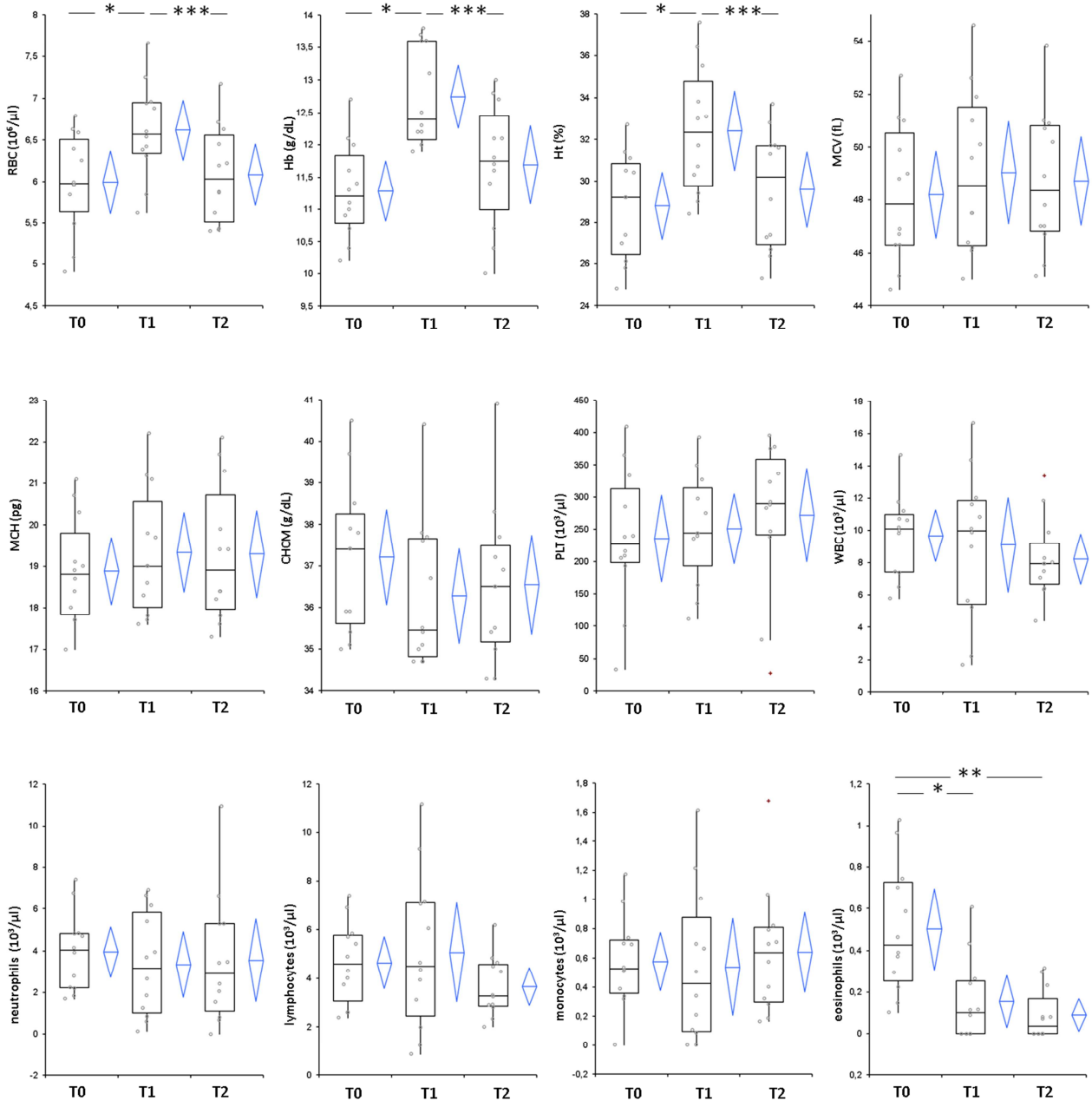

Figure 6.2.2: comparison of hematological parameters according to sampling time in cows with retained placenta. Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median, vertical lines are the limits of outlier distribution according to the Tukey rule. Near outliers are indicated by the symbols "x" and far outliers with asterisks outside the boxes. T0=2 to 7 days before parturition; T1=within 12 h from calving; T2=between 48 and 72 hours after parturition. *= T0 versus T1(P<0.05); **= T0 versus T2 (P<0.05); *= T1 versus T2 (P<0.05).**

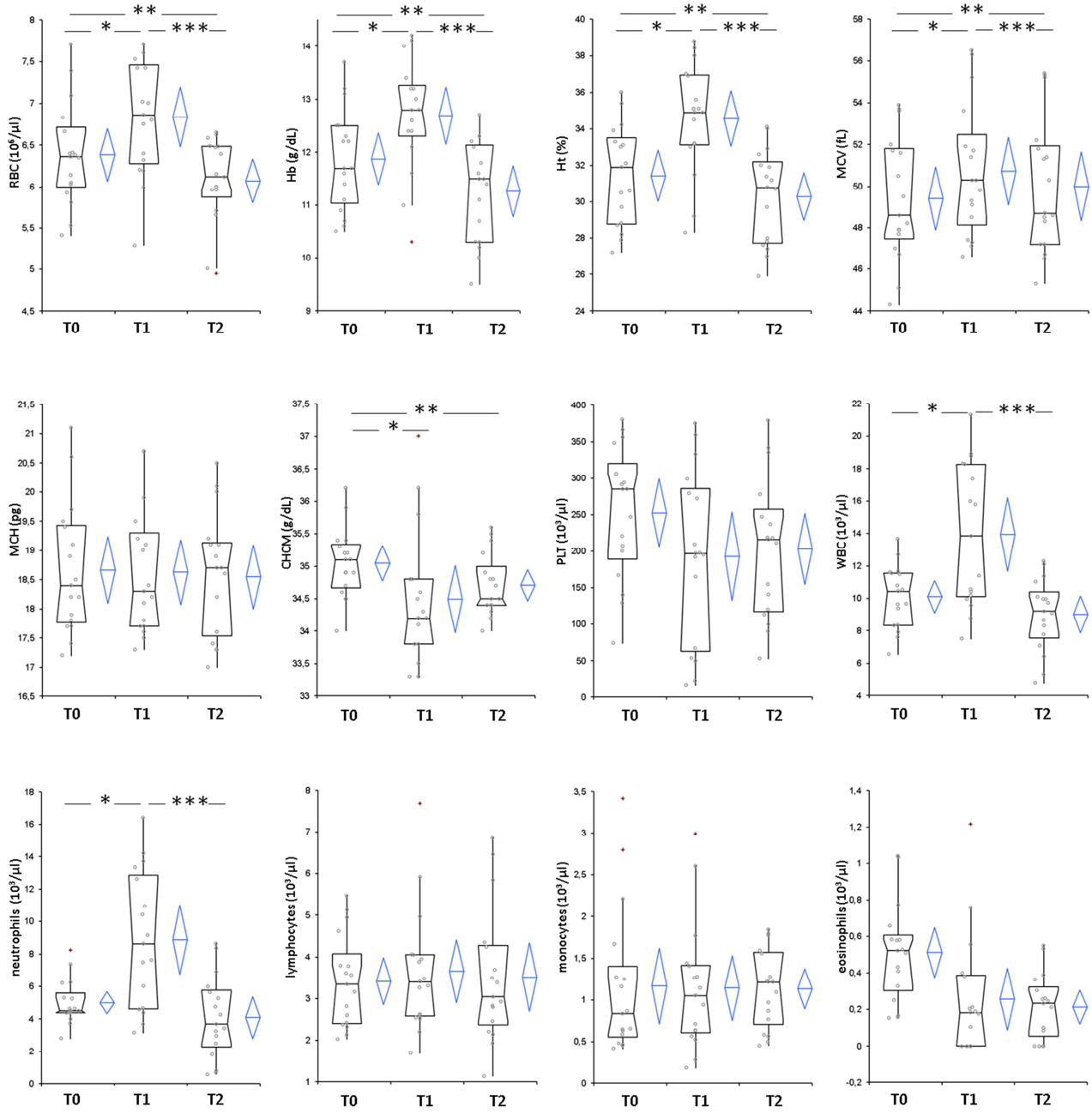

Figure 6.2.3: comparison of hematological parameters according to sampling time in CTRL cows. Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median, vertical lines are the limits of outlier distribution according to the Tukey rule. Near outliers are indicated by the symbols "x" and far outliers with asterisks outside the boxes. T0=2 to 7 days before parturition; T1=within 12 h from calving; T2=between 48 and 72 hours after parturition. *= T0 versus T1(P<0.05); **= T0 versus T2 (P<0.05); *= T1 versus T2 (P<0.05).**

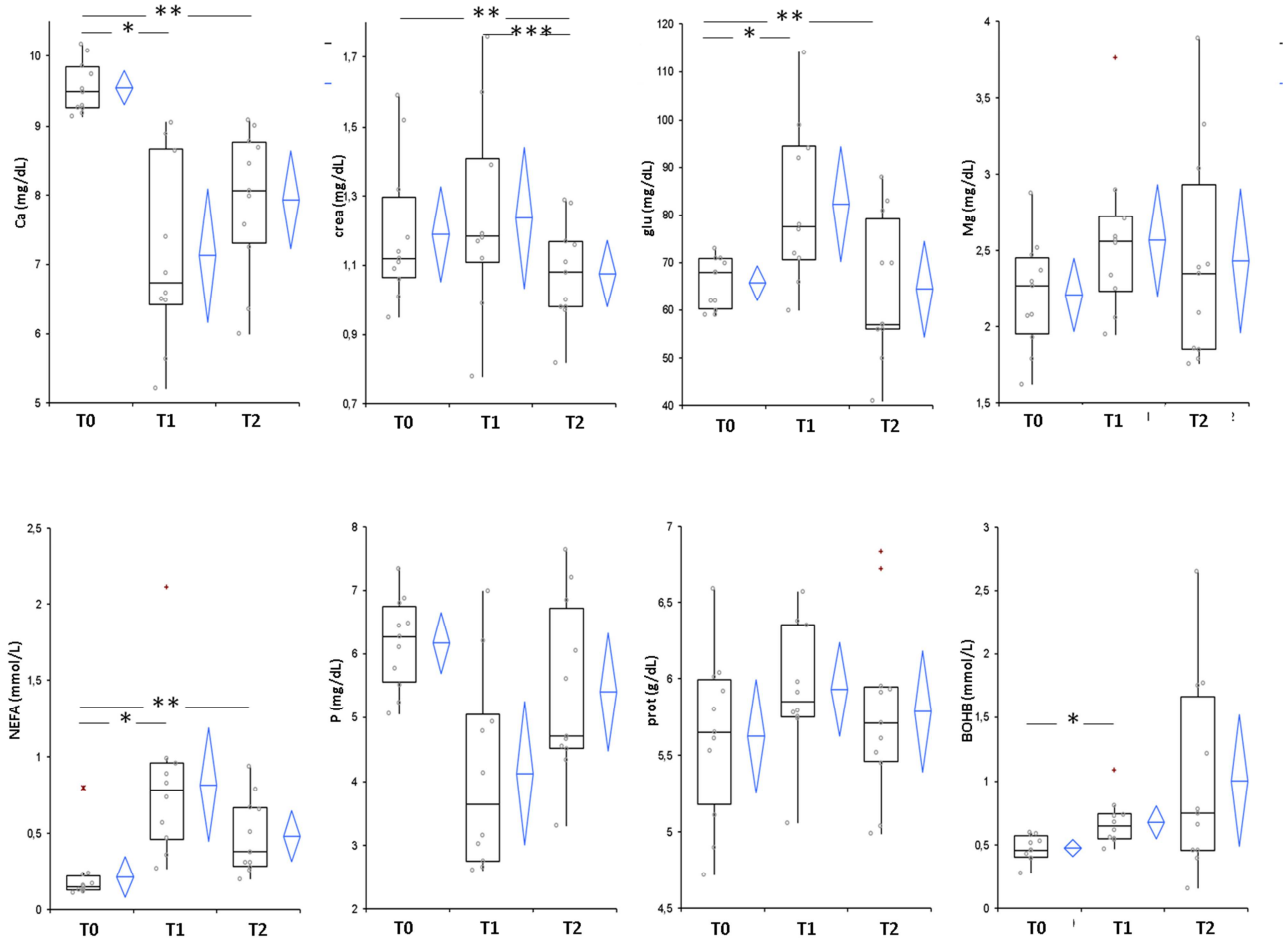

Figure 6.2.4: comparison of clinical chemistry results over time in cows with retained placenta. Boxes indicate theI-III interquartile interval, the horizontal line corresponds to the median, vertical lines are the limits of outlier distribution according to the Tukey rule. Near outliers are indicated by the symbols "x" and far outliers with asterisks outside the boxes. T0=2 to 7 days before parturition; T1=within 12 h from calving; T2=between 48 and 72 hours after parturition. *= T0 versus T1(P<0.05); **= T0 versus T2 (P<0.05); *= T1 versus T2 (P<0.05).**

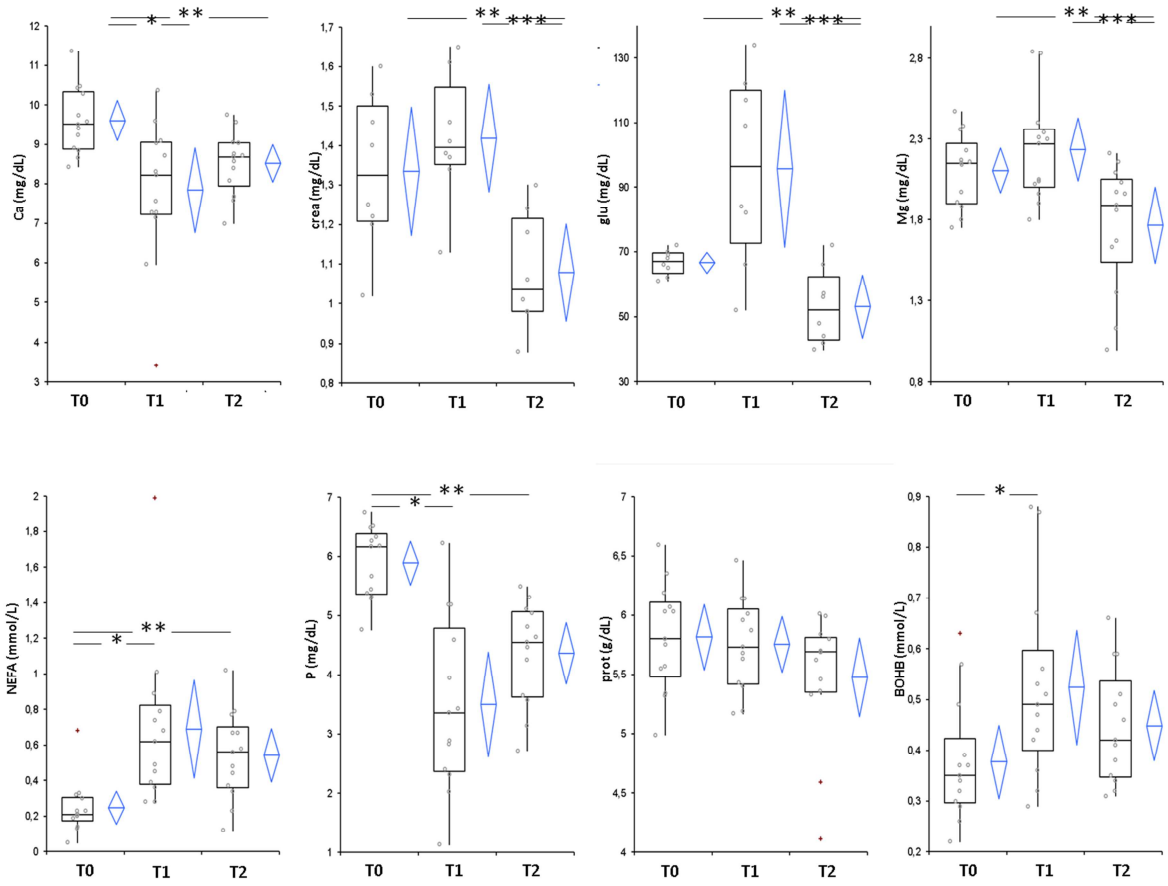

Figure 6.2.5: comparison of clinical chemistry results according to sampling time in CTRL cows. Boxes indicate theI-III interquartile interval, the horizontal line corresponds to the median, vertical lines are the limits of outlier distribution according to the Tukey rule. Near outliers are indicated by the symbols "x" and far outliers with asterisks outside the boxes. T0=2 to 7 days before parturition; T1=within 12 h from calving; T2=between 48 and 72 hours after parturition. *= T0 versus T1(P<0.05); **= T0 versus T2 (P<0.05); *= T1 versus T2 (P<0.05).**

6.2.4 Discussion and conclusions

Both animals with RP and normal cows manifested an increase of the erythroid mass at parturition, probably as a consequence of stress and reduction of water and food assumption around this time (Bell, 1995). Only in CTRL cows that was followed by a drop in the days after parturition with values that appeared lower than pre-partum. It may be speculated that this difference could reflect a delay in the access to water and food or a more “stressful” post-partum in cows that experience RP. However, in both the groups and in the three sampling times, RBC values were within the reference intervals, thus this difference is most likely attributable to an individual variation and/or a statistical artifact due to the low number of animals.

The leukocyte dynamics observed in CTRL cows may be justified by a normal leukogram in response to the inflammatory and stressful stimuli necessary for the correct parturition course (Beagley et al., 2010). In fact, stress leukogram changes in cattle include mild mature neutrophilia, lymphopenia, eosinopenia and variable monocytosis (Tornquist and Rigas, 2010). All these parameters returned to pre-partum values in the days after parturition, when probably inflammatory and stressful stimuli are progressively decreasing. It is well known that corticosteroids increase in the last weeks of gestation (Beagley et al., 2010) and is supposed to maintain a status of immune suppression that is needed to avoid the early detachment of the link between the cow and the foetus which is considered as non-self (Davies et al., 2000; Davies et al., 2004). The majority of cows with RP, in contrast, did not show a tendency to these changes, demonstrating the presence of a less responsive immune system at parturition. Differently to what happen in CTRL cows, mean values of leukocyte count did not change across time in RP cows that, however, have a lower number of circulating neutrophils and monocytes at parturition than cows without RP. The absence of significant differences in the number of circulating neutrophils before parturition between the two groups did not support the role of neutropenia as

an early predisposing factor to RP even if the low number of animals in this study should be increased to support this hypothesis. The lower number in RP cows of circulating neutrophils and monocytes at the time of parturition, both parts of the phagocytic system, may lead to an insufficient availability of neutrophils and monocytes in the endometrium at parturition thus reflecting a less efficient phagocytic system, likely affecting the proper dissolution of the fetal-maternal link and thus the expulsion of the fetal membranes.

Around parturition, high producing cows are subject to an increased negative energy balance (NEB) that favour an increased lipomobilization and the accumulation of NEFA in the blood (Opsomer, 2015). The results of this study showed that the rate of NEB was similar in the two groups (similar changes over time and concentration of NEFA and glucose) but cows that subsequently successfully expelled the fetal membranes were able to maintain BOHB concentration at lower levels compared to cows that retained the placenta. The possible pathogenesis of RP involves the impaired number and activities of endometrial leukocytes (Beagley et al., 2010). Different studies have proposed the role of hyperketonaemia, often observed in peri-parturient cows, as a cause of leukocyte disfunction although in literature is reported that very high concentrations of ketone bodies are necessary to interfere with leukocyte activities in ruminants (Sartorelli et al., 1999 and 2000; Scalia et al., 2006). In the two groups, both concentrations of NEFA and BOHB before and at parturition, when the mechanism responsible for the release of the placenta must be activated, were found within the reference range adopted in our laboratory for normal cows at 3±1 days. Even if huge increases of ketone bodies were not observed in RP cows, the higher BOHB may have influenced the number of circulating leukocytes as BOHB and AcAc at concentrations observed after parturition were shown to inhibit the proliferation of hematopoietic cells (Hoeben et al., 2000).

Glucose and calcium had similar temporal changes in both the groups. These findings may be explained with physiological changes occurring around parturition. In both groups, glucose

increased at parturition and decreased in the days after. This finding may be explained as the effect of the stress or of the hemoconcentration due to the reduced access to water at parturition followed by a return to water intake in the days after.

In the present study, both in RP and CTRL cows, calcium had the lowest mean concentration soon after parturition and the values remained lower in the days after. This finding may be related to the increased secretion of calcium in colostrum and the inadequate ability of the cow to mobilize bone calcium to restore blood concentration (Martinez et al., 2012). In contrast with the study from Melendez et al. (2004), where lower calcium concentrations 6 hours after parturition were found only in cows that retained the placenta, compared to cows that normally expelled, in the present study, this peculiarity was common for both the groups.

The very few differences observed and the similarity in variation over time between RP and CTRL cow likely reflect the similar metabolic and hormonal changes that cows in both groups had to deal with during the peri-partum. However, cows with RP were found to have some important peculiarities around parturition that may give an insight in the unknown pathogenesis of this condition. The most important finding in this part of the thesis was the observation of a lack of neutrophil peak in the RP group at parturition. This finding, especially if supported by a higher number of cases may have relevant diagnostic and prognostic implications. In fact, a complete blood cell count, which is an easy to perform and cheap analysis, may provide, alone, a helpful information. When performed at parturition, the finding of a neutropenia could predict which cows are at high risk of retain fetal membranes, thus allowing a very early medical intervention and possibly reduce economic and productive costs.

CHAPTER 7:

An attempt to prevent production diseases in dairy cows by intense monitoring and ad hoc treatment.

CHAPTER 7: AN ATTEMPT TO PREVENT PRODUCTION DISEASES IN DAIRY COWS BY INTENSE MONITORING AND AD HOC TREATMENT.

Preprint version of:

Coiatelli M., Giordano A., Sicilia F., **Moretti P.**, Durel L. An attempt to prevent production diseases in dairy cows by intense monitoring and ad hoc treatment. *Italian Journal of Animal Science.* 2015; 14:3918

7.1 Introduction

During the past two decades, considerable progress has been made in understanding the physiology of the transition towards lactation in dairy cows (Drackley et al., 2006). During the transition period, and particularly during the two weeks following parturition, dairy cows are faced with an inevitable energy deficit (NEB), which will play a crucial role for the remainder of lactation (Wensing et al., 1997).

Relations have already been reported between the duration and severity of periods of NEB, on the one hand, and the incidence of certain diseases, on the other hand, particularly digestive disorders and lameness (Collard et al., 2000). Between 30 and 50% of cows may suffer from a metabolic or infectious disease just before or after calving (Ingvartsen et al., 2003; LeBlanc, 2010). The disorders associated with inadequate energy intake predispose cows to metabolic or infectious diseases such as milk fever (MF), metritis (MET), acetoneamia (CK), displaced abomasum (DA) and retention of the placenta (RP) (Esposito et al., 2014). The reciprocal relations between the disorders referred to above have long since been demonstrated (Morrow, 1976; Curtis et al., 1985; Peeler et al., 1994; Heuer et al., 1999; Chapinal et al., 2011). Uncomplicated CK, RP MET and MF are risk factors for DA (Shaver, 1997).

It is now an accepted fact that an extreme rate of mobilisation of fatty tissue is related to a high incidence of metabolic diseases (Drackley, 1999). Such intense lipomobilisation leads to an elevation in the serum concentration of non-esterified fatty acids (NEFA) and then to their uptake by the liver, and to the accumulation of triglycerides in the latter. This accumulation of fatty acids in the liver predisposes the cow to the induction of ketosis accompanied by an elevation of serum beta-hydroxybutyrate (BOHB). Various authors have already demonstrated that the blood levels of BOHB, NEFA and calcium (Ca) are closely correlated with the incidence of production diseases (Hoedemaker et al., 2004; Goff, 2006; Stengärde et al., 2010; Chapinal et al., 2011; Chapinal et al., 2012).

The idea of compensating a NEB by the daily administration of propylene glycol or glycerol, starting from the last days of gestation and for 2 or 3 weeks after calving, is inconsistently practiced in modern dairy farms although it has already been experimented by various authors (Miettinen, 1995; Formigoni et al., 1996; Hoedemaker et al., 2004; Castañeda-Gutiérrez et al., 2009; McArt et al., 2011; Lomander et al., 2012). In addition to the practical and economic constraints involved, and although propane 1,2-diol is a permitted additive for animal feed in Europe (EU Regulation 892/2010) and in the United States, the adjunction of a non-natural additive to the ration of dairy cows may be questioned. It may be judicious to use propylene glycol sparingly in the form of a prescription drug. Some authors have already suggested the strategic use of blood tests for the detection of animals suffering from sub-clinical ketosis. The measurement of NEFA should be used during the last week of gestation and that of BOHB during the first week post-partum (McArt et al., 2013).

The purpose of this study was to verify whether systematic screening for sub-clinical ketosis during the transition period (2 weeks before and after calving) via the measurement of the blood levels of BOHB and NEFA, followed by *ad hoc* treatment with propylene glycol, calcium propionate, betaine, niacin and molasses (ENERGAN KETOSIS, Virbac) and by selective treatment with calcium (ENERGAN CALCIUM) for as long as necessary to restore the normal

values of these metabolites could have an impact on the morbidity and incidence of MF, RP, MET and DA during the immediate post-partum period.

7.2 Material and methods

The study was conducted in specialised dairy farms in the region of Milano (Italy) which had volunteered to take part in the trial. The farms were monitored by the same veterinary practice and were located close to the laboratory. All samplings points aimed to monitor the transition period disturbances and were performed under informed consent of the breeders. Blood (10 mL) were taken from all the animals during or just after the morning meal as animals were restrained for feeding, placed in plain tubes and immediately delivered to the laboratory. After centrifugation (2,200 g X 10 minutes), serum was separated and analysed soon after. Concentration of NEFA, BOHB and calcium were measured by means of an automated spectrophotometer (ILAB 300 plus; Instrumentation Laboratory S.p.a., Milan, Italy) using commercially available kits: NEFA (Acetyl CoA synthetase colorimetric method, Randox Laboratories Ltd., Crumlin, Co. Antrium, UK) and BOHB (D-3-Hydroxybutyrate dehydrogenase method, Randox Laboratories Ltd., Crumlin, Co. Antrium, UK), calcium (orthocresoftaleine method; Instrumentation Laboratory S.p.a., Milan, Italy).

The investigating veterinarian was responsible for evaluating the annual incidence of DA, RP, MF, MET and CK, one of which must exceed 5, 15, 10, 10, 10% respectively.

Monitored animals were gestating dairy cows. The size of the population was set at about 200 animals. Animals were recruited according to their foreseeable due date. No animal was excluded *a priori* if gestation was confirmed. Animals were maintained in permanent open housing, with individual bedding cubicles. Towards the end of gestation, the animals were fed a diet of corn silage, straw and concentrates. Glucogenic treatments were prohibited and their use was grounds for exclusion.

7.2.1 Follow-up protocol

The animals were included starting at 2 weeks before the presumed date of parturition. The study was continued until 15 days after calving. A computerised data-collection form provided, for each animal, the dates of the blood samples and the tests to be performed. Sampling on day d±1 was tolerated, according to the day of the week on which the animals were included. Overall, blood samples were taken 3 times a week from the animals monitored. The results from the laboratory were available late in the afternoon. The sampling plan was as follows:

- First week of the study (14-7days pre-partum): all the animals in both groups had samples taken for the measurement of BOHB and NEFA.
- Second week of the study (7 days pre-partum up to the date of parturition), all the animals had samples taken for the measurement of BOHB, NEFA and Ca.
- Third week of the study (first week post-partum): all the animals were monitored for BOHB and Ca.
- Fourth week (final sample) of the study (7-15 days post-partum): all the animals were monitored for BOHB.

7.2.2 Treatment given the animals

Animals presenting levels of BOHB>1.2 mmol/L or of NEFA>0.5 mmol/L for the first time were considered as POSITIVE animals. Positive animals with an odd-numbered ear tag (last digit) were included in the treatement group (group T) and were then drenched on a daily basis, with a glucogenic medication containing propylene glycol (122 g), calcium propionate (11 g), betaine (7 g), niacin (5 g) and molasses (~150 g) until the biological values returned to normal. Others POSITIVE animals (even-numbered ear tag) were left untreated and served as control (group C). Animals with BOHB and NEFA within the physiological range all over the study period were declared NEGATIVE (Fig. 1).

Hypocalcemia, even if mild, was judged to be a potential bias, liable in itself to jeopardise the possible beneficial effect of the glucogenic treatment. In an attempt to control it, calcium was therefore administered selectively at the time of parturition. The effects of the two treatments were therefore merged. Animals with an odd-numbered ear tag (last digit), including POSITIVE and NEGATIVE animals were also equipped with a parturition detector (VEL'PHONE, Medria, France), in order to be able to treat them with calcium in due time. When the parturition detector made it possible to predict the hour at which calving would occur, the animals involved received 41.4 g of elemental calcium 12 h prior to parturition and 12 hours after it, in the form of a commercial preparation containing calcium propionate and chloride associated with disodium phosphate and magnesium oxide (ENERGAN CALCIUM, Virbac).

7.2.3 Data collection and processing

The occurrence of DA, MF, RP, CK and MET during the study period was recorded for all the animals in order to compare the incidence of those diseases in each group.

In order to allow the processing of the blood tests, the calving dates had to be made to coincide and the values relating to dates -1, -2, -3, and so on had to be processed n-1 day prior to parturition and those relating to dates +1, +2, +3, and so on, had to be processed n+1 day after that date. However, while 80% of calvings in artificially inseminated Holstein cows occur 272±10 days after insemination (Matthews and Morton, 2012), predicting the exact date of parturition in an individual remains problematic. As a result, and without even taking into account the uncertainties regarding the presumed date of term, the date of the animals' incorporation into the follow-up may be somewhat distant from the date of calving. In addition, since blood samples were taken in each animal about 1 out of every 3 days (d±1), the number of values able to be processed for each of the three groups and for each day prior to calving may be expected to possibly be small. Moreover, in cows, the physiological distribution of BOHB serum concentrations may be very broad (<0.4 to >1.2 mmol/L) (Enjalbert et al., 2001). The

combination of such phenomena may lead to a significant loss of statistical power over the pre-partum period, which would not do justice to the importance of the work done. We thus propose to group the pre-partum data together, on the basis of the following principles:

- Metabolic changes having a significant impact on the health of a cow are unlikely more than 2 weeks before calving. They may then appear gradually and possibly accelerate suddenly during the days prior to parturition.
- The performance of a blood test a week before the presumed date of calving, for the purpose of anticipating a possible metabolic problem, would already constitute a practical advance. The test could be renewed a week later, if the cow had not calved. It is unreasonable to expect farmers to agree to additional tests.
- Consecutive daily data that are not significantly different have been grouped together.
- Data series that remained incomplete following consolidation have been excluded.

The samples were thus grouped together as follows:

- Pre-partum: 3 periods that are meaningful from a biological and operational viewpoint: [-30;-13], [-12;-7] and [-6;-1] days.
- Post-partum: the situation was simpler. The periods chosen were 1, 4, 7, 10, 13 days ±1 day ([0-2], [3-5], etc.

The data were analysed at DIVET using the Analyse-it software (Analyse-it Ltd, Leeds, UK). The relative incidence of the diseases monitored were compared via a chi-2 test. The evolution of the relative blood levels of various metabolites over time was analysed via the non-parametric ANOVA test for paired measurement, followed by a Wilcoxon signed rank test to assess the difference between single groups.

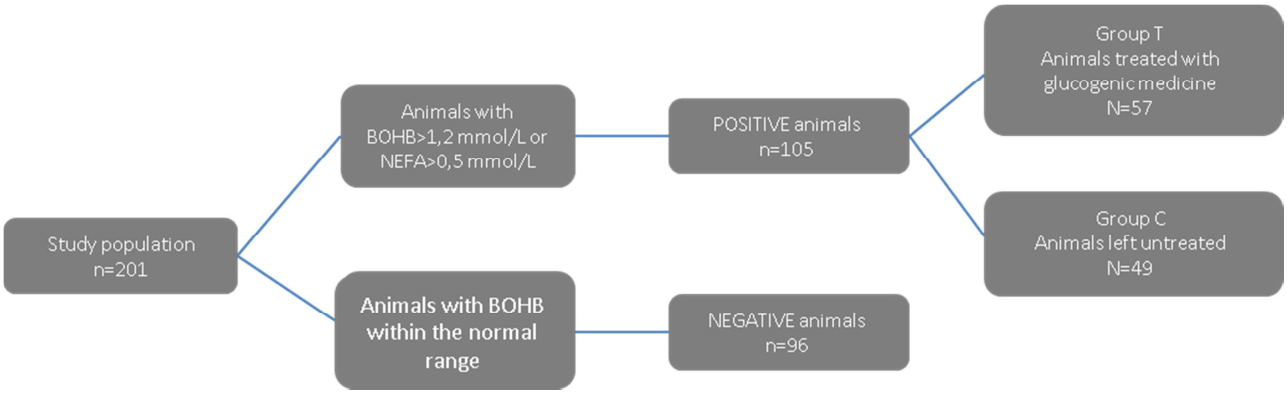

Figure 7.1: Study design and experimental groups, group definition and size (n). POSITIVE animals are animals with BOHB or NEFA values out of the physiological range (>1.2 or 0.5 mmol/L respectively). POSITIVE animals are therefore treated with a glucogenic medicine (group T) or left untreated (group C), according to final digit of their ear tag (even number=C; odd number=T). Positive animals were also drenched with an oral
calcium solution at calving time.

7.3 Results

Two dairy farms and 201 animals were included in this trial. The final study population is represented in figure 7.1.

7.3.1 Application of glucogenic treatments

Fifty-seven animals received glucogenic treatment during the study. The average treatment duration was 4.6 days, involving one dose per day of the commercial product. All treatment took place before parturition. Forty-three animals (75%) did not require any other treatment. Ten animals relapsed and had to be treated again for a mean duration of 6.2 days.

7.3.2 Prevalence of the diseases monitored

A total of 57 animals (28.4%) were diagnosed as having one of the monitored diseases during the trial period (SICK animals). The total numbers of animals (and percentages) presenting DA, MF, RP or CK was 9 (4.5%), 4 (2.0%), 20 (10.0%) and 38 (18.9%), respectively. No puerperal metritis (MET) was reported in any group. The incidence of the various conditions in all experimental groups is reported in Table 7.1.

A comparison between negative and control animals in one hand, and negative and treated animals in the other hand, revealed the following points:

- Animals belonging to group C (tests+ but untreated) were significantly ($p<0.01$) more SICK than NEGATIVE animals (test-), and presented significantly more CK ($p<0.01$) and DA ($p<0.05$).
- Animals belonging to group T (tests+ and treated) were significantly ($p<0.01$) more SICK than negative animals and presented significantly more CK ($p<0.05$). Regarding DA and other conditions differences are not significant.
- There is no significant difference between group C and group T animals for any of the monitored conditions.

Table 7.1: Statistical comparison of the distribution of animals affected by diseases (sick) or by specific a condition in negative animals (BOHB and NEFA within the range), in positive and treated animals (group T) and in positive and untreated controls (group C) *Yate's correction applied. **Fisher's exact test. Threshold of significance: p<0.05

	Overall	Negative animals	Positive animals (C)	Positive animals (T)	Negative vs positive animals	Negative animals vs C	Negative animals vs T
	Affected/ not affected	affected/ non affected (%)	affected/ non affected (%)	affected/ non affected (%)		P values	
Sick	57/144 (28.3)	15/81 (15.6)	22/26 (45,8)	20/37 (35,1)	<0.001	<0.001	0.006
Displaced abomasum (DA)	9/192 (4.5)	2/94 (2.1)	5/43 (10.4)	2/55 (3,5)	0.116	0.028	0.593
Milk fever (MF)	4/197 (2.0)	2/94 (2.1)	1/47 (2,0)	1/56 (1,8)	0.928	1.000	0.887
Retention of the placenta(RP)	20/181 (9.9)	6/90 (6.2)	7/41 (14,6)	7/50 (12,3)	0.093	0.099	0.196
Clinical ketosis (CK)	38/163 (18.9)	9/87 (9.4)	17/31 (35,4)	12/45 (21,1)	<0.001	<0.001	0.042

7.3.3 Blood chemistry tests pre-partum

Dispersion of calving

The median time elapsing between the inclusion of the animals and calving was 18 days, 50% of cows having calved between 15 and 21 days. The rest of the animals calved between 49 and 0 days after inclusion.

Dispersion of blood chemistry results

Although 2459 blood tests were performed on the animals, the number of samples available for each day prior to calving varied from 59 (NEFA, -12 days) to 0 (Ca, -27 days).

The samples from 3 pre-partum periods, i.e. [-30;-13], [-12;-7] and [-6;-1] days, were grouped together as explained in the *materials & methods* section. After eliminating the cases for which the data set was incomplete, the final number of animals for which statistical analysis was possible is presented in Table 7.2. Concerning post-partum, most of the blood test results could not be included for comparison with the pre-partum results due to the problem of heterogeneity between the groups, as referred above. The numbers of samples that could be compared, both for Ca and for BOHB, are indicated in Table 7.3.

Table 7.2. Number of cases included in the statistical comparison of results BOHB grouped by 3 consecutive days from day -29 to day -2 pre-partum.

Three-days sampling period	N of samples
relative to calving date°	
Day -29 (±1 day)	12
Day -26 (±1 day)	26
Day -23 (±1 day)	49
Day -20 (±1 day)	88
Day -17 (±1 day)	134
Day -14 (±1 day)	170
Day -11 (±1 day)	165
Day -8 (±1 day)	143
Day -5 (±1 day)	100
Day -2 (±1 day)	57

°Day-*n*: *n* days before calving.

Table 7.3: number of cases included in the statistical comparison of results recorded post-partum

	number		
Controls	100		
Treated	91	T-	37
		T+	54
TOTAL	191		

BOHB results

The results recorded for BOHB during the pre-partum period (Figures 7.2 and 7.3) showed that serum levels of BOHB increased during this period in all groups. For all the samples collected after d-12, those levels were significantly higher than during the -30/-13 days period, and as early as day -6 they were significantly higher than during the -12/-7 days period. At no time during monitoring, were significant differences between groups C, T and negative animals noted (data not presented). Post-partum, the comparison of results recorded in the two groups day by day did not revealed significant differences between controls and treated animals (Figures 7.2 and 7.3).

NEFA results

Results concerning NEFA concentration are shown in figures 7.4 and 7.5. The results recorded for NEFA during the pre-partrum period showed significant differences ($p<0.001$) in group T. In this group NEFA blood serum concentration dramatically increased from day 30 before calving to the very last days of gestation.

Calcium results

Pre-partum and post-partum results from calcium are graphically reported in figure 7.6. In the pre-partum period, due to the low number of animals in the experimental groups, it was not possible to assess a possible difference between these groups nor to investigate the trend over time detectable in groups. Also in cows treated with calcium no relevant changes were found.

Concerning the post-partum samples no significant differences between treated and untreated animals were observed

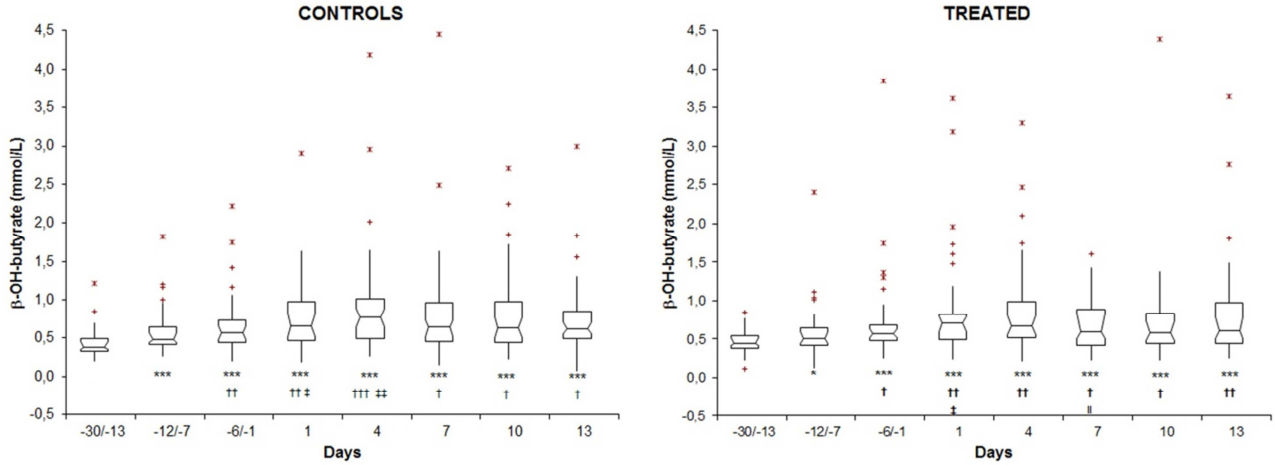

Figure 7.2: distribution of serum BOHB values and statistical comparison of results recorded pre-and post-partum in controls(C) and in the whole group of treated animals (T+ and T-). Boxes indicate the I-II interquartile interval, the horizontal line corresponds to the median, the vertical lines are the limits of outlier distribution according to the Tukey rule. Near outliers are indicated by the symbols "+"; far outliers are indicated by the orange asterisks outside the boxes. The black bolded symbols reported below each box and whiskers indicate significant differences between groups as follows: * = P<0.05 vs days -30 -13; *** = P<0.001 vs days -30 -13; † = P<0.05 vs days -12 -7; †† = P<0.01 vs days -12 -7; ††† = P<0.001 vs days -12 -7; ‡ = P<0.05 vs days -6 -1; ǂ = P<0.01 vs days -6 -1; ǀ = P<0.05 vs day 1.

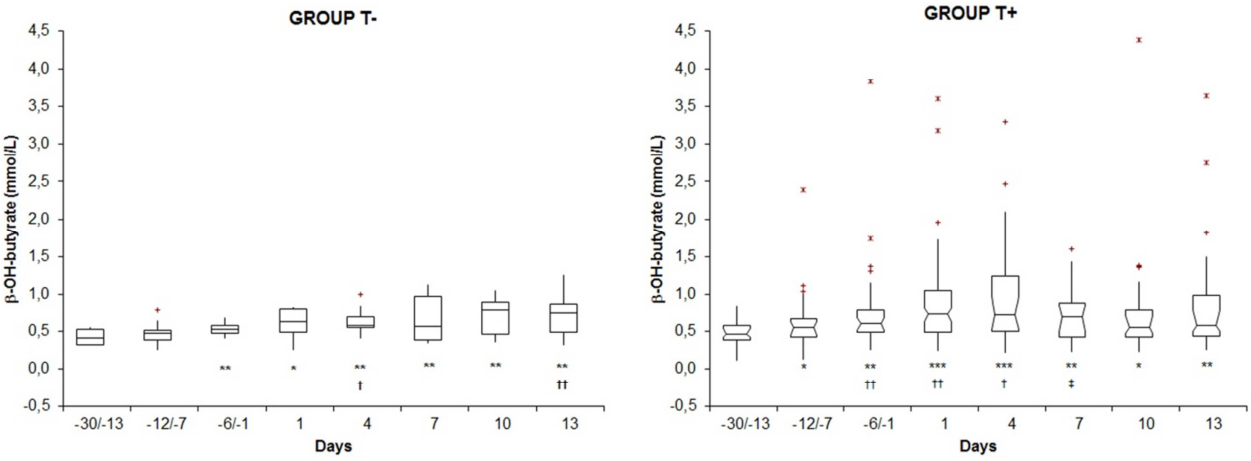

Figure 7.3: distribution of serum BOHB values and statistical comparison of results recorded pre-and post-partum in animals from groups T+ and T-. See figure 7.2 for interpretation of box and whiskers. The black bolded symbols reported below each box and whiskers indicate significant differences between groups as follows: * = P<0.05 vs days -30 -13; ** = P<0.01 vs days -30 -13; *** = P<0.001 vs days -30 -13; † = P<0.05 vs days -12 -7; †† = P<0.01 vs days -12 -7; ††† = P<0.001 vs days -12 -7; ‡ = P<0.05 vs days -6 -1.

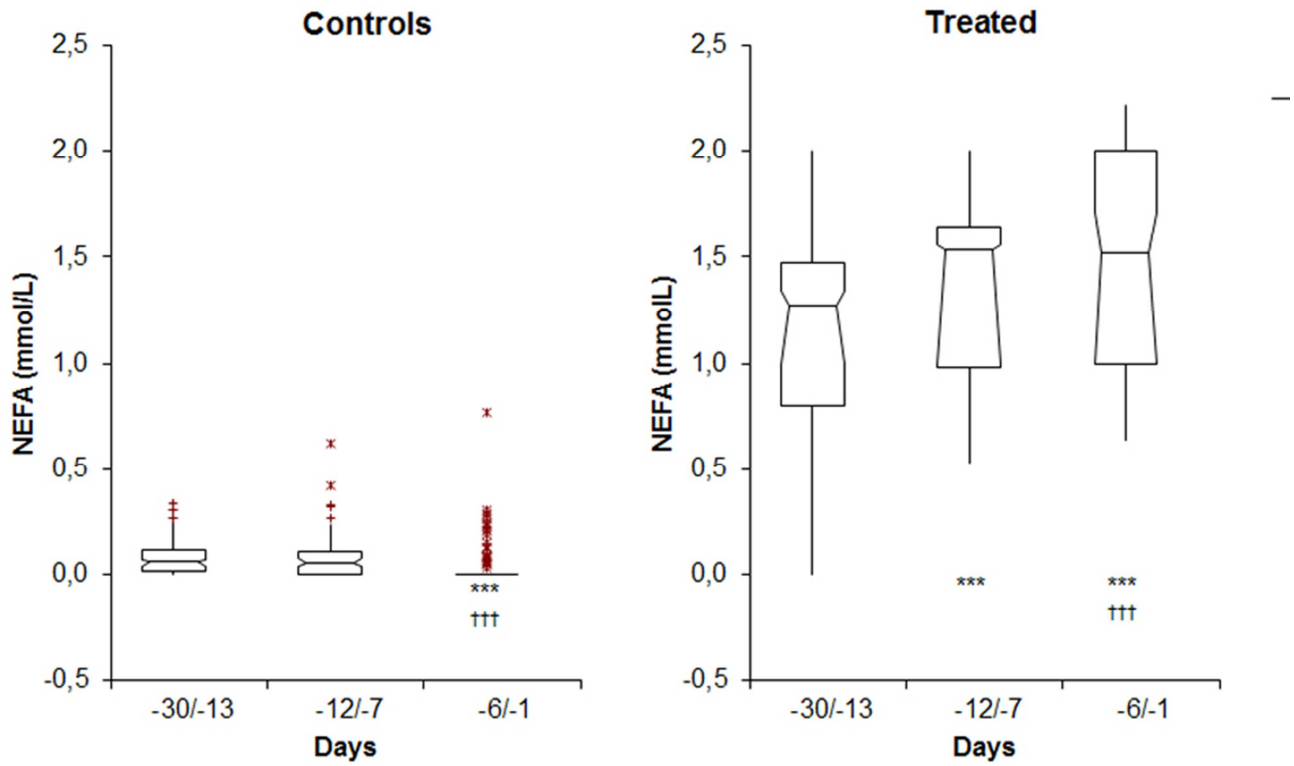

Figure 7.4: distribution of serum NEFA values and statistical comparison of results recorded pre- partum in controls (C) and in treated animals (T+ and T-). See figure 7.2 for interpretation of box and whiskers. The black bolded symbols reported below each box and whiskers indicate significant differences between groups as follows: *** = P<0.001 vs days -30 -13; ††† = P<0.001 vs days -12 -7.

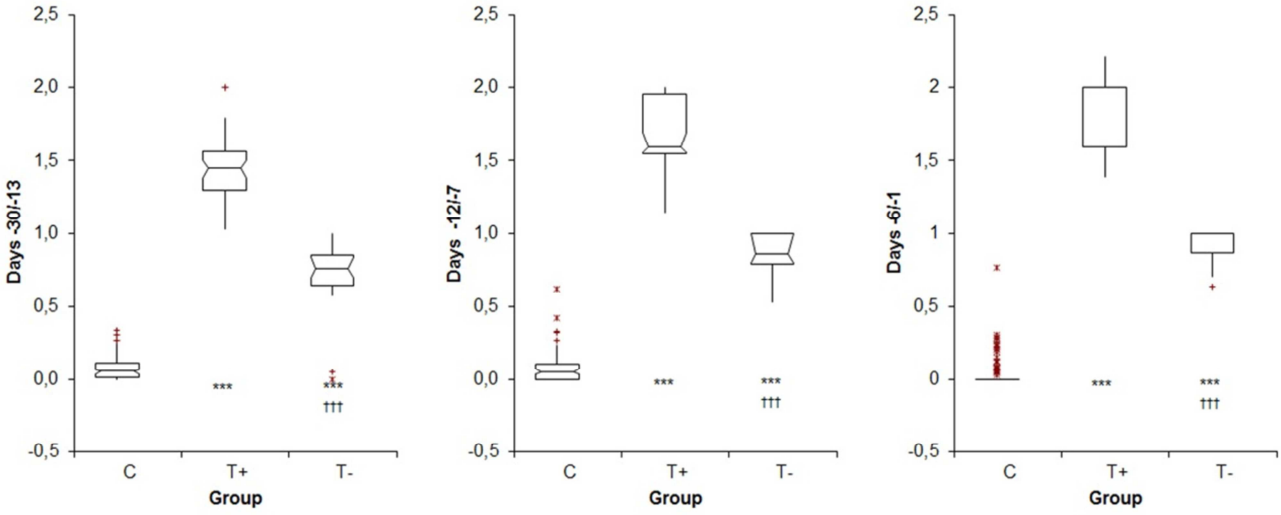

Figure 7.5: comparison of results from NEFA recorded pre-partum in each group of sampling days in Controls (C) and in animals from group T+ and T-. See figure 7.2 for interpretation of box and whiskers. *** = P<0.001 vs group C; ††† = P<0.001 vs group T+

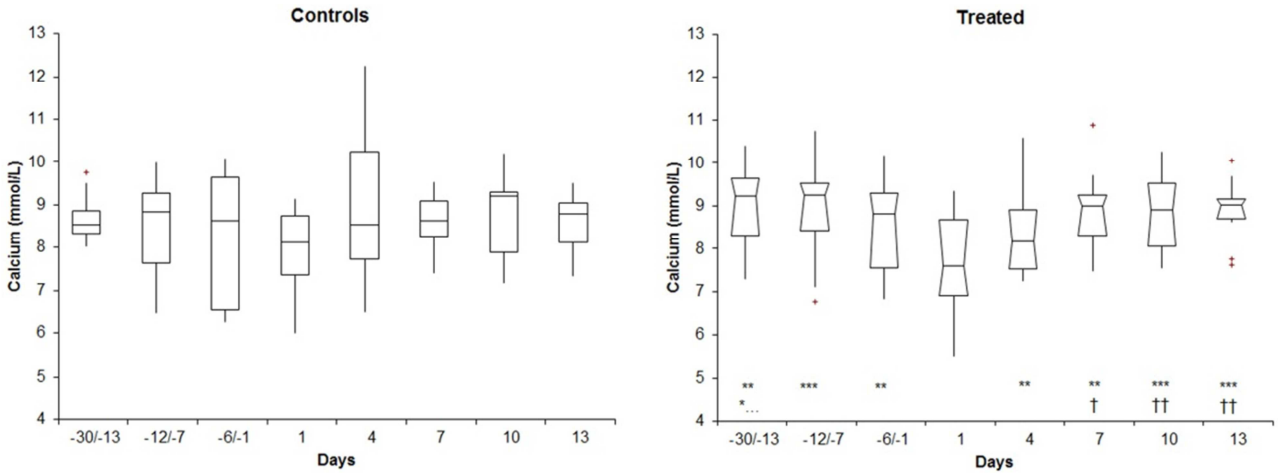

Figure 7.6: distribution of serum Ca and statistical comparison of results recorded pre-and post-partum in controls and in treated animals (T+ and T-). See figure 7.2 for interpretation of box and whiskers. The black bolded symbols reported below each box and whiskers indicate significant differences between groups as follows: ** = P<0.01 vs day 1; *** = P<0.001 vs day 1; † = P<0.05 vs day 4; †† = P<0.01 vs day 4.

7.4 Discussion

This study was conducted in order to verify whether it would be possible to decrease the incidence of metabolic related diseases in dairy cows presenting metabolic abnormalities during the transition period (altered blood concentration of BOHB, NEFA and to a lesser extent Ca), by administering a commercial product containing molasses, propylene glycol, calcium propionate and niacin and selective treatment with calcium at the time of calving.

The main result of this study shows that animals that always had levels of NEFA<0.5 and of BOHB<1.2 mmol/L (negative animals) during the pre-partum period are significantly ($p<0.01$) unlikely to develop one of the diseases monitored (DA, MF, RP or CK) than positive animals. Specifically, positive animals were more likely to get CK ($p<0.01$) and in a lesser extend RP ($p=0.09$).

Group T animals were always less sick and always presented less incidence of DA, MF, RP and CK than those belonging to group C. Animals in which the correction of those transition diseases was not attempted (group C) were more likely to get sick ($p<0.01$), to get CK ($p<0.01$) as well, to get DA ($p=0.03$) and in a lesser extend to get RP ($p=0.09$), than negative animals. In contrast,

treated animals (group T) were more likely to get sick ($p<0.01$) and to get CK ($p=0.04$) than negative individuals. Differences with negative animals were no longer significant for DA and RP, pointing out a possible interest of treating dairy cows with a glucogenic medicine as soon as they display elevated blood serum BOHB or NEFA. Based on the design of the study, we cannot exclude the possibility that some of the animals of this group developed one of the diseases mentioned above after the end of this study. However, this is unlikely since most of these conditions usually occur just after parturition. Moreover, in the case of the animals presenting a distinct elevation of BOHB or NEFA at any time prior to calving, the daily treatment with PG (group T) allowed a permanent return to normal values in 75% of them within 4 to 5 days.

The biochemical results for the pre-partum period were grouped together into weekly or fortnightly periods ([-1;-6], [-7;-13] and [-13;-30] days before calving), such periods being clinically and practically significant. For the post-partum period, the exact date of the blood sample was chosen. Whatever the period considered, it was not possible to observe any significant difference between the various groups of animals. This difficulty is ascribed to the great variability of the individual data and to the small size of each group, for each period. However, a significant ($p<0.001$) increase in BOHB levels was observed during the pre-partum period. That increase continued during the post-partum period, after which the values returned to normal. Non-esterified fatty acid levels increased significantly ($p<0.001$) during the pre-partum period. Ca levels remained rather equal during the study period, except for a significant drop 1 and 4 days after calving.

The incidences of the four diseases monitored are those usually reported by the literature (Goff, 2006; Mulligan et al., 2006; Parker Gaddis et al., 2012), except in the case of clinical ketosis (CK), For the negative animals the incidence of CK is slightly higher than those reported in the literature (0 to 5%), but for the other groups the incidence is more comparable to that reported for sub-clinical ketosis (20 to 40% or even more) (McArt et al., 2013, 2011).

Various blood parameters have been used as advanced indicators of an energy imbalance (Stengärde et al., 2010; Hailemariam et al., 2014). However, NEFA and BOHB measurements are among the most convenient, as blood BOHB can also be measured next to the cow by means of small portable devices (Optium Xceed, Abbott Laboratories) (Voyvoda and Erdogan, 2010). The threshold values chosen for this study are the same as those used by others (LeBlanc et al., 2005; Mulligan et al., 2006; McArt et al., 2011; McArt et al., 2012; McArt et al., 2013). NEFA and BOHB levels provide two measures of the severity of energy imbalance. It is now well known that this imbalance affects both cows at the start of lactation and dry cows at the end of gestation and predisposes them to LDA, RP, dystocia, fatty liver, CK and other problems (Mulligan et al., 2006). LeBlanc (LeBlanc, 2010) reports that NEFA>0.4 mmol/L during the 7 to 10 days before calving multiplies the risk of LDA by 2 to 4 fold and the risk of RP by 2 fold. Likewise, BOHB>1,2 mmol/L after calving multiplies the risk of LDA by 3 to 8 fold and the risk of CK by 4 to 6 fold. The observations reported here fully agree with those findings, since the negative animals were less (or significantly less) affected than the positive animals.

The early detection of sub-clinical ketosis on the basis of measurements of BOHB and NEFA, for the purpose of instituting corrective treatment and subsequently reducing the incidence of metabolic related diseases, has already been proposed by other authors (Hoedemaker et al., 2004; Overton and Waldron, 2004; Lomander et al., 2012; McArt et al., 2012). While it is possible, generally speaking, to have an effect on the serum concentrations of BOHB and NEFA, the results in regards the cows' health, are however, diverse. Some have ascribed the lack of effectiveness of such treatments to the fact that the studies are conducted on herds of high-producing cows, an objective that can be attained only thanks to excellent management of the stock, and the cows can then go through the NEB period without any particular consequences (Hoedemaker et al., 2004). Overton et Waldron (Overton and Waldron, 2004) have stated that, in

the absence of proven positive benefits, the routine administration of propylene glycol is not recommended.

7.5 Conclusion

This study confirms that an aggressive monitoring of pre-partum blood level would help to identify animals at increased risk to develop a production disease in the early post-partum. Although the proposed on demand glycogenic treatment shows a sharp decrease in the incidence of DA in animals with biochemical abnormalities and results in an absence of statistical difference between treated and negative animals, other studies involving more aggressive therapeutic diets and more animals would be worth conducting.

CHAPTER 8

General discussion and conclusions

CHAPTER 8: GENERAL DISCUSSION AND CONCLUSIONS

Ideally, due to the variation in methods, procedures, sampling sites and population of animals sampled among different laboratories, each diagnostic laboratory should establish a set of reference intervals for all the parameters and all the species analysed. Even if the transference of existing RIs may be possible in some circumstances, this may be not applicable when all the information concerning possible sources of variation are not available. In accordance to the guidelines recently published by the American Society for Veterinary Clinical Pathology (ASVCP) (Friedrichs et al., 2012), reference intervals for healthy high producing dairy cows around 3 and 30 days after calving were established as shown in the study presented in **chapter 5**. The adoption of separated RIs for these two different moments of lactation was mandatory due to the remarkable metabolic, hormonal, and immune changes occurring during the post-partum period but was also supported by the statistical analysis as shown in the study. The statistical analysis confirmed also some known differences concerning the variation occurring in the early post-partum period, such as the higher degree of negative energy balance (higher NEFA and BOHB), increased inflammatory status (higher ceruloplasmin and haptoglobin, and lower paraoxonase-1), and the increased oxidative stress (higher dROMs) near parturition. The establishment of RIs may be based on an *a priori* study, characterized by the determination of inclusion and exclusion criteria preceding the sampling or, as happened in this case, through the selection of data from an existing database, with an *a posteriori* method (Friedrichs et al. 2012). In the present case, the availability of a database derived from a large field study allowed us to select a sufficient number of reference values (results obtained from a selected reference individuals selected according to the inclusion and exclusion criteria) belonging to cows that were clinically healthy for the whole lactation period and that had production and fertility consistent with the normal standard of dairy herds of our geographical area. This allowed also to reduce the variability of results, leading to the establishment of narrower reference intervals

compared to the previously adopted reference intervals in many cases. According to the aims, in the same work, the possible effects of herd, days of sampling and parity were evaluated, evidencing only rare variations mainly regarding the samples obtained 30 days post-partum. Even if the low number of samples in each subgroup did not allow to establish specific RIs for the affected variables, some important information can be derived from these results. In particular, the differences observed among the herds one month after parturition highlighted that during a period less influenced by huge metabolic and hormonal variations as occurs one month after parturition, also slight differences in dietary and management strategy may reflect in changes of some metabolic and inflammatory variables, with a higher magnitude compared to the peri-parturient period.

Moreover, the metabolic, hormonal, and inflammatory variations occurring during transition, point out the importance to find possible markers that early indicate the presence of a subclinical condition in cows. Based on the published literature available, this is the first time that RIs for the main acute phase proteins (APPs) in cows (paraoxonase-1, haptoglobin, ceruloplasmin) (Giordano et al., 2004; Eckersall and Bell, 2010) and for some markers of oxidative stress (dROM and thiol groups) were provided. APPs are promising early markers of inflammation due to their rapid increase or decrease during this condition (Bertoni et al., 2008; Huzzey et al., 2011). Markers of oxidative stress are of relatively recent adoption in dairy monitoring but, as stated in the general introduction of this thesis, their role may be crucial to understand the relationship between metabolism and inflammation during the transition period (Sordillo and Mavangira, 2014).

The RIs established in the present thesis were generated for laboratory methods that are commonly available in routine practice. Commonly applied biochemical parameters, but also recently adopted inflammatory and oxidative parameters, were all performed on automated biochemistry analyzers, helping the standardization of the procedures and reducing the cost of analyses, a primary issue for dairy industry. In practice, these RIs may be useful for practitioners

to interpret results from cows in different lactation stages, especially in our geographical area. Moreover, applying a transference method (schematically represented in **figure 8.1**), every laboratory could adopt these RIs, provided that the laboratory's animal patient population and laboratory methods of the 'receiving' laboratory are appropriate (Friedricks et al., 2012).

Concerning the second aim of the thesis, even if a prognostic role of the investigated parameters in predicting the occurrence of retained placenta (RP) was not completely confirmed (based on the low number of affected cows enrolled in the study), some peculiarities in the biochemical, hematological and inflammatory patterns associated with the peri-partum in dairy cows with and without retained placenta had been shown in **chapter 6**. Concerning differences between healthy and diseased animals, the combined results from the two works evidenced a lack in peripheral neutrophils increase and lower neutrophil and monocyte concentrations at parturition in cows that retained placenta compared to not affected cows. Thus, although a similar pattern of NEB was present in the two groups, as shown by the presence of metabolic parameters within reference intervals before and soon after parturition in both the groups, a lower mass of phagocytes was present at parturition in blood of cows that subsequently developed RP. This deficiency occurs when one of the mechanisms responsible of the disruption of the feto-maternal link that involves the local presence of these cellular elements should be working efficiently (Beagley et al., 2010). In addition to the alteration of the normal leukocyte functions, found associated with retained placenta in different studies (Gunnink, 1984a, Gunnink, 1984b, Gunnink, 1984c, Gunnink, 1984d; Kimura et al., 2002b), the insufficient number of peripheral leukocytes may reflect a decreased availability of cells for migration into the endometrium. Due to the primary role of the aspecific immune response during transition, attempts to increase the number and functionality of leukocytes at parturition, in order to improve the ability of the cow to prevent clinical production diseases are in course (Kimura et al., 2014).

It is well known that disease prevention requires the adoption of a multidisciplinary team approach involving the farmer, the veterinarian, the nutritional and animal breeding consultants

(Mulligan and Doherty, 2008). The present thesis highlights the important role that the veterinary clinical pathologist may have in the strategic prevention and monitoring of dairy herd diseases. According to this topic, the study reported in **chapter 7** confirmed the utility of the monitoring of blood level of specific metabolic parameters to identify cows at increased risk of disease. In this study, the use of cut-off values for NEFA and BOHB was applied in conjunction with clinical signs in order to identify animals requiring intervention. The absence of significant differences of these metabolites across time in both groups with low and high incidence of diseases, once more highlights the limited power of the adoption of thresholds to identify animals requiring intervention to prevent production diseases. Different thresholds, also called decision limits, concerning NEFA and BOHB have been established experimentally and by consensus, in order to discriminate between individuals or groups of cows with and without subclinical diseases (Opsomer, 2015). However, the analytical variability among methods and instruments used in different laboratories become even more relevant when these thresholds are applied by practitioners which often use cow-side tests that are characterized by lower sensitivity and specificity compared to the gold standards (Oetzel, 2004).

The execution of laboratory tests in dairy herds is often hampered by economical and practical factors that limit the monitoring to only few parameters (namely NEFA and BOHB). Based on the results achieved in this thesis, it could be suggested that, in order to find possible subclinical conditions associated to metabolic, immune and oxidative disturbances during the transition period, a wider panel of analytes, from a consistent number of the herd individuals, should be applied. Moreover, each animal should be considered individually, taking in mind the possible sources of variability deriving from the effect of parity, distance from parturition, season of production, and management of the herd. The combination of this knowledge with those derived from clinicians, nutritionists and farmers is the gold standard in the final monitoring of the health of dairy cows.

In conclusion, the generation of RIs specific for 3±1 days after parturition, characterized by the start of lactation, and 30±3 days from parturition, when dairy cow metabolism is reaching a more stable phase, is of primary importance in the interpretation of laboratory results, since the application of non specific RIs may lead to the incorrect classification of animals as diseased or healthy, in absence of clear clinical or productive issues. All these considerations need to be kept in mind for the proper use of laboratory data in the management of the dairy herd.

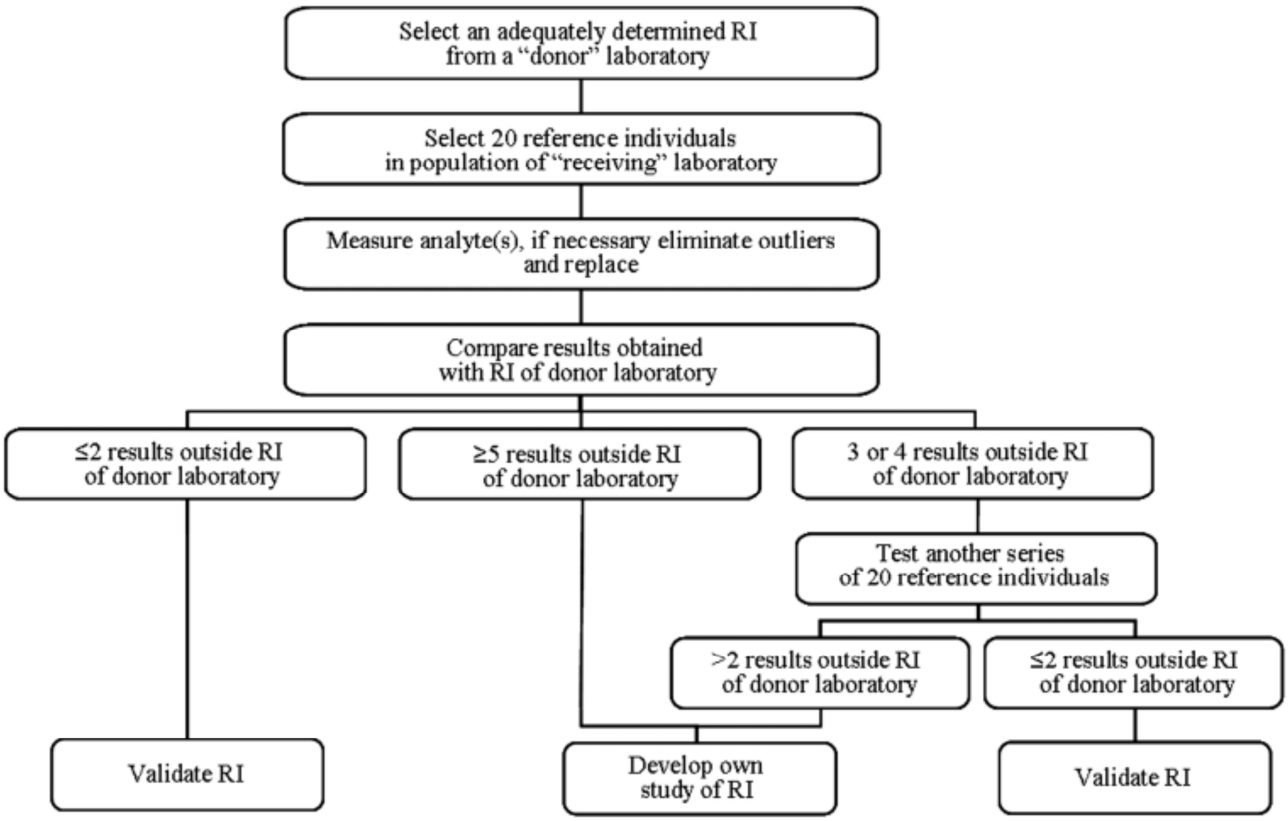

Figure 8.1: Algorithm of actions to validate the RIs provided in the present thesis for the use in other laboratories. From Geffré et al. (2009)

REFERENCES

Arthington, J.D., Qiu, X., Cooke, R.F., Vendramini, J.M.B., Araujo, D.B., Chase, C.C., Coleman, S.W., 2008. Effects of preshipping management on measures of stress and performance of beef steers during feedlot receiving. J. Anim. Sci. 86, 2016–2023.

Barkema, H.W., von Keyserlingk, M.A.G., Kastelic, J.P., Lam, T.J.G.M., Luby, C., Roy, J.-P., LeBlanc, S.J., Keefe, G.P., Kelton, D.F., 2015. Invited review: Changes in the dairy industry affecting dairy cattle health and welfare. J. Dairy Sci. 98 (11), 7426-45.

Beagley, J.C., Whitman, K.J., Baptiste, K.E., Scherzer, J., 2010. Physiology and treatment of retained fetal membranes in cattle. J. Vet. Intern. Med. 24, 261–268.

Bell, A.W., 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J. Anim. Sci. 73, 2804–2819.

Bertoni, G., Trevisi, E., 2013. Use of the Liver Activity Index and other metabolic variables in the assessment of metabolic health in dairy herds. Veterinary Clinics of North America Food Animal Practice: 29(2), 413–431.

Bertoni, G., Trevisi, E., Han, X., Bionaz, M., 2008. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. J. Dairy Sci. 91, 3300–3310.

Bertoni, G., Trevisi, E., Lombardelli, R., 2009. Some new aspects of nutrition, health conditions and fertility of intensively reared dairy cows. Ital. J. Anim. Sci. 8, 491–518.

Bionaz, M., Trevisi, E., Calamari, L., Librandi, F., Ferrari, a, Bertoni, G., 2007. Plasma paraoxonase, health, inflammatory conditions, and liver function in transition dairy cows. J. Dairy Sci. 90, 1740–1750.

Bobe, G, Young, J.W., Beitz, D.C., 2004. Invited review: Pathology, etiology, prevention, and treatment of fatty liver in dairy cows. J Dairy Sci. 87, 3105–3124.

Bochsler, P., Slauson, D., 2002. Inflammation and repair of tissues, in: Slauson, D., Cooper, B. (Eds.), Mechanisms of Disease. Mosby, St. Louis, MO, pp. 140–245.

Bolla, A., Fantini, A., 2003. Incidenza di alcune patologie post-parto nella vacca da latte: risultati preliminari. Atti della Soc. Ital. di Buiat. 25, 361–368.

Boos, A., Janssen, V., Mülling, C., 2003. Proliferation and apoptosis in bovine placentomes during pregnancy and around induced and spontaneous parturition as well as in cows retaining the fetal membranes. Reproduction 126, 469–480.

Bourne, N., Laven, R., Wathes, D.C., Martinez, T., McGowan, M., 2007. A meta-analysis of the effects of Vitamin E supplementation on the incidence of retained foetal membranes in dairy cows. Theriogenology 67, 494–501.

Bradley, P.P., Priebat, D.A., Christensen, R.D., Rothstein, G., 1982. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J. Invest. Dermatol. 78, 206–209.

Braun J.P., Lefebvre H.P., 2008. Kidney function and damage. In: Kaneko J.J., Harvey J.W., Bruss M.L. (eds) Clinical Biochemistry of Domestic Animals. 6th ed, Burlington, MA: Academic Press, 485-528.

Brscic, M., Cozzi, G., Lora, I., Stefani, A.L., Contiero, B., Ravarotto, L., Gottardo, F., 2015. Short communication: Reference limits for blood analytes in Holstein late-pregnant heifers and dry cows: Effects of parity, days relative to calving, and season. J. Dairy Sci. 98, 7886–7892.

Bruss, M.L., 2008. Lipids and Ketones. In: Kaneko JJ, Harvey JW, Bruss ML, eds. Clinical Biochemistry of Domestic Animals. 6th ed. Burlington, MA: Academic Press, 81-115.

Butler, W.R. 2005. Nutrition, negative energy balance and fertility in the postpartum dairy cow. Cattle Pract.13, 13–18.

Cai, T.Q., Weston, P.G., Lund, L.A., Brodie, B., McKenna, D.J., Wagner, W.C., 1994. Association between neutrophil functions and periparturient disorders in cows. Am. J. Vet. Res. 55, 934–943.

Carlson, G.P. and Bruss, M., 2008. Fluid, electrolyte, and acid-base balance. In: Kaneko JJ, Harvey JW, Bruss ML, eds. Clinical Biochemistry of Domestic Animals. 6th ed. Burlington, MA: Academic Press, 529-559

Castañeda-Gutiérrez, E., Pelton, S.H., Gilbert, R.O., Butler, W.R., 2009. Effect of peripartum dietary energy supplementation of dairy cows on metabolites, liver function and reproductive variables. Anim. Reprod. Sci. 112, 301–315.

Chapinal, N., Carson, M., Duffield, T.F., Capel, M., Godden, S., Overton, M., Santos, J.E.P., LeBlanc, S.J., 2011. The association of serum metabolites with clinical disease during the transition period. J. Dairy Sci. 94: 4897–4903.

Chapinal, N., LeBlanc, S.J., Carson, M.E., Leslie, K.E., Godden, S., Capel, M., Santos, J.E.P., Overton, M.W., Duffield, T.F., 2012. Herd-level association of serum metabolites in the transition period with disease, milk production, and early lactation reproductive performance. J. Dairy Sci. 95, 5676–5682

CLSI, Clinical and Laboratory Standards Institute. 2008. Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guidelines. 3rd ed. Wayne, PA

Collard, B.L., Boettcher, P.J., Dekkers, J.C., Petitclerc, D., Schaeffer, L.R., 2000. Relationships between energy balance and health traits of dairy cattle in early lactation. J. Dairy Sci. 83: 2683–2690.

Contreras, G.A., Raphael, W., Mattmiller, S.A., Gandy, J., Sordillo, L.M. 2012. Nonesterified fatty acids modify inflammatory response and eicosanoid biosynthesis in bovine endothelial cells. Journal of Dairy Science 95, 5011–5023.

Cozzi, G., Ravarotto, L., Gottardo, F., Stefani, L., Contiero, B., Moro, L., Brscic, M., Dalvit, P., 2011. Short communication: reference values for blood parameters in Holstein dairy cows: effects of parity, stage of lactation, and season of production. J. Dairy Sci. 94, 3895–901

Curtis, C.R., Erb, H.N., Sniffen, C.J., Smith, R.D., Kronfeld, D.S., 1985. Path analysis of dry period nutrition, postpartum metabolic and reproductive disorders, and mastitis in Holstein cows. J. Dairy Sci. 68, 2347–2360.

Davies, C.J., Fisher, P.J., Schlafer, D.H., 2000. Temporal and regional regulation of major histocompatibility complex class I expression at the bovine uterine/placental interface. Placenta 21, 194–202.

Davies, C.J., Hill, J.R., Edwards, J.L., Schrick, F.N., Fisher, P.J., Eldridge, J.A., Schlafer, D.H., 2004. Major histocompatibility antigen expression on the bovine placenta: its relationship to abnormal pregnancies and retained placenta. Anim. Reprod. Sci. 82-83, 267–280.

Drackley, J.K., 1999. Biology of dairy cows during the transition period: the final frontier? J. Dairy Sci. 82, 2259–2573.

Drackley, J.K., Dann, H.M., Douglas, G.N., et al., 2005. Physiological and pathological adaptations in dairy cows that may increase susceptibility to periparturient diseases and disorders. Ital J. Anim. Sci. 4, 323–44.

Drackley, J., Donkin, S., Reynolds, C., 2006. Major advances in fundamental dairy cattle nutrition. J. Dairy Sci. 89, 1324–1336.

Drackley, J.K., Dann, H.M., Douglas, G.N., Janovick, Guretzky N.A., Litherland, N.B., Underwood, J.P., Loor, J.J., 2010. Physiological and pathological adaptations in dairy cows that may increase susceptibility to periparturient diseases and disorders. Ital. J. Anim. Sci. 4, 323-344.

Dubreuil, P., Lapierre, H., 1997. Biochemistry reference values for Quebec lactating dairy cows, nursing sows, growing pigs and calves. Can. J. Vet. Res. 61, 235–239.

Dubuc, J., Duffield, T.F., Leslie, K.E., Walton, J.S., LeBlanc, S.J., 2010. Risk factors for postpartum uterine diseases in dairy cows. J. Dairy Sci. 93, 5764–5771.

Eckersall, P.D., Bell, R., 2010. Acute phase proteins: Biomarkers of infection and inflammation in veterinary medicine. Vet. J. 185, 23–7.

Enemark, J.M.D., Schmidt, H.B., Jakobsen, J. Enevoldsen, C., 2009. Failure to improve energy balance or dehydration by drenching transition cows with water and electrolytes at calving. Veterinary research communications vol. 33 (2), 123-37

Enjalbert, F., Nicot, M.C., Bayourthe, C., Moncoulon, R., 2001. Ketone bodies in milk and blood of dairy cows: relationship between concentrations and utilization for detection of subclinical ketosis. J. Dairy Sci. 84, 583–589.

Erb, H.N., Smith, R.D., Hillman, R.B., Powers, P.A., Smith, M.C., White, M.E., et al. 1984. Rates of diagnosis of six diseases of Holstein cows during 15-day and 21-day intervals. Am J Vet Res. 45, 333–335.

Esposito, G., Irons, P.C., Webb, E.C., Chapwanya, A., 2014. Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. Anim. Reprod. Sci. 144, 60–71

Ferré, N., Camps, J., Prats, E., Vilella, E., Paul, A., Figuera, L., Joven, J., 2002. Serum paraoxonase activity: a new additional test for the improved evaluation of chronic liver damage. Clin. Chem. 48, 261–268.

Formigoni, A., Cornil, M.C., Prandi, A., Mordenti, A., Rossi, A., Portetelle, D., Renaville, R., 1996. Effect of propylene glycol supplementation around parturition on milk yield, reproduction performance and some hormonal and metabolic characteristics in dairy cows. J. Dairy Res. 63, 11–24.

Fourichon, C., Seegers, H., Malher, X., 2000. Effect of disease on reproduction in the dairy cow: a meta-analysis. Theriogenology 53, 1729–1759.

Friedrichs, K.R., Harr, K.E., Freeman, K.P., Szladovits, B., Walton, R.M., Barnhart, K.F., Blanco-Chavez, J., 2012. ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. Vet. Clin. Pathol. 41, 441–53

Fronk, TJ, Schultz, LH, Hardie, AR., 1980 Effect of dry period overconditioning on subsequent metabolic disorders and performance of dairy cows. J Dairy Sci. 63:1080–90.

Geffré, A., Friedrichs, K., Harr, K., Concordet, D., Trumel, C., Braun, J.P., 2009. Reference values: a review. Vet. Clin. Pathol. 38, 288–98

Geffré, A., Concordet, D., Braun, J.P., et al. 2011. Reference Value Advisor: a new freeware set of macroinstructions to calculate reference intervals with Microsoft Excel. Vet. Clin. Pathol.; 40: 107–112

George, J.W., Snipes, J., Lane, V.M., 2010. Comparison of bovine hematology reference intervals from 1957 to 2006. Vet. Clin. Pathol. 39, 138–48.

Gilbert, R.O., Gröhn, Y.T., Guard, C.L., Surman, V., Neilsen, N., Slauson, D.O., 1993. Impaired post partum neutrophil function in cows which retain fetal membranes. Res. Vet. Sci. 55, 15–19.

Giordano, A., Veronesi, M.C., Rossi, G., Pezzia, F., Probo, M., Giori, L., Paltrinieri, S., 2013. Serum paraoxonase-1 activity in neonatal calves: age related variations and comparison between healthy and sick animals. Vet. J. 197, 499–501.

Goff, J.P., 2006. Major advances in our understanding of nutritional influences on bovine health. J. Dairy Sci. 89, 1292–1301.

Goff, J.P. 2008. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. Vet. J. 176, 50–57

Goff, J.P., Horst, R.L., 1997. Physiological changes at parturition and their relationship to metabolic disorders. Journal of dairy science. 80 (7), 1260-1268.

Goff, J.P., Stabel, J.R., 1990. Decreased plasma retinol, alpha-tocopherol, and zinc concentration during the periparturient period: effect of milk fever. J. Dairy Sci. 73, 3195.

Goff, J.P., Kimura, K., and Horst, R.L., 2002. Effect of mastectomy on milk fever, energy, and vitamin A, E, and β-carotene status at parturition. J. Dairy Sci. 85, 1427–1436

Grummer, R.R., Mashek, D.G., Hayirli, A., 2004. Dry matter intake and energy balance in the transition period. Vet. Clin. N. Am. Food Anim. Pract. 20 (3), 447–470.

Gunnink, J.W., 1984a. Retained placenta and leucocytic activity. Vet. Q. 6, 49–51.

Gunnink, J.W., 1984b. Pre-partum leucocytic activity and retained placenta. Vet. Q. 6, 52–54

Gunnink, J.W., 1984c. Post-partum leucocytic activity and its relationship to caesarian section and retained placenta. Vet. Q. 6, 55–57

Gunnink, J.W., 1984d. Influence of dilution on the chemotactic properties of cotyledon suspensions. Vet. Q. 6, 57–59

Hailemariam, D., Mandal, R., Saleem, F., Dunn, S.M., Wishart, D.S., Ametaj, B.N., 2014. Identification of predictive biomarkers of disease state in transition dairy cows. J. Dairy Sci. 97: 2680–2693.

Hammon, D.S., Evjen, I.M., Dhiman, T.R., Goff, J.P., Walters, J.L., 2006. Neutrophil function and energy status in Holstein cows with uterine health disorders. Vet. Immunol. Immunopathol. 113, 21–29.

Hammon, H. M., G. Stürmer, F. Schneider, A. Tuchscherer, H. Blum, T. Engelhard, A. Genzel, R. Staufenbiel, and W. Kanitz. 2009. Performance and metabolic and endocrine changes with emphasis on glucose metabolism in high-yielding dairy cows with high and low fat content in liver after calving. J. Dairy Sci. 92, 1554–1566.

Harris, E.K., Boyd, J.C., 1990. On dividing reference data into subgroups to produce separate reference ranges. Clin. Chem. 36, 265–70.

Harvey, J.W., 2012. Evaluation of Leukocytic Disorders, in: Harvey, J.W. (Ed.), Veterinary Hematology: A Diagnostic Guide and Color Atlas. Elsevier Saunders, St. Louis, MO, pp. 122–176.

Hemingway, R. G. 2003. The influences of dietary intakes and supplementation with selenium and vitamin E on reproduction diseases and reproductive efficiency in cattle and sheep. Veterinary Research Communications 27, 159 – 174.

Herdt, T.H., 2000. Variability characteristics and test selection in herd-level nutritional and metabolic profile testing. Vet. Clin. North Am. Food Anim. Pract. 16, 387–403

Herdt, T.H., Smith, J.C., 1996. Blood-lipid and lactation-stage factors affecting serum vitamin E concentrations and vitamin E cholesterol ratios in dairy cattle. J. Vet. Diagn. Invest. 8, 228–232

Heuer, C., Schukken, Y.H., Dobbelaar, P., 1999. Postpartum body condition score and results from the first test day milk as predictors of disease, fertility, yield, and culling in commercial dairy herds. J. Dairy Sci. 82: 295–304.

Hoeben, D., Monfardini, E., Opsomer, G., Burvenich, C., Dosogne, H., De Kruif, A., Beckers, J.F., 2000. Chemiluminescence of bovine polymorphonuclear leucocytes during the periparturient period and relation with metabolic markers and bovine pregnancy-associated glycoprotein. J. Dairy Res. 67, 249–59.

Hoedemaker, M., Prange, D., Zerbe, H., Frank, J., Daxenberger, A., Meyer, H.H.D., 2004. Peripartal propylene glycol supplementation and metabolism, animal health, fertility, and production in dairy cows. J. Dairy Sci. 87: 2136–2145.

Hoffman, W.E. and Solter, P.F., 2008, Diagnostic Enzymology of Domestic Animals. In: Kaneko J.J., Harvey J.W., Bruss M.L., eds. Clinical Biochemistry of Domestic Animals. 6th ed. Burlington, MA: Academic Press; 2008:351-378.

Horn, P.S., Pesce, A.J.. Reference intervals: a user's guide, AACC Press, Washington DC, 2005.

Huzzey, J.M., Nydam, D.V, Grant, R.J., Overton, T.R., 2011. Associations of prepartum plasma cortisol, haptoglobin, fecal cortisol metabolites, and nonesterified fatty acids with postpartum health status in Holstein dairy cows. J. Dairy Sci. 94, 5878–5889.

Ingvartsen, K.L., 2006. Feeding-and management-related diseases in the transition cow: physiological adaptations around calving and strategies to reduce feeding-related diseases. Anim. Feed Sci. Technol. 126, 175–213.

Ingvartsen, K., Dewhurst, R., Friggens, N., 2003. On the relationship between lactational performance and health: is it yield or metabolic imbalance that cause production diseases in dairy cattle? A position paper. Livest. Prod. Sci. 83, 277–308.

Johnston, L. A., and Chew, B. P. 1984. Peripartum changes of plasma and milk vitamin A and β-carotene among dairy cows with or without mastitis. Journal of Dairy Science 67, 1832 – 1840.

Jorritsma, R., Wensing T., Kruip T.A.M., Vos P.L.A.M., Noordhuizen J. P. T. M. 2003. Metabolic changes in early lactation and impaired reproductive performance in dairy cows. Vet. Res. 34:11–26

Kaneko, J.J., 2008. Carbohydrate Metabolism and Its Diseases. In: Kaneko JJ, Harvey JW, Bruss ML, eds. Clinical Biochemistry of Domestic Animals. 6th ed. Burlington, MA: Academic Press; 45-80

Kaneko, J.J., Harvey J.W., Bruss M.L. eds. Clinical Biochemistry of Domestic Animals. 6th ed. Burlington, MA: Academic Press; 2008

Kehrli, M.E., Nonnecke, B.J., Roth, J.A., 1989. Alterations in bovine neutrophil function during the periparturient period. Am. J. Vet. Res. 50, 207–14

Kehrli, Jr., M., Neil, J., Burvenich, C., Goff, J., Lippolis, J., Reinhardt, T., Nonnecke, B., 2006. Energy and Protein Effects on the Immune System: Ruminant Physiology: Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress. Wageningen Academic Publishers, The Netherlands, pp. 455–471

Kelton, D.F., Lissemore, K.D., Martin, R.E., 1998. Recommendations for recording and calculating the incidence of selected clinical diseases of dairy cattle. J. Dairy Sci. 81, 2502–2509.

Kimura, K., Goff, J.P., Kehrli, M.E., 1999. Effects of the presence of the mammary gland on expression of neutrophil adhesion molecules and myeloperoxidase activity in periparturient dairy cows. J. Dairy Sci. 82, 2385–92.

Kimura, K., Goff, J.P., Kehrli, M.E., Harp, J., Nonnecke, B.J., 2002a. Effects of mastectomy on composition of peripheral blood mononuclear cell populations in periparturient dairy cows. J. Dairy Sci. 85, 1437–1444.

Kimura, K., Goff, J.P., Kehrli, M.E., Reinhardt, T. A., 2002b. Decreased neutrophil function as a cause of retained placenta in dairy cattle. J. Dairy Sci. 85, 544–550.

Kimura, K., Goff, J.P., Canning, P., Wang, C., Roth, J., 2014. Effect of recombinant bovine granulocyte colony-stimulating factor covalently bound to polyethylene glycol injection on neutrophil number and function in periparturient dairy cows. J. Dairy Sci. 97, 4842–51.

Kinsel, ML, Etherington, WG., 1998. Factors affecting reproductive performance in Ontario dairy herds. Theriogenology 50:1221–38

Kjelgaard-Hansen, M., Jensen, A.L., 2006. Is the inherent imprecision of manual leukocyte differential counts acceptable for quantitative purposes? Vet. Clin. Pathol. 35, 268–70.

Lacetera, N., Scalia, D., Franci, O., Bernabucci, U., Ronchi, B., Nardone, A., 2004. Short communication: Effects of nonesterified fatty acids on lymphocyte function in dairy heifers. J. Dairy Sci. 87:1012–1014.

Laven, R. A., Peters, A. R., 1996. Bovine retained placenta: aetiology, pathogenesis and economic loss. Vet. Rec. 139, 465–471.

LeBlanc, S., 2010. Monitoring metabolic health of dairy cattle in the transition period. J. Reprod. Dev. 56 Suppl, S29–S35.

LeBlanc, S.J., Herdt, T.H., Seymour, W.M., Duffield, T.F., Leslie, K.E., 2004. Peripartum serum vitamin E, retinol, and beta-carotene in dairy cattle and their associations with disease. J. Dairy Sci. 87, 609–619

LeBlanc, S.J., Leslie, K.E., Duffield, T.F., 2005. Metabolic predictors of displaced abomasum in dairy cattle. J. Dairy Sci. 88: 159–170.

Lefebvre, H.P., 2011. Greyhound-specific reference intervals: a good start to a long race. Vet. Clin. Pathol. 40, 405–6

Lomander, H., Frössling, J., Ingvartsen, K.L., Gustafsson, H., Svensson, C., 2012. Supplemental feeding with glycerol or propylene glycol of dairy cows in early lactation--effects on metabolic status, body condition, and milk yield. J. Dairy Sci. 95: 2397–2408.

Lucy, M., Jiang, H., Kobayashi, Y., 2001. Changes in the somatotrophic axis associated with the initiation of lactation. J. Dairy Sci. 84, E113–E119.

Lumsden, JH, Mullen, K, Rowe, R.. 1980. Hematology and biochemistry reference values for female holstein cattle. Can J Comp Med, 44: 24–31

Martinez, N., Risco, C., Lima, F.S., Bisinotto, R.S., Greco, L.F., Ribeiro, E.S., Maunsell, F., Galvão, K., Santos, J.E.P., 2012. Evaluation of peripartal calcium status, energetic profile, and neutrophil function in dairy cows at low or high risk of developing uterine disease. J. Dairy Sci. 95, 7158–72

Matthews, B.J., Morton, J.M., 2012. Accuracy of predicted calving dates in Holstein-Friesian dairy cows based on fetal ages estimated using manual rectal palpation. N. Z. Vet. J. 60: 234–240.

McArt, J.A.A., Nydam, D.V, Ospina, P.A., Oetzel, G.R., 2011. A field trial on the effect of propylene glycol on milk yield and resolution of ketosis in fresh cows diagnosed with subclinical ketosis. J. Dairy Sci. 94: 6011–6020.

McArt, J.A.A., Nydam, D.V, Oetzel, G.R., 2012. A field trial on the effect of propylene glycol on displaced abomasum, removal from herd, and reproduction in fresh cows diagnosed with subclinical ketosis. J. Dairy Sci. 95: 2505–12.

McArt, J.A.A., Nydam, D.V, Oetzel, G.R., Overton, T.R., Ospina, P.A., 2013. Elevated non-esterified fatty acids and β-hydroxybutyrate and their association with transition dairy cow performance. Vet. J. 198: 560–570.

Melendez, P., Donovan, G.A., Risco, C.A., Goff, J.P., 2004. Plasma mineral and energy metaboite concentrations in dairy cows fed an anionic prepartum diet that did or did not have retained fetal membranes after parturition. Am. J. Vet. Res. 65, 1071–1076.

Miettinen, P.V.A., 1995. Prevention of bovine ketosis with glucogenic substance and its effect on fertility in Finnish dairy cows. Berl. Munch. Tierarztl. Wochenschr. 108, 14–19

Mittal, M., Siddiqui, M.R., Tran, K., Reddy, S.P., Malik, A.B., 2014. Reactive oxygen species in inflammation and tissue injury. Antioxid. Redox Signal. 20, 1126–1167.

Moretti, P., Probo, M., Morandi, N., Trevisi, E., Ferrari, A., Minuti, A., Venturini, M., Paltrinieri, S., Giordano, A., 2015. Early post-partum hematological changes in Holstein dairy cows with retained placenta. Anim. Reprod. Sci. 152, 17–25.

Morrow, D.A., 1976. Fat cow syndrome. J. Dairy Sci. 59, 1625–1629.

Moyes, K.M., Larsen, T., Friggens, N.C., Drackley, J.K., Ingvartsen, K.L., 2009. Identification of potential markers in blood for the development of subclinical and clinical mastitis in dairy cattle at parturition and during early lactation. J. Dairy Sci. 92, 5419–5428.

Mulligan, F.J., O'Grady, L., Rice, D.A., Doherty, M.L., 2006. A herd health approach to dairy cow nutrition and production diseases of the transition cow. Anim. Reprod. Sci.: 331–353.

Mulligan, F.J., Doherty, M.L., 2008. Production diseases of the transition cow. Vet. J. 176, 3–9.

National Research Council, 2001. Nutrient Requirements of Dairy Cattle, 7 revised. ed. National Academy Press, Washington, DC.

Nonnecke, B.J., Kimura, K., Goff, J.P., Kehrli, M.E., 2003. Effects of the mammary gland on functional capacities of blood mononuclear leukocyte populations from periparturient cows. Journal of Dairy Science 86, 2359–2368.

Oetzel, G.R., 2004. Monitoring and testing dairy herds for metabolic disease. Vet. Clin. North Am. Food Anim. Pract. 20, 651–74.

Opsomer, G., 2015. Interaction between metabolic challenges and productivity in high yielding dairy cows. Jpn. J. Vet. Res. 63 Suppl 1, S1–14.

Ospina, P.A., Nydam, D. V, Stokol, T., Overton, T.R., 2010. Evaluation of nonesterified fatty acids and beta-hydroxybutyrate in transition dairy cattle in the northeastern United States: Critical thresholds for prediction of clinical diseases. J. Dairy Sci. 93, 546–554.

Overton, T.R., Waldron, M.R., 2004. Nutritional Management of Transition Dairy Cows: Strategies to Optimize Metabolic Health. J. Dairy Sci. 87: E105–E119.

Paisley, L.G., Mickelsen, W.D., Anderson, P.B., 1986. Mechanisms and therapy for retained fetal membranes and uterine infections of cows: A review. Theriogenology 25, 353–381.

Paltrinieri, S., Ibba, F., Rossi, G., 2014. Hematological and biochemical reference intervals of four feline breeds. Journal of Feline Medicine and Surgery, 16, pp. 125–136

Papich, M., Riviere, J., 2009. Chemotherapy of microbial diseases, in: Riviere, J., Papich, M. (Eds.), Veterinary Pharmacology and Therapeutics. Wiley-Blackwell, Ames, IA, pp. 835–1013.

Parker Gaddis, K., Cole, J., Clay, J., Maltecca, C., 2012. Incidence validation and relationship analysis of producer-recorded health event data from on-farm computer systems in the United States. J. Dairy Sci. 95: 5422–5435.

Peeler, E., Otte, M., Esslemont, R., 1994. Inter-relationships of periparturient diseases in dairy cows. Vet. Rec. 134: 129–132.

Peter, A.T., Bosu, W.T.K., 1987. Peripartal endocrine changes associated with retained placenta in dairy cows. Theriogenology 28, 383–394.

Petersen, H.H., Nielsen, J.P., Heegaard, P.M.H., 2004. Application of acute phase protein measurements in veterinary clinical chemistry. Vet. Res. 35, 163–187

Quiroz-Rocha, G.F., LeBlanc, S.J., Duffield, T.F., Wood, D., Leslie, K.E., Jacobs, R.M., 2009. Reference limits for biochemical and hematological analytes of dairy cows one week before and one week after parturition. Can. Vet. J. 50, 383–388.

Raboisson, D., Mounié, M., Maigné, E., 2014. Diseases, reproductive performance, and changes in milk production associated with subclinical ketosis in dairy cows: A meta-analysis and review. J. Dairy Sci. 97, 7547–7563.

Ramberg C.F., Mayer G.P., Kronfeld D.S., Phang J.M., Berman M., 1970. Calcium kinetics in cows during late pregnancy, parturition, and early lactation. Am J Physiol. 219, 1166–1177.

Raphael, B.C., Dimick, P.S., Puppione, D.L., 1973. Lipid characterization of bovine serum lipoproteins throughout gestation and lactation. J. Dairy Sci. 56, 1025–32

Roland, L., Drillich, M., Iwersen, M., 2014. Hematology as a diagnostic tool in bovine medicine. J. Vet. Diagn. Invest. 26, 592–598.

Rosol, T.J. and Capen, C.C., 1997. Calcium-regulating hormones and diseases of abnormal mineral (calcium, phosphorous, magnesium) metabolism. In: Kaneko JJ, Harvey JW, Bruss ML, eds. Clinical Biochemistry of Domestic Animals. 5th ed. London: Academic Press 610-702

Roussel, J.D., Aranas, T.J., Seybt S.H., 1982. Metabolic profile testing in holstein cattle in Louisiana reference values. Am. J. Vet. Res. 43:1658–1660.

Russell, K.E., Roussel, A.J., 2007. Evaluation of the Ruminant Serum Chemistry Profile. Vet. Clin. North Am. - Food Anim. Pract. 23, 403–426.

Sartorelli, P., Paltrinieri, S., Agnes, F., 1999. Non-specific immunity and ketone bodies. I: In vitro studies on chemotaxis and phagocytosis in ovine neutrophils. Zentralbl. Veterinarmed. A 46, 613–619.

Sartorelli, P., Paltrinieri, S., Comazzi, S., 2000. Non-specific immunity and ketone bodies. II: In vitro studies on adherence and superoxide anion production in ovine neutrophils. J. Vet. Med. A. Physiol. Pathol. Clin. Med. 47, 1–8.

Scalia, D., Lacetera, N., Bernabucci, U., Demeyere, K., Duchateau, L., Burvenich, C., 2006. In vitro effects of nonesterified fatty acids on bovine neutrophils oxidative burst and viability. J. Dairy Sci. 89, 147–154.

Schlafer, D.H., Fisher, P.J., Davies, C.J., 2000. The bovine placenta before and after birth: placental development and function in health and disease. Anim. Reprod. Sci. 60-61, 145–160.

Seifi, H.A., Dalir-Naghadeh, B., Farzaneh, N., Mohri, M., Gorji-Dooz, M., 2007. Metabolic changes in cows with or without retained fetal membranes in transition period. J. Vet. Med. A. Physiol. Pathol. Clin. Med. 54, 92–97.

Shaver, R., 1997. Nutritional risk factors in the etiology of left displaced abomasum in dairy cows: a review. J. Dairy Sci. 80: 2449–2453.

Sheldon, M.I., Williams, E.J., Miller, A., Nash, D.M., Herath, S., 2008. Uterine diseases in cattle after parturition. The Veterinary Journal 176 (1), 115–121

Shi, X., Li, X., Li, D., Li, Y., Song, Y., Deng, Q., Wang, J., Zhang, Y., Ding, H., Yin, L., Wang, Z., Liu, G., 2014. Beta-hydroxybutyrate activates the NF-kappaB signaling pathway to promote the expression of pro-inflammatory factors in calf hepatocytes. Cellular Physiology and Biochemistry: International Journal of Experimental Cellular Physiology. Biochemical Pharmacology 33, 920–932.

Skinner, J.G., Brown, R.A., Roberts, L., 1991. Bovine haptoglobin response in clinically defined field conditions. Vet. Rec. 128, 147–149.

Smith, K. L., Hogan, J. S., & Weiss, W. P. 1997. Dietary vitamin E and selenium affect mastitis and milk quality. Journal of Animal Science 75, 1659 – 1665

Sordillo, L.M., Aitken, S.L., 2009. Impact of oxidative stress on the health and immune function of dairy cattle. Vet. Immunol. Immunopathol. 128, 104–109.

Sordillo, L.M., Mavangira, V., 2014. The nexus between nutrient metabolism, oxidative stress and inflammation in transition cows. Anim. Prod. Sci. 1204–1214

Sordillo, L.M., Raphael, W., 2013. Significance of Metabolic Stress, Lipid Mobilization, and Inflammation on Transition Cow Disorders. Vet. Clin. North Am. Food Anim. Pract. 29, 267–278.

Sordillo, L.M., O'Boyle, N., Gandy, J.C., Corl, C.M., Hamilton, E., 2007. Shifts in thioredoxin reductase activity and oxidant status in mononuclear cells obtained from transition dairy cattle. J. Dairy Sci. 90, 1186–1192.

Stengärde, L., Holtenius, K., Tråvén, M., Hultgren, J., Niskanen, R., Emanuelson, U., 2010. Blood profiles in dairy cows with displaced abomasum. J. Dairy Sci. 93: 4691–4699.

Ster, C., Loiselle, M.C., Lacasse, P. 2012. Effect of postcalving serum nonesterified fatty acids concentration on the functionality of bovine immune cells. Journal of Dairy Science 95, 708–717.

Stockham, S.L., Scott, M.A., 2008a. Introductory concepts, in: Stockham, S.L., Scott, M.A. (Eds.), Fundamentals of Veterinary Clinical Pathology. Blackwell Publishing Ltd, Ames, IA, pp. 3–52

Stockham, S.L., Scott, M.A., 2008b. Leukocytes, in: Stockham, S.L., Scott, M.A. (Eds.), Fundamentals of Veterinary Clinical Pathology. Blackwell Publishing Ltd, Ames, IA, pp. 53–106.

Stockham, S.L., Scott, M.A., 2008c. Lipids, in: Stockham, S.L., Scott, M.A. (Eds.), Fundamentals of Veterinary Clinical Pathology. Blackwell Publishing Ltd, Ames, IA, pp. 763–782.

Stockham, S.L., Scott, M.A., 2008d. Enzymes, in: Stockham, S.L., Scott, M.A. (Eds.), Foundamentals of Veterinary Clinical Pathology. Blackwell Publishing Ltd, Ames, IA, pp. 639–674.

Stockham, S.L., Scott, M.A., 2008e. Liver function, in: Stockham, S.L., Scott, M.A. (Eds.), Fundamentals of Veterinary Clinical Pathology. Blackwell Publishing Ltd, Ames, IA, pp. 675–706.

Stockham, S.L., Scott, M.A., 2008f. Platelets. In: Stockham, S.L., Scott, M.A. (Eds.), Fundamentals of Veterinary Clinical Pathology. Blackwell Publishing Ltd., Ames, IA, pp. 223-257

Stockham, S.L., Scott, M.A., 2008g. Proteins. In: Stockham, S.L., Scott, M.A. (Eds.), Fundamentals of Veterinary Clinical Pathology. Blackwell Publishing Ltd., Ames, IA, pp. 369-413

Sunderman, F.W., Nomoto, S., 1970. Measurement of human serum ceruloplasmin by its p-phenylenediamine oxidase activity. Clin. Chem. 16, 903–910.

Tornquist, S.J., Rigas, J., 2010. Interpretation of Ruminant Leukocyte Responses, in: Weiss, D.J., Wardrop, K.J. (Eds.), Shalm's Veterinary Hematology. Blackwell Publishing Ltd, Ames, IA, pp. 307–313.

Trevisi, E., Ferrari, A. R., Bertoni, G., 2008. Productive and metabolic consequences induced by the retained placenta in dairy cows. Vet. Res. Commun. 32 Suppl 1, S363–366.

Trevisi, E., Amadori, M., Bakudila, A.M., Bertoni, G., 2009. Metabolic changes in dairy cows induced by oral, low-dose interferon-alpha treatment. J. Anim. Sci. 87, 3020–3029.

Trevisi, E., Amadori, M., Cogrossi, S., Razzuoli, E., Bertoni, G., 2012. Metabolic stress and inflammatory response in high-yielding, periparturient dairy cows. Res. Vet. Sci. 93, 695–704.

Trevisi, E., G. Bertoni, R. Lombardelli, Minuti, A., 2013. Relation of inflammation and liver function with the plasma cortisol response to ACTH in early lactating dairy cows. J. Dairy Sci. 96:5712–5722.

Turk, R., Juretic, D., Geres, D., Turk, N., Rekic, B., Simeon-Rudolf, V., Svetina, A., 2004. Serum paraoxonase activity and lipid parameters in the early postpartum period of dairy cows. Res. Vet. Sci. 76, 57–61.

Turk, R., Juretic, D., Geres, D., Turk, N., Rekic, B., Simeon- Rudolf, V., Robic, M., Svetina, A., 2005. Serum paraoxonase activity in dairy cows during pregnancy. Res. Vet. Sci. 79, 15–18.

van der Veen, B.S., de Winther, M.P.J., Heeringa, P., 2009. Myeloperoxidase: molecular mechanisms of action and their relevance to human health and disease. Antioxid. Redox Signal. 11, 2899–2937.

van Dorland, H.A., Richter, S., Morel, I., Doherr, M. G., Castro, N., Bruckmaier, R. M., 2009. Variation in hepatic regulation of metabolism during the dry period and in early lactation in dairy cows. J. Dairy Sci. 92:1924–1940.

Voyvoda, H., Erdogan, H., 2010. Use of a hand-held meter for detecting subclinical ketosis in dairy cows. Res. Vet. Sci. 89, 344–351.

Wathes, D., Cheng, Z., Bourne, N., Taylor, V., Coffey, M., Brotherstone, S., 2007. Differences between primiparous and multiparous dairy cows in the inter-relationships between metabolic traits, milk yield and body condition score in the periparturient period. Domest. Anim. Endocr. 33, 203–225.

Wensing, T., Kruip, T., Geelen, M.J.H., Wentink, G.H., van den Top, A.M., 1997. Postpartum fatty liver in high-producing dairy cows in practice and in animal studies. The connection with health, production and reproduction problems. Comp. Haematol. Int. 7, 167–171.

Wilde, D., 2006. Influence of macro and micro minerals in the peri-parturient period on fertility in dairy cattle. Anim. Reprod. Sci. 96, 240–249.

Zerbe, H., Schneider, N., Leibold, W., Wensing, T., Kruip, T.A., Schuberth, H.J., 2000. Altered functional and immunophenotypical properties of neutrophilic granulocytes in postpartum cows associated with fatty liver. Theriogenology 54, 771–786.

ANNEX:

Scientific production during the PhD

ANNEX: SCIENTIFIC PRODUCTION DURING THE PhD

The list of the publications produced during the course of this thesis is presented in the following section. Concerning the aim developed in **chapter 5**, related to the establishment of RIs in cows, one oral communication and one poster (**publication 2.3 and 3.1**, respectively) were produced, and a manuscript is under submission (**publication 4.1)**. Related to the same topic, but with the application to a wild species, a poster (**publication 3.2**) concerning the establishment of RI for Egyptian fruit bats was presented at the 17th ESVCP annual congress. The topic presented in **chapter 6**, concerning the peri-partum laboratory changes associated with retained placenta, was the subject of one peer reviewed article (**publication 1.4**) and two oral communications (**publication 2.1 and 2.2**). The last topic, presented in **chapter 7**, concerning the prevention of production diseases in dairy cows, was the subject of a peer reviewed article (**publication 1.1**) and a poster (**publication 3.3**).

Besides the main topics addressed in this thesis, during this PhD course I dealt also with some aspects of veterinary clinical pathology in calves. In particular, a peer reviewed article concerning the hematological profile of calves associated with different mode of delivery was produced (**publication 1.7**). Other two studies, one concerning the biochemical profiles of calves associated with different mode of delivery (**publication 4.2**) and another one aimed to elucidate the characteristics of calves lipidograms and his correlation with inflammatory conditions, are under submission (**pubblication 4.3)**.

A latere of the above mentioned research activities, during this PhD course, due to the involvement in the clinical pathology laboratory diagnostic service at the Large Animal Hospital (Azienda "Polo Veterinario di Lodi"), I dealt also with some aspects of veterinary clinical pathology in small animals. In particular, concerning different aspects of veterinary oncology, three peer reviewed articles (**publication 1.2, 1.3 and 1.6**), one oral communication (**publication 2.4**) and one poster (**publication 3.5**) were produced. Finally I've actively participated to the preparation of a manuscript concerning biochemical variations associated with contrast medium administration in dogs (**publication 1.5**), and to the preparation of a poster concerning the method validation for paraoxonase-1 determination in feline samples (**publication 3.4).**

List of publications

1. Peer-reviewed publications

1.1. Coiatelli M., Giordano A., Sicilia F., **Moretti P.**, Durel L. An attempt to prevent production diseases in dairy cows by intense monitoring and ad hoc treatment. *Italian Journal of Animal Science.* 2015; 14:3918.

1.2. **Moretti, P.**, Giordano, A., Stefanello, D., Ferrari, R., Castellano, S., Paltrinieri, S.: Nucleated erythrocytes in blood smears of dogs undergoing chemotherapy. *Veterinary and Comparative Oncology*. Epub ahead of print. 2015 n/a–n/a. doi:10.1111/vco.12156

1.3. Marconato, L., Martini, V., Stefanello, D., **Moretti, P.,** Ferrari, R., Comazzi, S., Laganga, P., Riondato, F., Aresu, L.: Peripheral blood lymphocyte/monocyte ratio as a useful prognostic factor in dogs with diffuse large B-cell lymphoma receiving chemoimmunotherapy. *The Veterinary. Journal*. 2015. Epub ahead of print. doi:10.1016/j.tvjl.2015.07.009

1.4. **Moretti, P.**, Probo, M., Morandi, N., Trevisi, E., Ferrari, A., Minuti, A., Venturini, M., Paltrinieri, S., Giordano, A.: Early post-partum hematological changes in Holstein dairy cows with retained placenta. *Animal Reproduction Science*. 2015, 152, 17–25

1.5. Carotenuto A. M., Borghi L., Paltrinieri S., Giordano A., **Moretti P.**, Di Giancamillo M.:Serum biochemical response to contrast media administration in anaesthetised dogs. *Veterinary Record.* 2013, 172(4): 101

1.6. Paltrinieri S, Rossi G., Meregalli A., Stefanello D., Pecile A., **Moretti P**, Rondena M.: Sialic acid and sialyltransferase activity in serum and tissues of dogs with mammary tumors. *Veterinary Pathology*. July 2012, vol 49 n.4, pages 669-681

1.7. Probo M., Giordano A., Opsomer G., Fiems L., **Moretti P.**, Cairoli F.: Mode of delivery is associated with different hematological profiles in new born calf. *Theriogenology*. 2012, vol. 77, issue 55. pages 865-872

2. Oral communications at international congresses (*presented as speaker)

2.1. **Moretti P.**[*], Cantoni A., Probo M., Paltrinieri S., Giordano A.: Hematological changes around parturition in dairy cows with and without retained placenta. 16th ECVCP and ESVCP annual congress, Milan, Oct 1th-14th 2014

2.2. **Moretti P.**[*], Giordano A., Venturini M., Morandi N., Paltrinieri S.: Hematological changes associated with retained placenta in cattle. 15th congress ISACP-14th congress ESVCP. Ljiubljana (SLO) 3rd-7th July, 2012

2.3. Paltrinieri S., **Moretti P.**, Venturini M., Morandi N, Giordano A.: Association between hematological and electrophoretic profiles and prevalence of post-partum disease in dairy cows. 15th congress ISACP-14th congress ESVCP. Ljiubljana (SLO) 3rd-7th July, 2012

2.4. Giordano A., Stefanello D., Pastore M., Ferrari R., Giori L., **Moretti P.**, Paltrinieri S.: Lactate dehydrogenase (LDH) isoenzymes in dogs affected by lymphoma. 15th congress ISACP-14th congress ESVCP. Ljiubljana (SLO) 3rd-7th July, 2012

3. Posters at international congress

3.1. **Moretti P.**, Paltrinieri S., Probo M., Giordano A.: Laboratory reference intervals of holstein lactating cows at 3 and 30 days post-partum. 17th ESVCP annual congress, Lisbon, Sep 9th-12th 2015

3.2. Giordano A., Ravasio G., **Moretti P.**, Moretti P., Di Cesare F., Paltrinieri S., Pecile A.: Hematological, serum biochemical and electrophoretic values in captive Egyptian fruit bats (Rousettus Aegyptiacus). 17th ESVCP annual congress, Lisbon, Sep 9th-12th 2015

3.3. Coiatelli M., Giordano A., Sicilia F., **Moretti P.**, Durel L.: Prevention of production diseases in dairy cows by intense monitoring and ad hoc treatment. Preliminary results. 53rd annual meeting of the national mastitis council at Fourth Worth, Texas (USA), 26-28th January, 2014

3.4. Rossi G., Giordano A., Costarelli E., **Moretti P.**, Paltrinieri S.: Analytical validation of a paraoxon-based method to measure the activity of paraoxonase-1 in feline serum. 2014 ACVP and ASVCP annual meeting, Atlanta, Nov 8th-12th, 2014

3.5. **Moretti P.**, Ferrari R., Castellano S., Giordano A., Stefanello D., Paltrinieri S.: Nucleated erythrocytes in blood smears of dogs on chemotherapy. 15th ESVCP and ECVCP annual congress Berlin, Germany November 6th–9th November 2013

4. Manuscripts under submission

4.1. **Moretti P.**, Paltrinieri S.,Trevisi E., Probo M., Ferrari A., Minuti A., Giordano A. Reference intervals for hematological and biochemical parameters, acute phase proteins, and markers of oxidative stress in holstein dairy cows at 3 and 30 days post-partum. Submitted to *Journal of dairy science*

4.2. Probo M., Giordano A., **Moretti P.**, Opsomer G., Fiems L., Veronesi M.C.: Serum biochemical profile in newborn calves; a comparison between Belgian Blue and Holstein Friesian calves in the first 48 hours of age. Submitted to *The Veterinary Journal*.

4.3. Giordano A., Rossi G., **Moretti P.**, Probo M., Paltrinieri S.: Biochemical and electrophoretic evaluation of high density lipoproteins (HDL) in healthy neonatal calves: comparison with results of adult cows and of calves with inflammatory conditions. Submitted to *Acta Vecterinaria Scandinavica*.