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Abstract: Retained placenta (RP) occurs frequently in dairy cattle but little is known about the pathogenic or prognostic role of the hematological changes in this disease. This retrospective study was designed to investigate the hematological changes associated with RP in the immediate post-partum period and to assess whether these changes are associated with an acute phase reaction. Data concerning hematology, acute phase protein, marker of inflammation and biochemical profile performed on cows at 3±1 days in milk (DIM) from two intensive farms were extracted from the database of the ProZoo project, a research project aimed to investigate the relationship between genomic traits and bovine health and production. After application of restrictive inclusion criteria, data about 45 cows, 22 with RP and 23 controls, were statistically compared. RBC count, d-ROMs concentration, and AST activity were significantly higher in the RP group than controls. Conversely, neutrophils, thiol groups, and serum zinc concentration were significantly lower in the RP group than controls. In conclusion, although retained placenta has to be considered as a syndrome with multifactorial causes, neutropenia may be a co-factor involved in its pathogenesis. Further studies are needed to clarify whether neutropenia act as a contributor in the pathogenesis of RP or if it is a very early consequence of the syndrome, 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.

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- Information on hematological changes associated with retained placenta are scarce
- We retrospectively examined hematological changes associated with retained placenta
- Neutropenia is associated with retained placenta in cattle
- No changes of acute phase proteins are present when neutropenia occurs
- Neutropenia may be a co-factor involved in the pathogenesis of retained placenta

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EARLY POST PARTUM HEMATOLOGICAL CHANGES IN HOLSTEIN DAIRY COWS WITH RETAINED PLACENTA

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Abstract

Retained placenta (RP) occurs frequently in dairy cattle but little is known about the pathogenic or prognostic role of the hematological changes in this disease. This retrospective study was designed to investigate the hematological changes associated with RP in the immediate post-partum period and to assess whether these changes are associated with an acute phase reaction. Data concerning hematology, acute phase protein, marker of inflammation and biochemical profile performed on cows at 3±1 days in milk (DIM) from two intensive farms were extracted from the database of the ProZoo project, a research project aimed to investigate the relationship between genomic traits and bovine health and production. After application of restrictive inclusion criteria, data about 45 cows, 22 with RP and 23 controls, were statistically compared. RBC count, d-ROMs concentration, and AST activity were significantly higher in the RP group than controls. Conversely, neutrophils, thiol groups, and serum zinc concentration were significantly lower in the RP group than controls. In conclusion, although retained placenta has to be considered as a syndrome with multifactorial causes, neutropenia may be a co-factor involved in its pathogenesis. Further studies are needed to clarify whether neutropenia act as a contributor in the pathogenesis of RP or if it is a very early consequence of the syndrome, 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.

Keywords: dairy cows, retained fetal membranes, peripheral neutrophils, neutropenia, acute phase proteins

1. Introduction

Retention of fetal membranes or retained placenta (RP) occurs frequently in high-yielding dairy cows and has been proved to cause negative effects on reproductive performances (Kelton et al., 1998). A worldwide survey by Kelton et al. (1998) estimates an incidence 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, although 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 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; 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., 2002; 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., 2002). 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 (BHBA), 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 have 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 on hematological changes occuring soon after parturition in affected cows.

We designed this retrospective study 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.

2. Material and methods

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 visit (transrectal uterine palpation and ultrasonography) was conducted at 30 DIM on all the cows.

The database was searched 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 treatment administered 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.

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. Sample 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.

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.

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 instrument, 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) and β-hydroxybutyrate (BHBA, D-3-Hydroxybutyrate dehydrogenase method, Randox Laboratories Ltd., Crumlin, Co. Antrium, UK).

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).

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.

3. Results

3.1 Case selection and characteristics of the study population

Results of the retrospective search in the database are reported in figure 1. As shown in the figure, 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 by 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 by 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 control 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)

196 was performed in 15 cows with RP whereas a combination of manual removal of the fetal
197 membranes and intrauterine administration of 1.2 g of rifaximin (Fatroximin Pessari, Fatro s.p.a.,
198 Ozzano dell'Emilia, Bologna, Italy) was performed in 5 cows with RP. All the treatments were
199 administered on the same day of blood collection.
200 On the basis of the 30 DIM gynecological visit all animals with RP recovered completely.
201
202 *3.2 Hematology and clinical chemistry*
203
204 Results recorded at 3±1 DIM are reported in Figure 2 and revealed that RBC were significantly
205 higher in cows affected by RP compared with CTRL cows but values were within the reference
206 intervals in both groups. Conversely, neutrophils were significantly lower in the RP affected group,
207 in which about one third of the cows had values lower than the lower limit of the reference interval,
208 compared with controls, which, conversely, had values included in the reference interval, with rare
209 exceptions. No significant differences were found for total WBC, lymphocytes, and monocytes
210 (Fig. 1). Band neutrophils, basophils and eosinophils were only occasionally seen in both groups
211 but the total number of these cells was always within the reference interval, without differences
212 between the two groups (data not shown). Similarly, no significant differences regarding the
213 markers of inflammation and oxidation were recorded between CTRL and RP cows, except for a
214 significant increase of d-ROMs and for a significant decrease of thiol groups, which, however,
215 largely remained within the reference intervals in both groups (Fig. 1). Biochemistry was also
216 unremarkable, without significant differences between groups except for AST activity, which was
217 significantly higher in the RP group, and serum zinc concentration, that was significantly lower in
218 the RP group. However, for both these analytes values were largely within the reference intervals in
219 both groups. Interestingly, no significant differences between the groups were found regarding the
220 analytes that are considered as risk factors for RP such as Calcium, NEFA and BHBA.
221

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 function (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 latter may be particularly important in the management of animals that are at risk for or are affected by RP, since the depression of neutrophil function and the inflammatory state, both associated 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 to increased concentration of albumin and total proteins that, however, 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 allow to identify peculiar abnormalities at 3±1 DIM in animals with RP, in agreement with what 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 BHBA 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 function may be found only when severe increases of NEFA and BHBA 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. 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 to 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 (e.g. 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, 2008), significantly increased in cattle with RP compared with CTRL cows; anyway 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 it 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 not exceeding 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.

Conflict of interest statement

The Authors do not have any conflict of interest potentially influencing the results of this study

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Figure captions

Figure 1: flowchart summarizing the results of the retrospective search in the database and the final composition of the study group.

Figure 2: Results regarding hematological parameters, inflammatory markers and biochemical parameters of potential interest for placental retention or showing significant differences between groups. The figure displays the comparison between animals with retained placenta (RP) and controls (CTRL). 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. 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 1

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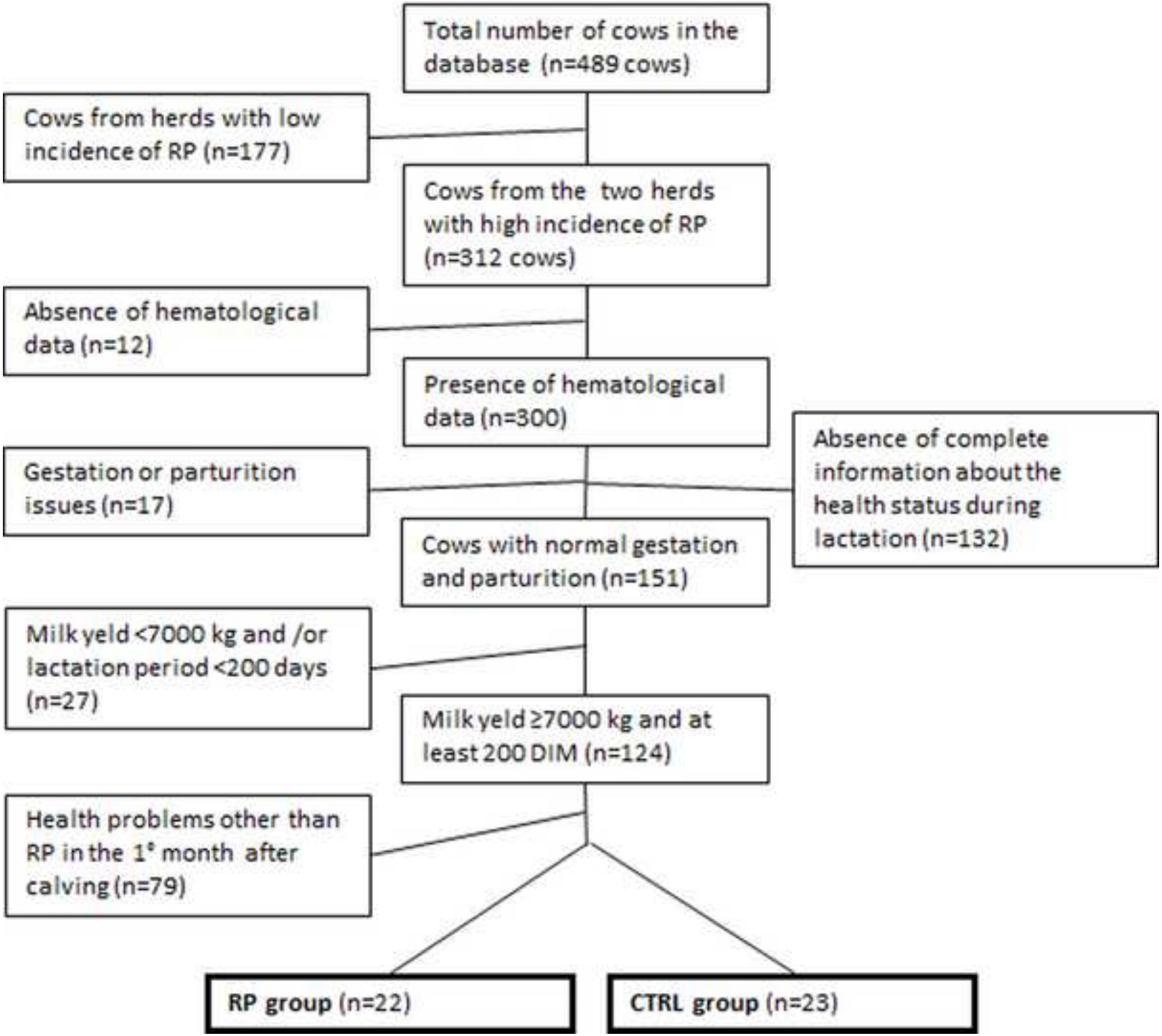

Figure 2

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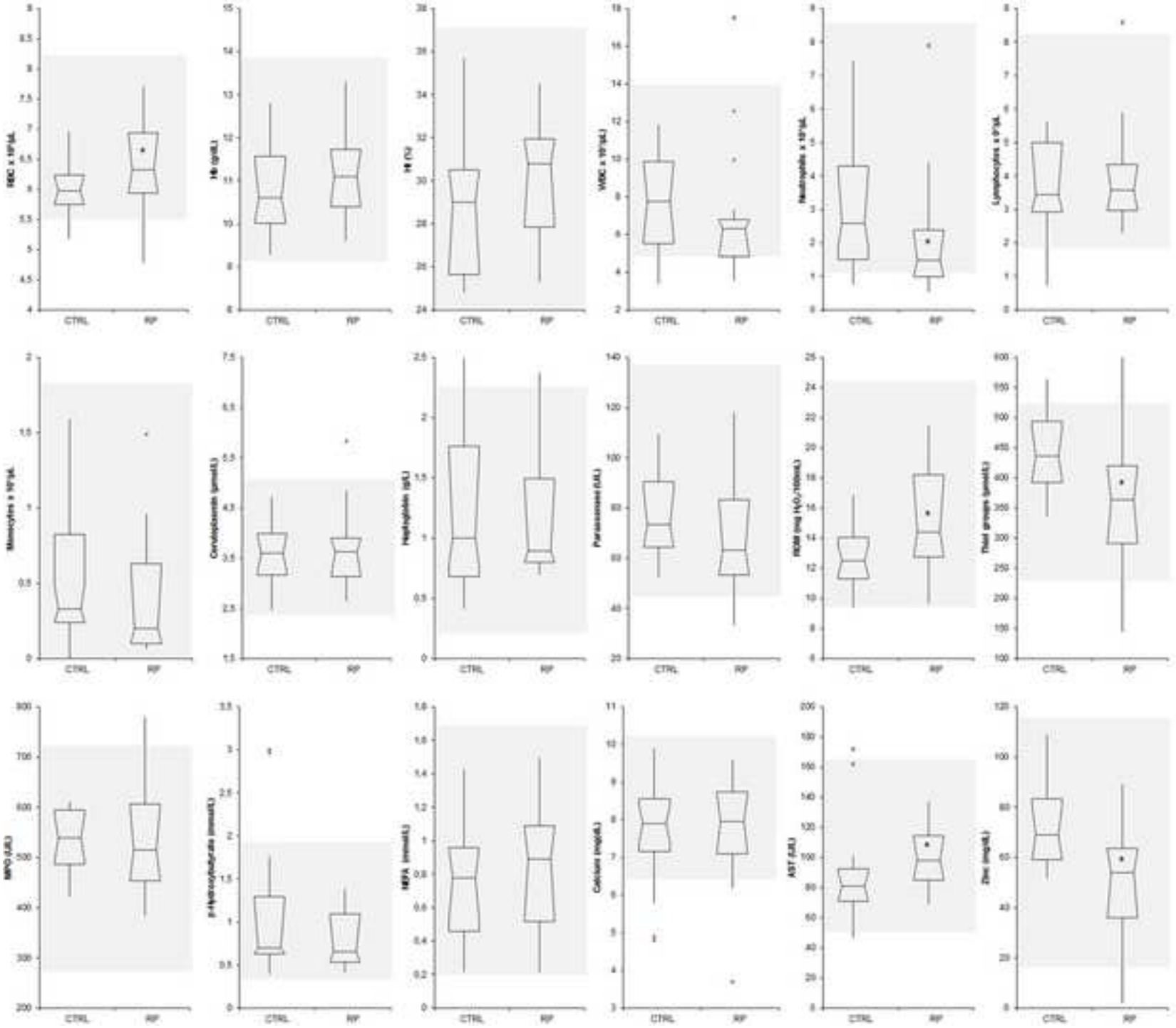

*Conflict of Interest Statement

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EARLY POST PARTUM HEMATOLOGICAL CHANGES IN HOLSTEIN DAIRY COWS WITH RETAINED PLACENTA

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We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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