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Early Detection of Bovine Respiratory Disease and Evaluation of the Efficacy of NSAID in Pre-Weaned Calves

Usefulness of the parameters: activity, rectal temperature, differential count on leukocytes, α_2- and γ-globulin fractions, in early diagnostics

Master Thesis in Veterinary Medicine

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Authors: Helene Ahlman Nielsen, lms914 and Dortea Dencker Hansen, zwv876

Supervisor: Associate Professor Nynne Capion

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Name of department:	Department of Veterinary Clinical Sciences
Faculty:	Faculty of Health and Medical Science, Copenhagen University
Authors:	BSc.med.vet. Helene Ahlman Nielsen, lms914 BSc.med.vet. Dortea Dencker Hansen, zwv876
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Helene Ahlman Nielsen Dortea Dencker Hansen

Front page picture by Dortea Dencker Hansen

PREFACE

This master thesis was made as a part of the master's degree in Veterinary Medicine at University of Copenhagen. The thesis was made as a part of the Ph.D. project "Evaluation of Diagnosis and Treatment of Early Signs of Pneumonia in Danish Dairy Calves by Clinical Score and Changes in Activity Parameters."

Our project took place from August 2020 to January 2021 and corresponds to 30 ECTS points.

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A special thanks to the farmer for lending the farm's calves to the project and always answering our questions.

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ABSTRACT

Bovine respiratory disease (BRD) is a great health problem in calves all over the world including Denmark. In Denmark, the antibiotic consumption for BRD in calves has increased over the last decade and with the worldwide discussion about antibiotic consumption it is important to look for alternatives to reduce the use of antibiotics. The aim of this study was to evaluate the treatment efficacy of meloxicam in calves with early signs of BRD. With the purpose to describe the various parameters (activity, rectal temperature, differential count and acute phase proteins) of sick and healthy calves. Fifty-one pre-weaned calves were followed in September and October 2020. The calves' activity was monitored with ear tag sensors (Sensehub Young Stock) and the calves' well-being was scored with Visual Analog Scale (VAS) score. Blood samples were drawn, and blood smears were made to count lymphocytes and neutrophils. Furthermore, capillary electrophoreses were made to look at protein fractions. We found a significant decrease in activity and increase in rectal temperature when calves became ill, but there was no change in lymphocytes, mature neutrophils, α_2-globulins and γ-globulins when comparing healthy and sick calves. When we looked at the efficacy of treating calves with NSAID we saw no significant change after treating the calves, but we saw a tendency in increased activity and decreased rectal temperature. Thirty-seven percent of the calves treated with NSAID recovered from two treatments with meloxicam injected 48 hours between. The rest of the calves needed more treatments with either NSAID or antibiotic. These results suggest that NSAID in some cases could be used alone to treated BRD, and, therefore, reduce the need of antibiotics. Furthermore, the study's results suggest that the VAS score, rectal temperature and activity from Sensehub Youngstock ear tag can be used to help find the calves at an early state of disease. Further investigation into NSAID treatment alone, SenseHub Young Stock ear tag and VAS score should be performed to be sure the results was not an isolated finding.

Keywords: Bovine Respiratory Disease, Leukocytes, Activity, Rectal Temperature, Protein Fractions

RESUMÉ

Luftvejsinfektioner (BRD) er et stort sygdomsproblem hos kalve over hele verden inklusive Danmark. I løbet af det sidste årti er antibiotikaforbruget til kalve mod BRD steget i Danmark. Som følge af den verdensomspændende diskussion om at få nedbragt brugen af antibiotika, er det vigtigt at undersøge alternativer til antibiotikabehandling og herved reducere antibiotikaforbruget.

Formålet med dette studie var at evaluere behandlingseffektiviteten af meloxicam hos kalve med tidlige tegn på BRD. Dette blev undersøgt ved at beskrive de forskellige parametre (aktivitet, rektal temperatur, differentiel tælling af leukocytter og akut fase proteiner) hos syge og raske kalve. Enoghalvtreds kalve blev fulgt før fravænning i september og oktober 2020. Kalvenes aktivitet blev registeret med aktivitets øremærker (Sensehub Young Stock), og kalvenes sundhedstilstand blev scoret med Visual Analog Skala (VAS). Vi udtog blodprøver fra kalvene, som vi lavede blodudstrygninger af. Blodudstrygningerne blev brugt til differentiel tælling af lymfocytter og neutrofile. Desuden blev blodprøverne brugt til kapillærer elektroforese for at undersøge proteinfraktionerne i blodet. Vi fandt et signifikant fald i aktivitet samt en signifikant stigning i rektal temperatur, når kalvene blev syge. Der blev dog ikke fundet nogle signifikante ændringer i niveauet af lymfocytter, mature neutrofile, α_2-globuliner og γ-globuliner ved sammenligning af raske og syge kalve. Da vi undersøgte behandlingseffekten af NSAID, fandt vi ingen signifikant ændring i parametrene efter endt behandling, men vi så en tendens til øget aktivitet og nedsat rektal temperatur hos de syge kalve. Syvogtredive procent af kalvene, der blev behandlet med NSAID, kom sig fra de to behandlinger med meloxicam, injiceret med 48 timer imellem. De resterende kalve havde brug for flere behandlinger med enten NSAID eller antibiotika.

Studiets resultater tyder på, at NSAID i nogle tilfælde kan bruges til behandling af BRD når kalven har tidlige tegn på BRD. Dette kan dermed være med til at reducere brugen af antibiotika. Desuden antyder studiets resultater, at VAS-score, rektal temperatur samt aktivitet fra Sensehub Youngstock-øremærkerne kan bruges til tidlig diagnostisk af BRD hos kalvene. Dog kræver det yderligere undersøgelser af NSAID-behandling, SenseHub Young Stock-øremærker og VAS-score for at være sikker på, at resultaterne i dette studie ikke var et isoleret fund.

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1. INTRODUCTION

1.1 Bovine Respiratory Disease

Bovine respiratory disease (BRD) is the biggest health problem in Danish calves. In 2015, 86% of all veal calves herds and 73% of all dairy calves herds were prescribed antibiotics for BRD (Jensen et al., 2018). BRD can cause major financial losses for the farmer due to decreased productivity, increased costs for treatment (Sacco et al., 2014) and a higher mortality rate in calves (Mahendran et al., 2017b). Furthermore, BRD can cause development of refractory sequelae such as chronic lung injury and pulmonary abscessation (McGuirk, 2008).

Most cases of BRD in calves start with a viral infection (Tuncer and Yeşilbağ, 2015), and are then complicated by a secondary bacterial infection. The diagnostic is problematic, and delayed diagnosis may result in prolonged use of antibiotics (McGuirk, 2008). When treating for BRD the goal is to eradicate the pathogen, reduce the severity of the inflammation and minimize the effect associated with pyrexia and depression (Fathi et al., 2013). It is common to use antibiotics for BRD, and in 2015 71% of the antibiotic use in Danish calves under one year of age were prescribed to BRD (Jensen et al., 2018). The overall antibiotic use for calves in Denmark has increased with 39% over the last decade, and it is primarily used for BRD (Statens Serums Institut, 2020). With the worldwide focus on reducing antibiotic consumption and resistance it is relevant to study the potential of using non-steroidal anti-inflammatory drugs (NSAID) to treat BRD. If NSAID alone should be a successful treatment it has to be used for early onset BRD, when the clinical signs are; loss of appetite, depression, fever, and nasal discharge (Mahaendran et al., 2017a). However, calves are rarely identified at the initial viral stage, but only at the onset of clinical signs associated with secondary bacterial infection often resulting in the need for antimicrobial treatment (Mahaendran et al., 2017a). The success of treating diseased calves, regardless of ethology, is related to how quickly the diseased calves are detected and treated (Mahendran et al., 2017b).

1.2 Paraclinical parameters for bovine respiratory disease

In addition to looking at clinical signs, it can be useful to study paraclinical signs, e.g., inflammatory response detected by capillary electrophoresis. Capillary electrophoresis is a method of separating charged molecules by their electrophoretic mobility at a specific pH in an alkaline buffer (abacusdx.com, 2020). Capillary electrophoresis divides the protein into six fractions: albumin, α_1-, α_2-,

β_1-, β_2- and γ-globulins. Many acute phase proteins (APPs) are in the α_2-globulin fraction, e.g. haptoglobin and serum amyloid A (SAA), therefore, this fraction will increase doing acute inflammation (Kaneko, 1997; O'Connell et al., 2005). Immunoglobulins are glycoproteins found in the γ-fraction. Calves are born with an immature immune system, and it is crucial calves get high quality colostrum within the first four to six hours post-partum (Goddon, 2008). From the colostrum, the maternal antibodies are passively transferred to the calf. This passive immunity helps the calves the first two to four weeks until their own immune system works properly (Chase et al., 2008). As shown in Figure 1 there is a gap in immunity between the passive immunity from the colostrum and the active immunity from the calf (Chase et al., 2008). The antibodies are divided into IgG, IgE, IgM and IgA (Kaneko, 1997; Tóthová et al., 2014). IgG antibodies contribute to the immune response, including neutralizing toxins and viruses. IgA is present in secret of the respiratory, genitourinary and gastrointestinal tract and is critical for protecting the mucosal surface from toxins, viruses and bacteria by neutralizing them or preventing bacteria or viruses from binding to the surface (Kaneko, 1997; Schroeder and Cavacini, 2010).

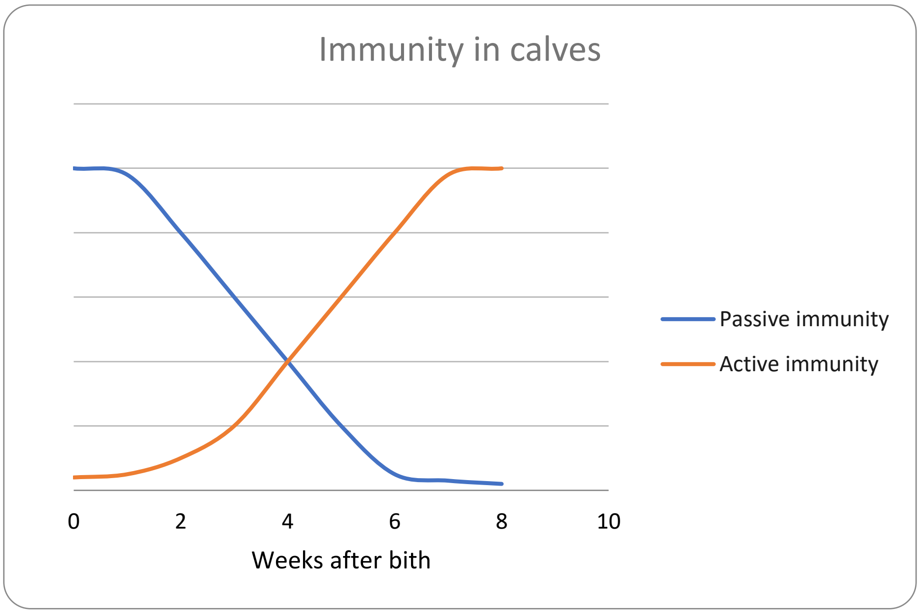

Figure 1. Immunity in calves after birth.
The passive immunity from the cow decrease with age of the calf.
The calf's active immunity develops in the first couple of weeks post-partum and replaces the passive immunity.
Modified from Chase et al. (2008).

Haptoglobin and SAA are a part of the acute phase response (APR) (Jones and Allison, 2007). APR is the inflammatory response occurring shortly after any tissue damage and is characterized by systemic inflammatory signs such as fever, anorexia and increasing pulsation and respiratory rate (Fathi et al., 2013). SAA and haptoglobin are produced by hepatocytes as a response to proinflammatory cytokines, and function as a part of the early nonspecific immune defense in the blood (Jones and Allison, 2007). SAA blood levels increase rapidly when calves have BRD (Gabay and Kushner, 1999; Akgul et al., 2019; El-Deeb et al., 2020) (Figure 2), and declines in the next seven days (Gabay and

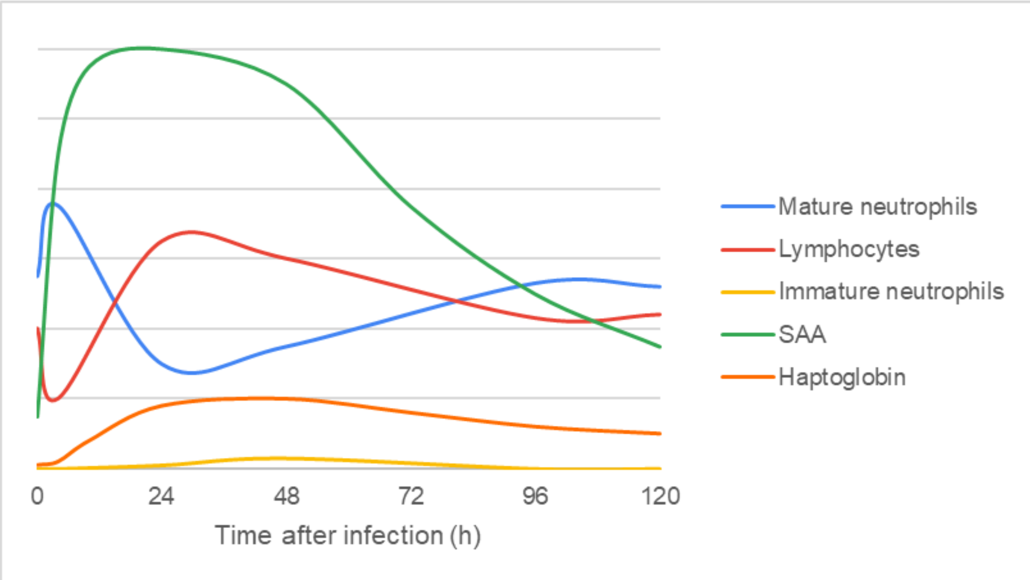

Figure 2. Acute phase proteins and leukocytes in cirkulation hours after infection.
SAA: Serum amyloid A.
Mature neutrophils, lymphocytes and immature neutrophil modified by Jones and Allison (2017).
SAA and haptoglobin modified by Gabay and Kushner (1999) and Peterson et al. (2004).

Kushner, 1999; El-Deeb et al., 2020). Haptoglobin levels increase in the blood 24 to 48 hours after tissue damage (Figure 2), and can remain high for up to two weeks (Jones and Allison, 2007).

Besides SAA and haptoglobin, leukocytes are a part of the APR (Heinrich et al., 1990). Leukocytes are divided into two groups; granulocytes which include neutrophils, eosinophils and basophils, and mononuclear cells which include monocytes and lymphocytes. All leukocytes, except lymphocytes, are produced and matured in the bone marrow. Besides producing neutrophils, the bone marrow has many neutrophils stored, which will be released when an infection occurs and raise the level of neu-trophils in the blood (Parham, 2009). Two hours after tissue damage an initial neutropenia will occur due to local composition (Jones and Allison, 2007; Parham, 2009). Within 24 hours a left shift will occur, characterized by the appearance of immature neutrophils in the circulation, which are released from the bone marrow (Jones and Allison, 2007). The bone marrow pool of neutrophils will normally be replenished in four to five days, returning cell count to normal if the inflammation is resolved. If the inflammation is ongoing there may still be neutrophilia, with or without a left shift (Jones and Allison, 2007). Lymphocytes originate from the stem cells of the bone marrow but are maturing and proliferating in other lymphoid tissues. Lymphocytes are the only leukocytes that routinely recirculate between blood and tissues (Jones and Allison, 2007).

Due to the variations, seen in Figure 2, of lymphocytes and mature neutrophils level in the blood, the leukogram will change with inflammation at various stages of the disease process (Jones and Allison, 2007). Therefore, manually classifying leukocytes on stained blood film is important because individual cell types can increase and decrease at the same time, leaving the total leukocyte count in the normal range (Jones and Allison, 2007).

1.3 Clinical signs of bovine respiratory disease and scoring

The clinical signs of BRD may vary from mild to severe, and can include increased rectal temperature and respiratory rate, abnormal pulmonary sound, nasal and ocular discharge, cough, declined appetite, depression and disinterest in their surroundings (Timsit et al., 2011; Toaff-Rosenstein and Tucker 2018; Akgul et al., 2019). As we can see in Figure 3 all these clinical signs of BRD occur at different times after infection. To evaluate the clinical signs the Wisconsin Calf Health Score (WI) (McGuirk and Peek, 2014) is often used. The WI score is based on rectal temperature, cough, ear position, nasal- and eye discharges. We wanted to find the sick calves early, and we, therefore, used a Visual Analog Scale (VAS) of general calf demeanor and a data collecting ear tag (SenseHub Young Stock).

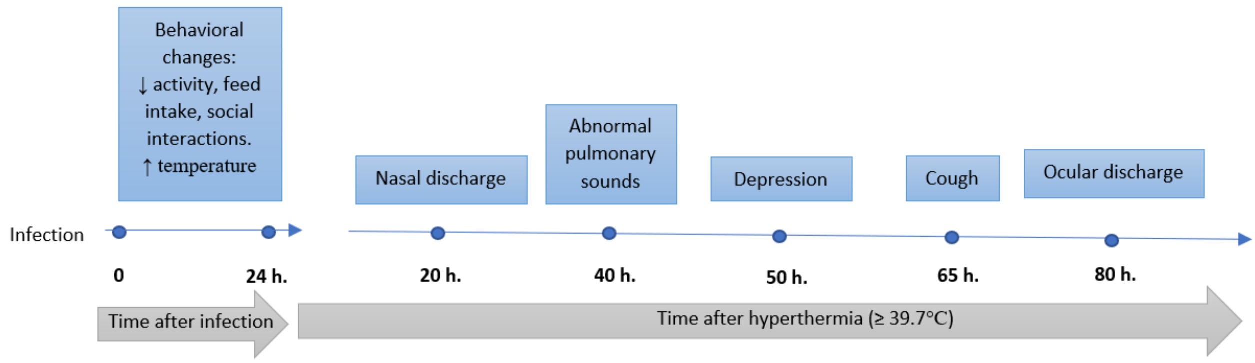

Figure 3. Timeline of clinical signs of bovine respiratory disease. Starts with infection at time 0 until fever is developed within the first 24 hours after infection. 20, 40, 50, 65 and 80 hours is time after onset of fever (≥ 39.7°C). Modified from Timsit et al., 2011 and Hixson et al., 2018.

VAS is a continuous scale from zero to 10 with clear verbal descriptors, usually “no pain” at zero and “worst imaginable pain” at 10. This version is widely used especially in human medicine where the patient is asked to use the scale to determine the degree of pain they experience (Williamson and Hoggart, 2005). A similar visual analog scale is used for gait score and lameness diagnosis of dairy cows, where the scale has been used in several studies in combination with a one-to-five numerical rating system (NRS) (Flower and Weary, 2006; Flower et al., 2007; Chapinal et al., 2010; Chapinal et al., 2012). The two scales, NRS and VAS, have been compared in studies where the results were similar regardless whether they were using the VAS or the NRS to score pain or lameness (Flower and Weary, 2006; Kielland et al., 2009; Chapinal et al., 2010). VAS can be used to score many different clinical assessments and is continuously, and are, therefore, preferred in this study.

The aim of this study is to evaluate the treatment efficacy of meloxicam in calves with early signs of bovine respiratory disease to minimize the use of antibiotics. The purpose is to describe the various parameters (activity, temperature, differential count and acute phase proteins) of sick and healthy calves from the specific objectives:

- To determine if there is a significant change in differential count (lymphocytes and mature neutrophils), acute phase proteins (α_2- and γ-fraction), activity (registered with SenseHub Young Stock), and temperature between healthy calves and calves with early clinical signs of bovine respiratory disease (based on general well-being).
- To investigate change in expression of bovine respiratory disease for calves treated with NSAIDs (meloxicam) based on differential count (lymphocytes and mature neutrophils), acute phase proteins (α_2- and γ-fraction), activity (registered with SenseHub Young Stock), and temperature.
- To investigate if there is a correlation between acute phase proteins (α_2- and γ-fraction) compared with activity (registered with SenseHub Young Stock), temperature, and differential count (lymphocytes and mature neutrophils).

2. MATERIALS AND METHODS

2.1 Study design and herd selection

Data for this thesis were collected in a field study as a part of a Ph.D. project, aiming to evaluate diagnosis and treatment of early signs of pneumonia in Danish dairy calves based on clinical score and changes in activity parameters. The data was obtained from September 2nd, 2020 until November 11th, 2020. The study was a prospective longitudinal cohort study conducted on a conventional dairy farm in Denmark, with about 660 dairy cows and 800 heifers of the breeds Danish Holstein and Danish Red Holstein. The selection of the herd used in this study was based on the farm's willingness to participate, the fact that it is a large-scale farm so as to ensure enough calves, the location and the use of SenseHub Activity Recording System for heat detection in dairy cows. All heifers born on the farm between the 7th of August and 25th of September were included in the study.

2.2 Animals and sampling

On the selected farm the newborn calves were separated from their cows as soon as an employee discovered the calf and had time to separate them. The calves received colostrum from cows that had been vaccinated with Rotavec® Corona (MSD Animal Health) about eight weeks before calving. The colostrum was given to the newborn calves with an esophageal tube. They got four liters of colostrum at the first possible moment and two liters extra at the next milk feeding, thereby, getting six liters of colostrum within the first 24 hours. Thereafter, calves were fed twice daily with four to eight liters of milk, depending on age, in feeding troughs. The calves in single pens were fed with high somatic cell count milk, and calves in group pens were fed with milk replacer. After milk feeding, they had free access to corn silage topped with homemade calf starter. The calves were housed in single pens with no physical contact to neighboring calves for the first 16-39 days (mean: 27 days SD: 6 days). There-after, they were grouped in pens of five to seven (one pen was fourteen) calves, located both inside and outside, until weaning. Both single and group pens were bedded with straw and got topped with straws every Monday, Wednesday and Friday. Both single and group pens were cleaned between calves' transfer and sprayed with calcium hydroxide. Dehorning took place when calves were be-tween 21 and 49 days (mean: 34 days, SD: 7 days), except three calves which were dehorned after the experiment had ended. When the calves were dehorned, they were sedated with xylazine and got a local analgesic with lidocaine. The calves on this farm were not treated with NSAID when dehorned.

Calves were between five and 25 days (mean: 13 days, SD: 5 days) when they got the SenseHub Young Stock (Allflex ®) ear tag in their left ear as described by the manufacturer. The calves were included in the experiment when they were between 17 and 25 days old and remained in the experiment for the following 30 days.

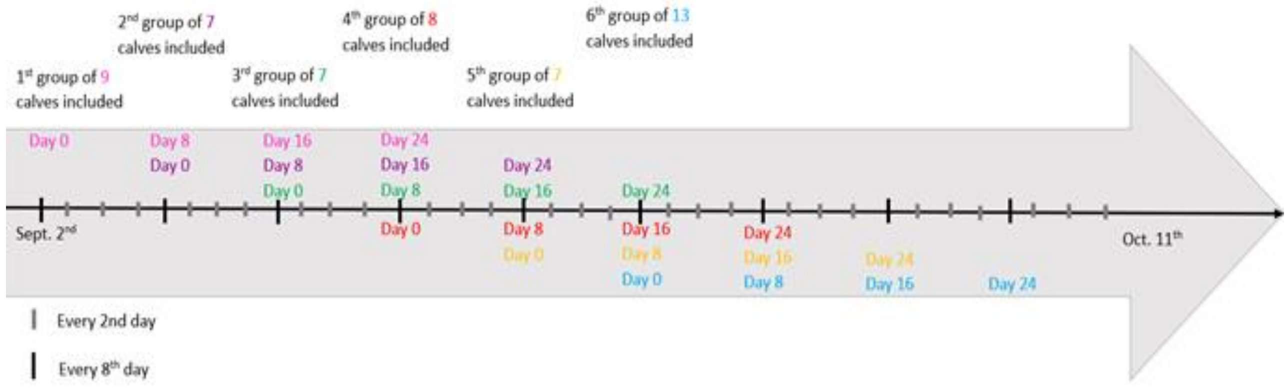

Figure 4. Timeline of the flow of calves included in the study through the data collection period from September 2nd to October 11th. Every group had a color showed at the number and on days in the period. They had their last day in the experiment on day 30.

As seen in Figure 4, the experiment consisted of four eight-days periods, where calves got a VAS score and had their rectal temperature taken every second day. The VAS score was the primary scoring system and was used to decide the calves' overall well-being and, thereby, as seen in Figure 5, if the calves should be treated.

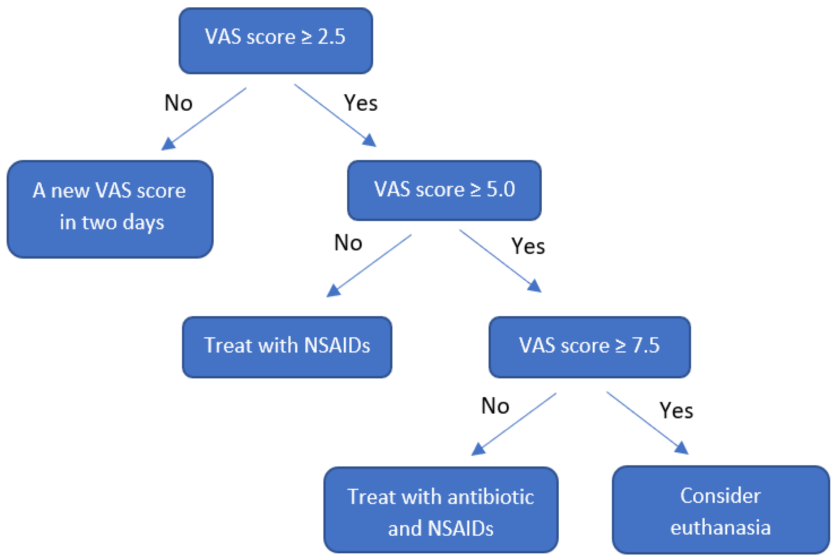

*Figure 5. Decision tree whether a calf should be treated including the treatment.
Calves with a VAS score between 2.5 and 5.0 received NSAID treatment.
Calves with a VAS score between 5.0 and 7.4 received an antibiotic treatment
Euthanasia was considered if calves had a VAS score ≥ 7.5*

Calves with a VAS score less than 2.5 were awake and interested in their surroundings, they were playful and would suckle on pen mates. The calves' ears were pointing up-forward, and they could have slight serous eyes or nasal discharge. Calves with a score between 2.5 and 4.9 were a little less active and playful. The calves' heads were lowered, and the ears were in a middle position. There could be a small amount of mucopurulent and cloudy nasal discharge. Eye discharge could be visible or in lines at the cheeks. Calves with a score above 5.0 and less than 7.5 were depressed. They would only stand up when being chased and had no interest in pen mates or suckling. Head and ears would hang. They could have a larger amount of purulent nasal discharge, runny eyes and spontaneous cough. Calves above 7.5 would not stand up, would often lie in lateral recumbency and would have heavy breathing.

Figure 6. If the calves received a VAS score between 2.5 and 4.9, they got treated twice with NSAID (Meloxicam) and had three blood samples drawn with a day in between.

As seen in Figure 6, if the calves, on any given day in the experiment, got a VAS score between 2.5 and 4.9 they were treated with NSAID twice with 48 hours between. Blood samples were drawn on the day of the score before treatment, two days after the score before the second treatment and four days after the score as a control sample.

Figure 7. If the calves received a VAS score between 5.0 and 7.4, they were treated once with both antibiotic (Tulathromycin) and NSAID (Meloxicam) and had two blood samples drawn with a day in between.

If the VAS score was between 5 and 7.4 (Figure 7) the calves were treated with NSAID and antibiotic once. Blood samples were drawn on the day of the score before treatment and two days after the score as a control sample. As seen in Figure 5, euthanasia was considered if the VAS score was greater than 7.5. We only had one calf with a score above 7.5 in this study and it received the same treatment as calves scored between 5.0 and 7.4.

Figure 8 shows our repeated sample circle consisting of an eight-day period.

On day 0/8 all calves in the experiment had a blood sample drawn. Only sick and control calves had blood samples drawn on day two, four and six. The VAS score was given by one of two observers (supervisors: NC or HM). Every day in the experiment we started between 8 am and 11 am and ended between 12 pm and 4 pm.

In this study we used Loxicom (meloxicam) (20 mg/ml) as the NSAID in a dosage of 1 ml per 40 kg body weight twice with 48 hours between (s.c.). The antibiotic used was Draxxin (tulathromycin) (100 mg/ml) 1 ml per 40 kg body weight given once (s.c.). All treatments were performed by the employees of the farm according to the protocol. If there were signs of diarrhea or other illness this was noted.

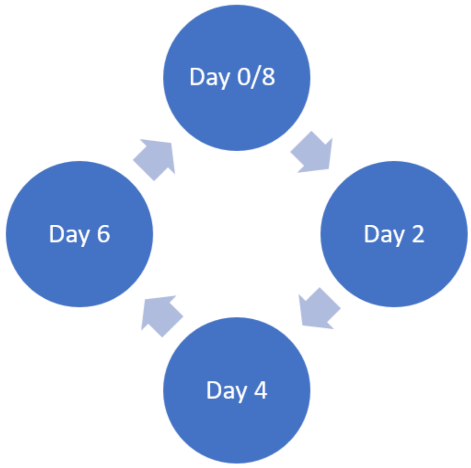

Figure 8. The repeated cycle of our 8-day period with data collecting every second day of the study. On all sample days calves got a VAS score and had a rectal temperature taken. On day 0/8 all calves had blood samples drawn. On day 2, 4 and 6 only sick or control calves had blood samples drawn.

2.3 Sample handling

All VAS scores and temperatures were typed into an Excel document. Data about rumination and activity from the SenseHub Young Stock ear tag was automatically collected on SenseHub's webpage (https://st.scrdairy.com/). For each day in the experiment we estimated the average activity and rumination for the previous 48 hours. The activity and rumination had a "score" for every hour, and due to time constraints, we only included 8 data points in the 48 hours average. As seen in Figure 9, we included the "score" at 4 pm and 10 pm two days earlier, 4 am, 10 am, 4 pm and 10 pm the day before as well as 4 am and 10 am on the day of the experiment.

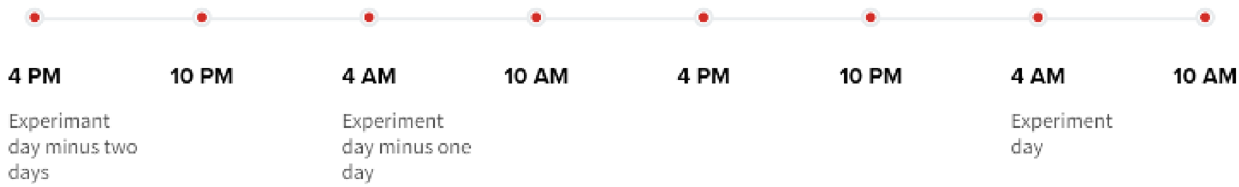

Figure 9. The previous two days' activity and rumination was collected from the SenseHub application at the times: 4 am, 10am, 4 pm and 10 pm.

To evaluate blood parameters, blood samples were drawn from the jugular vein into vacutainer tubes. We took serum (BD Vacutainer ®, SST™ II Advance) and EDTA (BD Vacutainer ®, K2E (EDTA) 7.2 mg, 4.0 mL) tubes in that order. All tubes were stored for one to four hours until arrival at the laboratory. Serum was centrifuged in Eppendorf Centrifuge 5810 R with 1800G at 20ºC for 10 minutes. Afterwards serum was pipetted into Cryotubes and stored at -80ºC until analyzing. The serum was analyzed with Sebia MINICAP, a capillary electrophoresis, at Veterinary Diagnostic Laboratory (University of Copenhagen, Dyrlaegevej 46, DK-1870 Frederiksberg C) in the fractions albumin, α_1-, α_2-, β_1-, β_2- and γ-globulins. The EDTA was used for blood smears, which were made the same day as the samples were collected. The blood smears were colored with Hemacolor and used for manual leukocyte differential count. The differential count was based on the count of 100 leukocytes, made by two persons. To test the agreement between the two counters, a Cohen's Kappa was performed on 10 blood smears with Kappa values as followed: ≤ 0 as no agreement, 0.01-0.20 as none to slight, 0.21-0.40 as fair, 0.41-0.60 as moderate, 0.61-0.80 as substantial and 0.81-1.00 as almost perfect agreement McHugh, 2012). The differential count was made for both lymphocytes and mature neutrophils and Kappa was, thereby, made on 20 observations with a substantial agreement (Table 1).

Table 1. Weighted Kappa: 0,703, SE: 0,097 (http://vassarstats.net/kappa.html).
Differential count made on 10 blood smears counted by two persons.
Lymphocytes and neutrophils were selected.
Kappa was made on 20 observations.

	≤ 25	26-35	36-45	46-55	56-65	≥ 66
≤ 25	1					
26-35	1	1				
36-45		1	2	2		
46-55			2	4	2	
56-65				1		
≥ 66						3

2.4 Data management and statistical analysis

The data was analyzed in statistical software R (version 4.0.3, 2020-10-10) with R studio (version 1.3.1073) and Microsoft Excel.

All data were used in different datasets depending on the objectives. To make the dataset, calves were divided into three groups according to what treatment they had received. The group of healthy calves were defined as calves never treated during the study period and with a VAS score ≤ 1.5. NSAID calves were defined as calves treated with NSAID at some point during the study period but never treated with antibiotics. AB calves were defined as calves treated with antibiotics at some point during the study period.

To describe our data, healthy calves were used to show the changes in parameters compared to age. Data from all 51 calves were made to illustrate VAS score compared to the parameters.

To determine if there was a change between healthy calves, NSAID calves and AB calves' observations from NSAID calves from the days they got a VAS score between 2.5 and 4.9 were used. For AB calves, observations from days they got a VAS score between 5.0 and 7.4 were used. They were also used for scatter plots with linear regression. This was made in RStudio and Microsoft Excel to compare activity, temperature, mature neutrophils, lymphocytes, α_2- and γ-globulin fractions with days in study. Boxplots were made to illustrate and compare the two groups, healthy and NSAID calves, and a t-test was calculated in RStudio to find if there was a significant change between healthy and NSAID calves on the chosen parameters.

To investigate the course of BRD we looked at calves treated with NSAID and set inclusion criteria. The calves had to have the wanted disease course (WDC) with blood samples from before, under and after treatment. Scatter plots with connection dots were made as a descriptive picture of the course of each calf. One-way repeated measure ANOVA with the calves as fixed effect was made in RStudio to find if there was a significant change in each course.

To investigate if there was a correlation between acute phase proteins (α_2- and γ-globulin fractions) compared with activity, temperature, lymphocytes and mature neutrophils, scatter plots with linear regression were made in RStudio. For this, observations from all 51 calves were used.

3. RESULTS

3.1 Descriptive statistics

This study had in total 816 observations based on 51 calves. Depending on the figure and which group of calves we looked at, the number of calves and observations would differ throughout the results. Table 2 presents the different groups and subgroups of calves.

Table 2. The distribution of 51 calves included in the study subdivided into different treatment groups depending on clinical scores.
Healthy calves were calves never treated in the study period.
Treated calves were calves treated with either NSAID or antibiotic combined with NSAID at some point in the study.
NSAID calves were defined as calves only treated with NSAID, after receiving a VAS score between 2.5 and 4.9, and no antibiotic treatment in the study period.
AB calves were defined as calves treated with antibiotic combined with NSAID after receiving a VAS score above 5.0, at some point in the study period.

	Number	Prevalence (%)
Calves	51	
Healthy calves	18	18/51 (35)
Treated calves	33	33/51 (65)
NSAID calves	22	22/33 (67)
AB calves	11	11/33 (33)

In this study 35% (18/51) of the calves never received a treatment (healthy calves). Sixty-five percent of our calves developed signs of BRD during the experiment. Out of the 33 calves treated in the study period, 30 calves received NSAID in some cases in combination with antibiotics. Sixty-seven percent (22/33) were treated only with NSAID (NSAID calves). Thirty-three (11/33) percent of the calves were treated with antibiotics (AB calves) (Table 2), three of the AB calves where only treated with antibiotics. In the following sections the groups: Healthy-, NSAID- and AB calves were used to calculate our results.

To provide an overview of treatments for each calf we created Figure 10. We had 18 healthy calves which were never treated in our study period e.g. calf #588 and #577. We had 30 calves treated with NSAID e.g. calf #573 and #550. Calf #516 and #518, for example, had more than one period with illness (≥ six days from last treatment). We had 11 calves which did recover after NSAID treatment (two treatments with 48 hours apart) e.g. calf #517 and #531. Eleven out of 30 NSAID calves received more than two treatments of NSAID in a row e.g. calf #536. Eight calves were treated with antibiotic after ineffective treatment with NSAID e.g. calf #550 and #536 and the three calves, #511, #565 and #586, was treated with antibiotics without prior NSAID treatment.

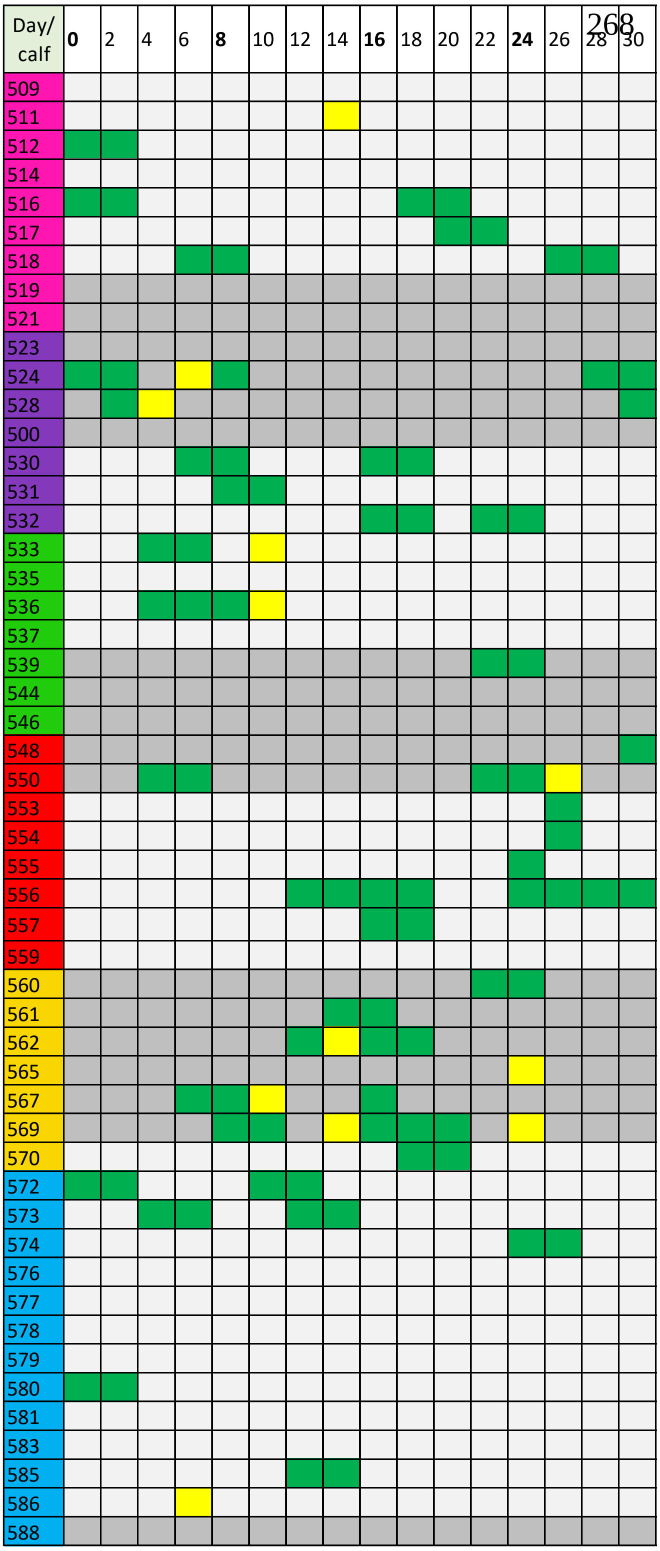

Figure 10. All 51 calves in the study and their treatment.
The colors in the left side are calves included at the same time.
The two shades of gray indicate group division of calves when housed in group pens.
Numbers from 0 to 30 were days in study for each calf.
Day 0, 8, 16 and 24 were days with blood samples from all calves.
Green days was NSAID treatment
Yellow days was antibiotic treatment (combined with NSAID)
Gray days is no treatment.

3.1.1 Parameters compared to age

Activity and rumination. Figures 11 and 12 present the SenseHub data on activity and rumination for seven healthy calves with at least 10 observations with a VAS score ≤ 1.5 on these days.

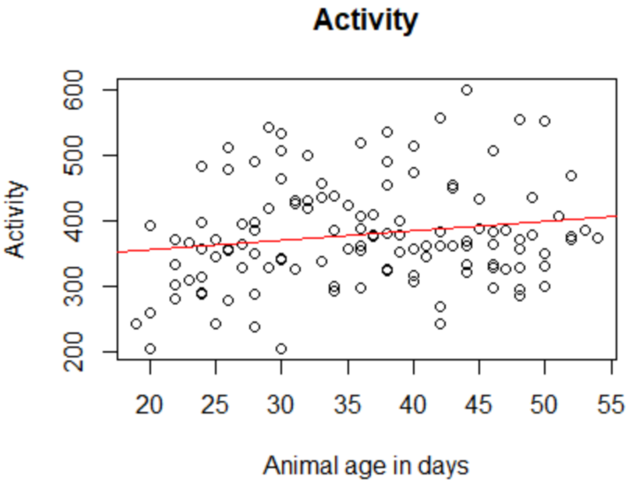

Figure 11. Scatter plots and linear regression line for activity compared with age in days based on 124 observations from 9 healthy calves with minimum 10 observations with VAS score ≤ 1.5.
F(x) = 1.4x + 327, R^2 = 0.03, P = 0.06

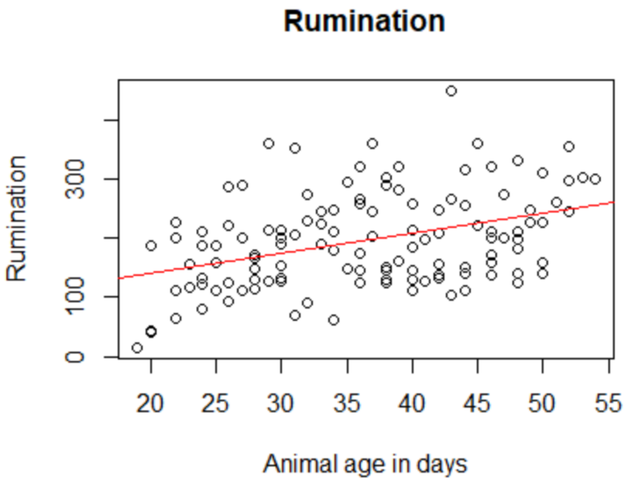

Figure 12. Scatter plots and linear regression line for rumination compared with age based on 124 observations from 9 healthy calves with minimum 10 observations with a VAS score ≤ 1.5.
F(x) = 3.4x + 71, R^2 = 0.16, P < 0.001

As we saw on Figure 11 activity had a slight increase with age with a slope of 1.4. The scatter plots showed a great variation (R^2 = 0.03) e.g. from 243 to 555 at age 42 days. Rumination had a greater increase with age than activity and a slope of 3.4. There was also a great difference in rumination between calves in the same age (R^2 = 0.16), e.g. 104 and 448 on age 43 days (Figure 12).

Lymphocytes and neutrophils. Figure 13 and 14 present the analysis of age compared with the differential count on lymphocytes and neutrophils from nine healthy calves with a VAS score ≤ 1.5 on day zero, eight, 16 and 24 in the study period

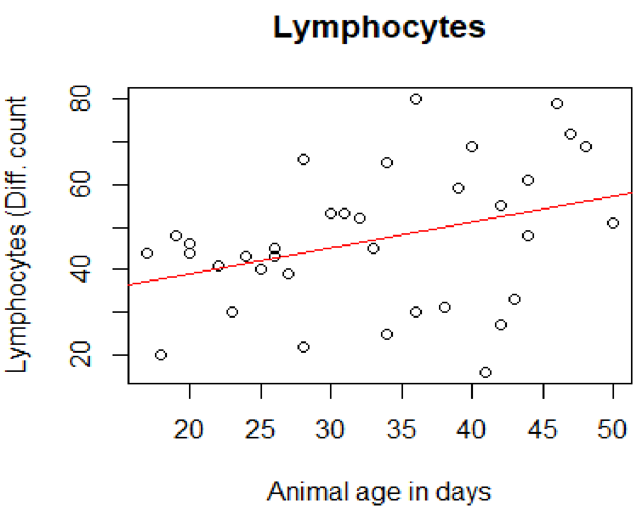

Figure 13. Scatter plots and linear regression line for lymphocytes from differential count compared with age in days based on 35 observations from 9 healthy calves with a VAS score ≤ 1.5.
$F(x) = 0.6x + 26.8$, $R^2 = 0.13$, $P = 0.04$

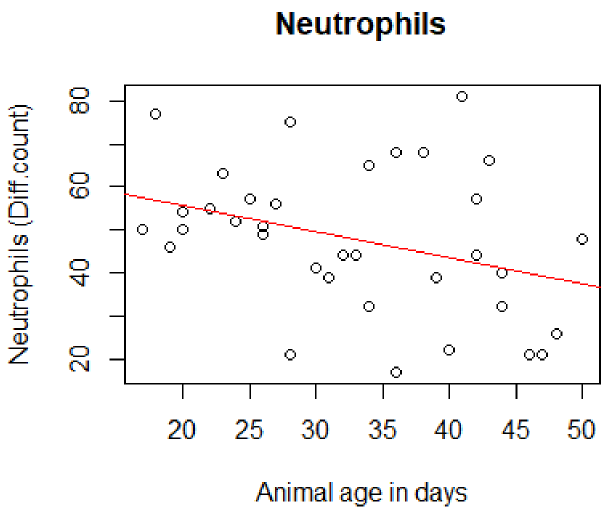

Figure 14. Scatter plots and linear regression line for neutrophils (mature + immature neutrophils) from differential count compared with age in days based on 35 observations from 9 healthy calves with a VAS score ≤ 1.5.
$F(x) = - 0.6x + 67.7$, $R^2 = 0.12$, $P = 0.05$

Lymphocytes also increased and had a slope of 0.6, still with a great variation ($R^2 = 0.13$) (Figure 13). Neutrophils was the only regression line with a decrease, and a negative slope of 0.6, still with a great variation between calves ($R^2 = 0.12$) (Figure 14).

α_2- and γ-globulins. Figure 15 and 16 present the analysis of α_2- and γ-globulin fractions from the capillary electrophoreses for nine healthy calves with a VAS score ≤ 1.5 on day zero, eight, 16 and 24 in the study. The data from the capillary electrophoreses were incomplete. For three out of the nine calves we had a complete dataset with data from all four days. However, from three out of the remaining six calves we only had data from three out of the four days, and for the last three calves we only had data from two out of the four days.

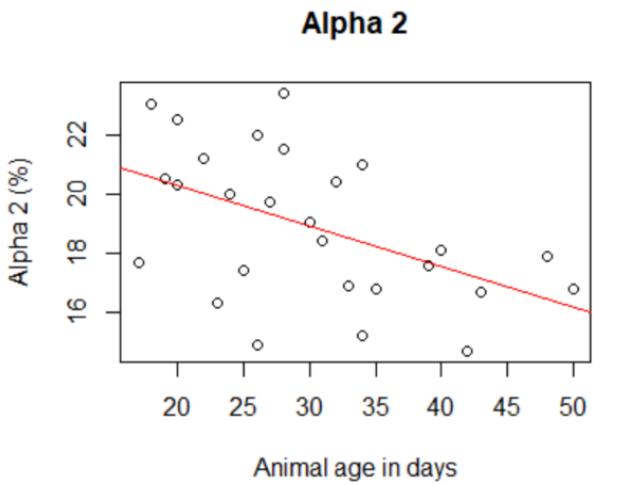

Figure 15. Scatter plots and linear regression line for α_2 -globulin fraction in percent, compared with age in days based on 27 observations from 9 healthy calves with a VAS score ≤ 1.5 from day 0, 8, 16 and 24 in the study period.
$F(x) = -0.14x + 23$, $R^2 = 0.24$, $P < 0.01$

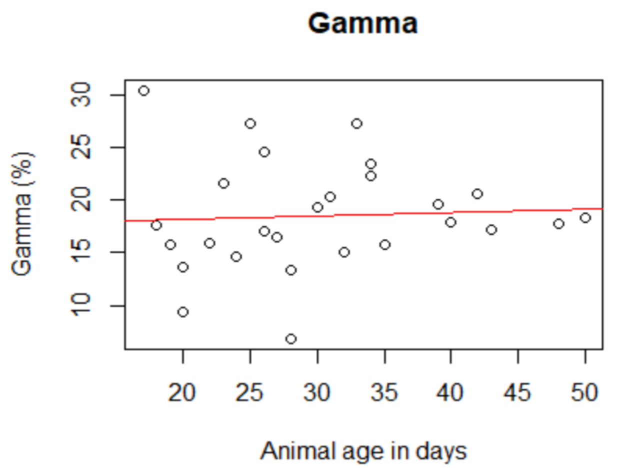

Figure 16. Scatter plots and linear regression line for γ -globulin fraction in percent, compared with age in days based on 27 observations from 9 healthy calves with a VAS score ≤ 1.5 from day 0, 8, 16 and 24 in the study period.
$F(x) = 0.03x + 17.6$, $R^2 = 0.003$, $P = 0.8$

As we saw on Figure 15, the α_2-globulin fraction levels had a slight decrease with age, with a slope of – 0.14. The scatter plots showed a great variation between the healthy calves ($R^2 = 0.24$). The γ-globulin fraction levels had no correlation with the age ($P = 0.8$). γ-globulin had as well a great difference between calves in the same age ($R^2 = 0.003$), e.g. 17.1% and 24.6% at age 26 days from two different calves (Figure 16).

Total protein. Figure 17 presents the analysis of total protein compared with days in age for the same nine healthy calves as in figure 15 and 16, with a VAS score ≤ 1.5 on day zero, eight, 16 and 24 in the study.

As we saw on Figure 17 the levels of total protein increased with age with a slope of 0.19. The scatter plot showed a great variation between the healthy calves e.g. one calf at age 26 days had a total protein on 63.6 g/L in comparison with another calf at 28 days with total protein levels on 49.9 g/L.

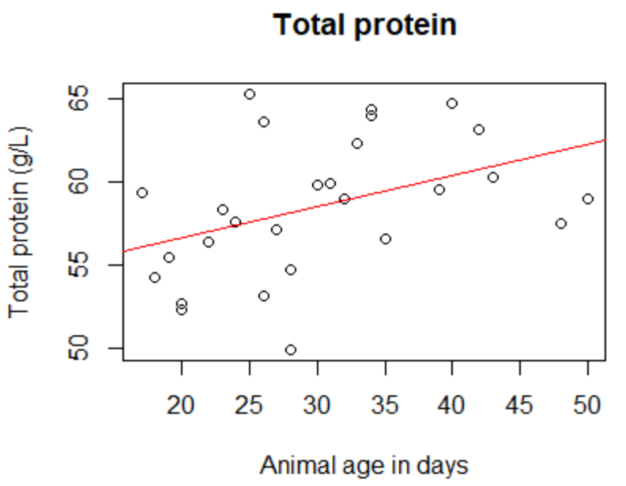

Figure 17. Scatter plots and linear regression line for total protein in gram per liter, compared with age in days based on 27 observations from 9 healthy calves with a VAS score ≤ 1.5 from day 0, 8, 16 and 24 in the study period.
$F(x) = 0.19x + 52.9$, $R^2 = 0.17$, $P = 0.03$

3.1.2 Parameters compared to VAS score

Activity and rectal temperature. Figures 18 and 19 present the VAS score compared with the SenseHub data on activity and rectal temperature from all calves.

The scatter plots showed poor correlation (P = 0.75) between activity and VAS score with values diffusely distributed for calves with VAS score between zero and 2.5 (Figure 18). As we saw in Figure 19 the values for temperature also showed great variation ($R^2 = 0.1$), but in general, the temperature had a positive correlation with VAS score (P < 0.001).

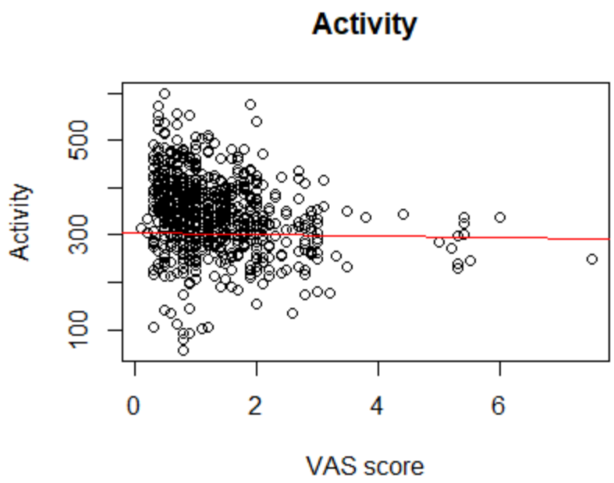

Figure 18. Scatter plot of VAS score compared to activity based on 710 observations from all 47 calves in the study. $R^2 < 0.001$, P = 0.75.

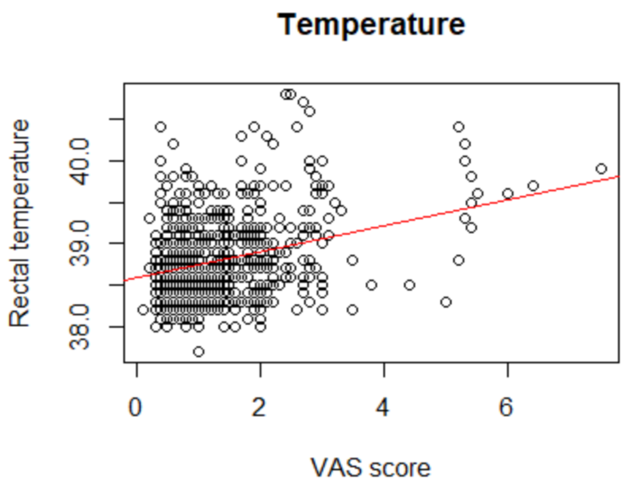

Figure 19. Scatter plot of VAS score compared to rectal temperature based on 816 observations from all 51 calves in the study. $R^2 = 0.1$, P < 0.001

Lymphocytes and neutrophils. Figure 20 and 21 present the analysis of VAS score compared with differential count on lymphocytes and neutrophils in all calves.

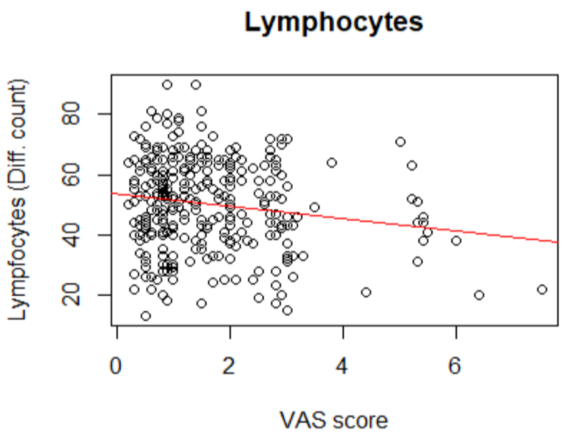

Figure 20. Scatter plot of VAS score compared to lymphocytes based on 305 observations from all 51 calves in the study. $R^2 = 0.03$, P < 0.01.

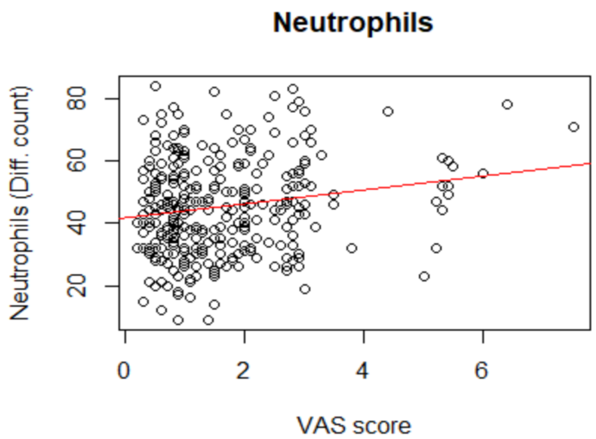

Figure 21. Scatter plot of VAS score compared to neutrophil based on 305 observations from all 51 calves in the study. $R^2 = 0.03$, P < 0.01.

Scatter plots from the differential count of lymphocytes (Figure 20) and neutrophils (Figure 21) showed a wide range in cell count from calves with a VAS score between zero and five ($R^2 = 0.03$). Scatter plots from calves with a VAS score above 5.0 had a low level of lymphocytes and a high number of neutrophils. Both neutrophils and lymphocytes showed a correlation ($P < 0.01$) with VAS score.

α_2- and γ-globulins. Figure 22 and 23 present the VAS score compared with α_2- and γ-globulin fractions from the capillary electrophoreses on all calves.

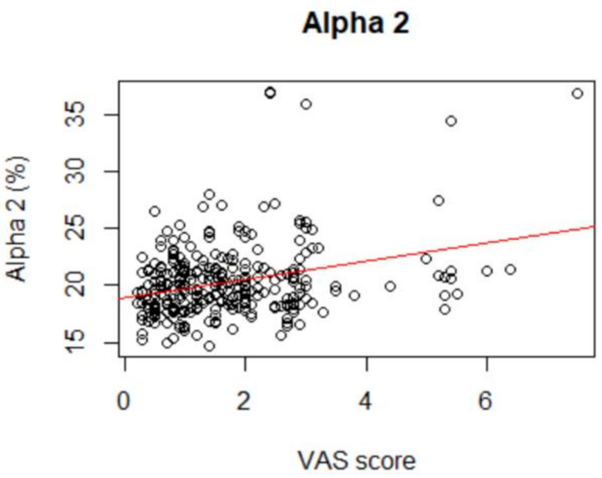

Figure 22. Scatter plot of VAS score compared to α_2-globulin fractions in percent, based on 285 observations from all 51 calves in the study.
$R^2 = 0.09$, $P < 0.001$

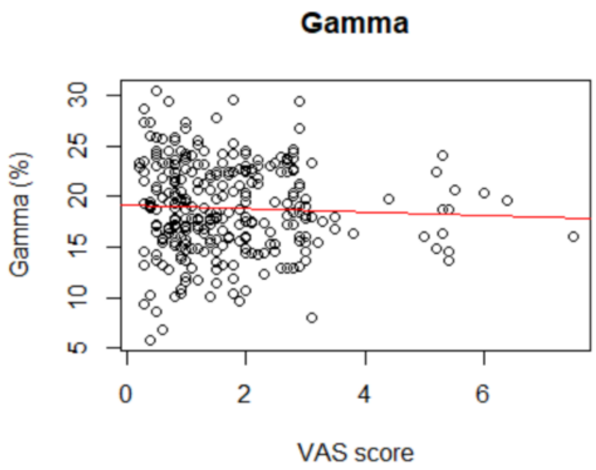

Figure 23. Scatter plot of VAS score compared to γ – globulin fractions in percent, based on 285 observations from all 51 calves in the study.
$R^2 = 0.002$, $P = 0.46$

The scatter plots showed a correlation ($P < 0.001$) between α_2-globulin and VAS score with values increasing with a higher VAS score (Figure 22). Values for the γ-globulin showed no correlation ($P = 0.46$) and with values diffusely distributed ($R^2 = 0.002$) (Figure 23).

Total protein. Figure 24 presents the analysis of total protein compared with VAS score on all calves.

As shown in Figure 24, there was a correlation ($P = 0.03$) between total protein and VAS score, with a decrease in total protein with a higher VAS score, but with values diffusely distributed ($R^2 = 0.016$).

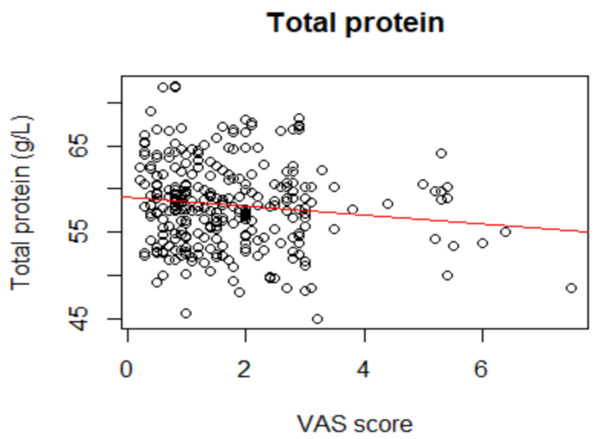

Figure 24. Scatter plot of VAS score compared to total protein in gram per liter, based on 285 observations from 51 calves.
$R^2 = 0.016$, $P = 0.03$

3.2 Healthy calves compared with NSAID calves

3.2.1 Parameters compared with days in study

Activity. Figure 25 presents and compares the activity data from the groups: Healthy-, NSAID- and AB calves with days in study. In the group of healthy calves, we had 18 calves but only 15 with activity data from SenseHub. Observations were from the days where they received a VAS score ≤ 1.5. The group of NSAID calves included 19 calves with observations from the days they received a VAS score between 2.5 and 4.9. In the group of AB calves we had 11 calves with observations from the days they received a VAS score above 5.0.

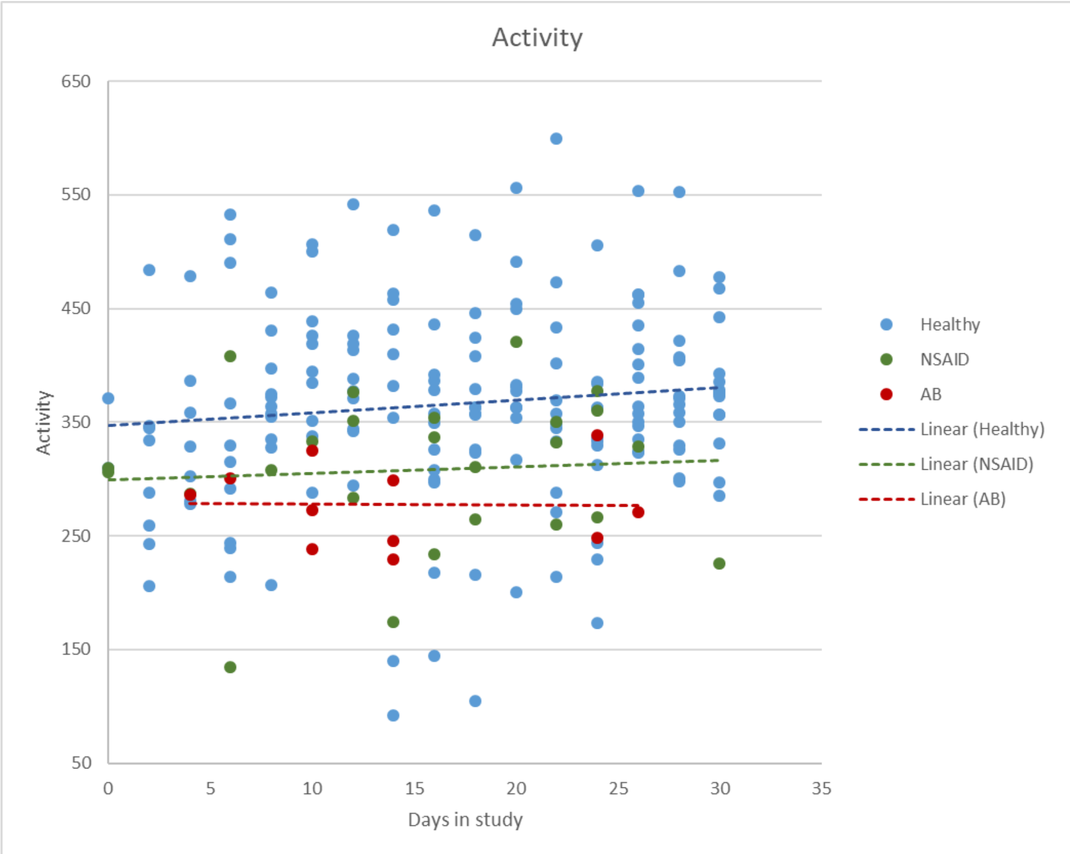

Figure 25. Scatter plots and linear regression line for activity of calves compared with days in study with different treatment regimens for bovine respiratory disease.
Blue: 172 observations from 15 healthy calves from the days they received a VAS score ≤ 1.5.
Green: 25 observations from 19 calves that only had NSAID and on the day they received a VAS score between 2.5 and 4.9.
Red: 11 observations from 11 calves treated with antibiotic on the day they received a VAS score > 5.0

In Figure 25 we saw that NSAID calves had a general lower activity than healthy calves regardless of days in the study. AB calves' activity were even lower than NSAID calves' activity. Though the activity varied a lot for all three groups of calves, there was a visible difference, e.g. no NSAID calves had an activity above 450, and no AB calves had activity above 350.

Rectal temperature. Figure 26 presents and compares days in study with rectal temperature from the 18 healthy calves, 20 NSAID calves and 11 AB calves. Observations were from days with a VAS score for healthy calves below 1.5, NSAID calves between 2.5 and 4.9 and for AB calves above 5.0.

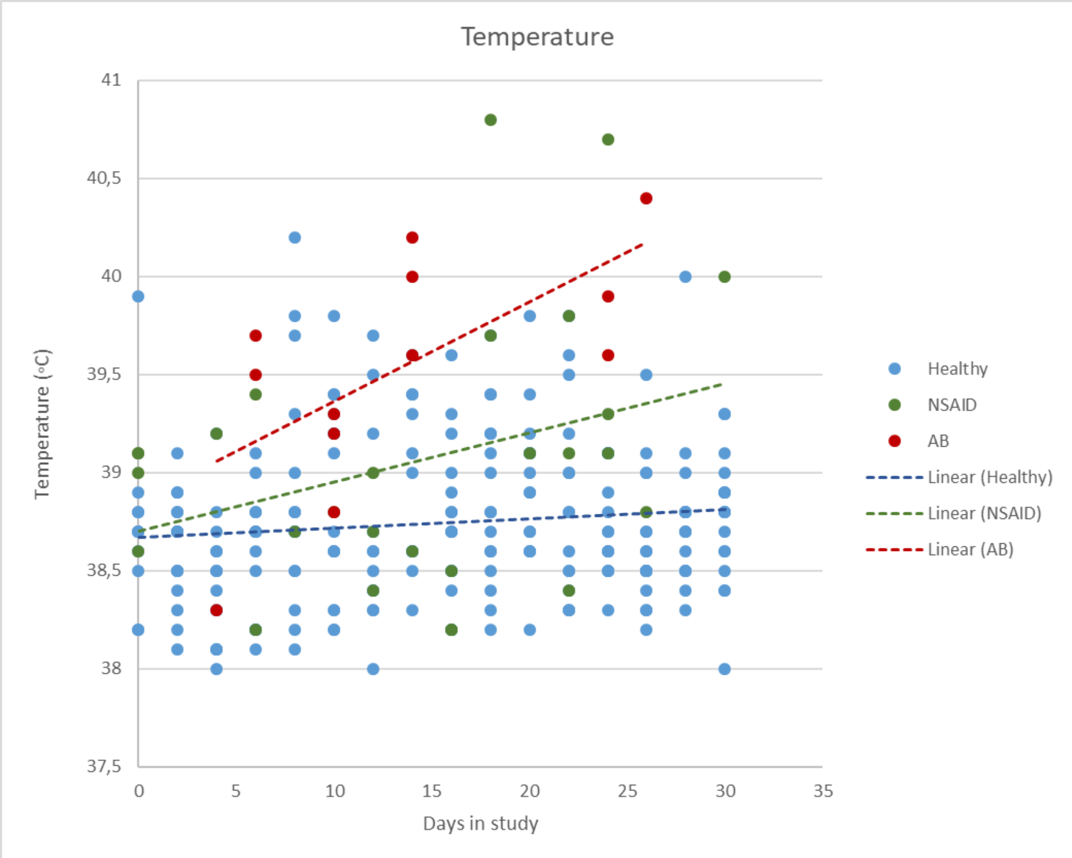

Figure 26. Scatter plots and linear regression line for rectal temperature in degrees Celsius from calves compared with days in study with different treatment regimens for bovine respiratory disease.
Blue: 243 observations from 18 healthy calves from the days they received a VAS score ≤ 1.5.
Green: 27 observations from 20 calves that only had NSAID and on the day they received a VAS score between 2.5 and 4.9.
Red: 12 observations from 11 calves treated with antibiotic on the day they received a VAS score > 5.0.
$F(x)_{Blue} = 0.0047x + 38.7$
$F(x)_{Green} = 0.0288x + 38.7$
$F(x)_{Red} = 0.0506x + 38.9$

The regression lines in Figure 26, showed that NSAID calves had the same temperature as healthy calves at the start of the study, but later in the study, NSAID calves had a higher temperature than healthy calves. Calculated by the equations from the linear regression lines we found that both healthy and NSAID calves started at 38.7°C. Healthy calves were only slightly higher at day 30 (38.8°C) whereas NSAID calves ended at 39.5°C, at day 30. Compared with the group of AB calves, we saw that the temperature started at 38.9°C and ended at 40.4°C, at day 30. Seven percent (16/243) of the observations from healthy calves had a temperature above 39.5°C. Eighty percent (21/26) of the observations from NSAID calves were below 39.5°C, and 33% (4/12) of the observations from AB calves were below 39.5°C.

Lymphocytes. Figure 27 presents and compares days in study with the number of lymphocytes from the differential count in calves from 18 healthy calves, 20 NSAID calves and 11 AB calves. Observations were from days with a VAS score for healthy calves below 1.5, NSAID calves between 2.5 and 4.9 and for AB calves a VAS score above 5.0.

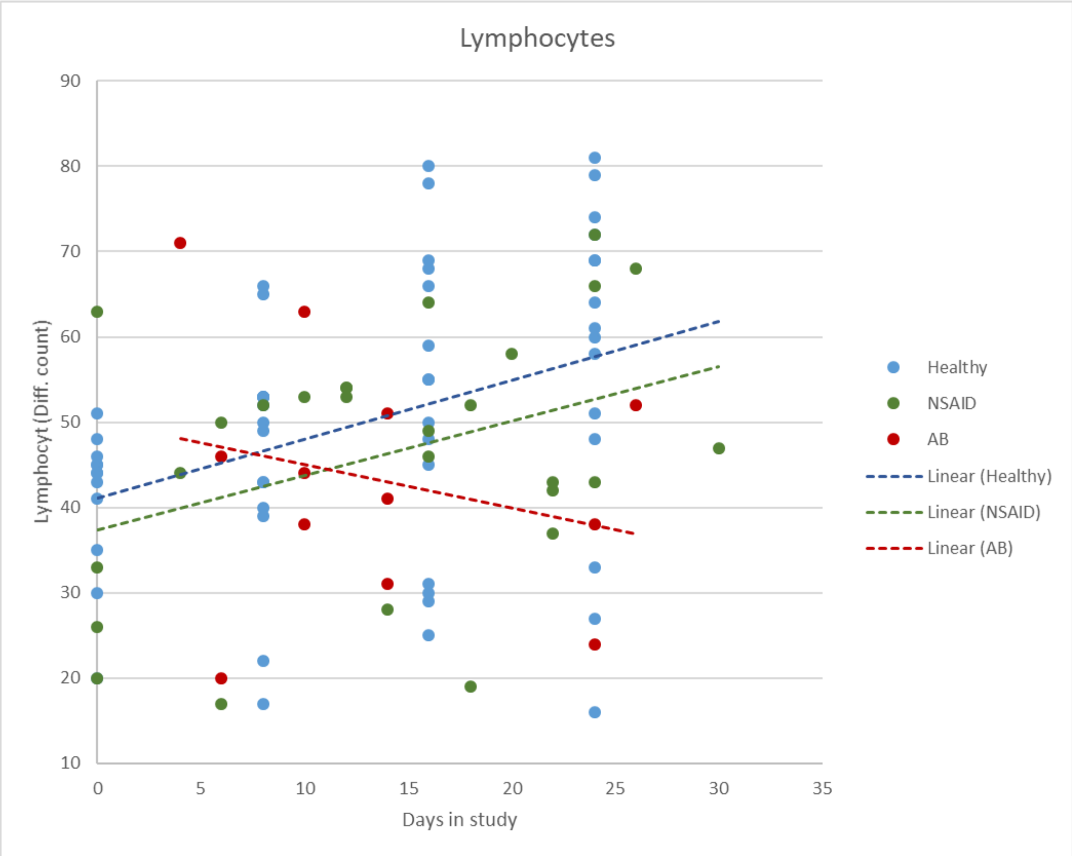

Figure 27. Scatter plots and linear regression line for lymphocytes of calves compared with days in study with different treatment regimens for bovine respiratory disease.
Blue: 57 observations from 18 healthy calves from the days they received a VAS score ≤ 1.5.
Green: 27 observations from 20 calves that only had NSAID and on the day they received a VAS score between 2.5 and 4.9.
Red: 12 observations from 11 calves treated with antibiotic on the day they received a VAS score > 5.0.

In Figure 27, we saw that the proportion of lymphocytes for both healthy and NSAID calves increased through the study. In general, NSAID calves had a lower number of lymphocytes than healthy calves, but there was a large variation in lymphocytes for both healthy and NSAID calves. Healthy calves' lymphocytes on day 24 varied between 16 and 81 per 100 counted cells, and NSAID calves' lymphocytes in general varied from 17 per 100 counted cells on day six to 72 per 100 counted cells on day 24.

Mature neutrophils. Figure 28 presents and compares days in study with the number of mature neutrophils from the differential count in calves from 18 healthy calves, 20 NSAID calves and 11 AB calves. Observations were from days with a VAS score for healthy calves below 1.5, NSAID calves between 2.5 and 4.9 and for AB calves a VAS score above 5.0.

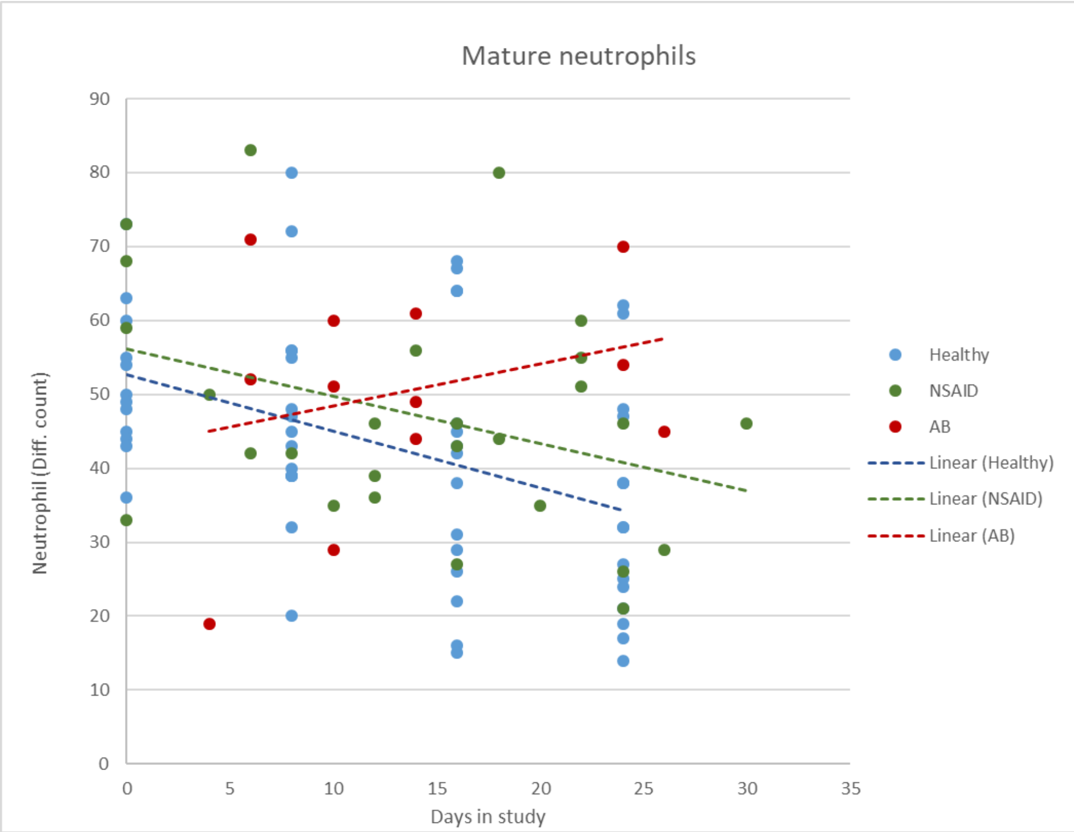

Figure 28. Scatter plots and linear regression line for mature neutrophils compared with days in study with different treatment regimens for bovine respiratory disease.
Blue: 57 observations from 18 healthy calves from the days they received a VAS score ≤ 1.5.
Green: 27 observations from 20 calves that only had NSAID and on the day they received a VAS score between 2.5 and 4.9.
Red: 12 observations from 11 calves treated with antibiotic on the day they received a VAS score > 5.0.

The mature neutrophils acted opposite to lymphocytes (Figure 27 and 28). As seen in Figure 28, the number of mature neutrophils from both healthy and NSAID calves decreased through the study. NSAID calves had in general a higher number of mature neutrophils than healthy calves no matter how long the calves had been in the study. Mature neutrophils had a great variation between calves just as the other parameters.

α_2- and γ-globulins. Figure 29 and 30 compared days in study with α_2- and γ-globulin fractions from the capillary electrophoreses from 17 healthy calves, 19 NSAID calves and 11 AB calves. Observations were from days with a VAS score for healthy calves below 1.5, NSAID calves between 2.5 and 4.9 and for AB calves a VAS score above 5.0.

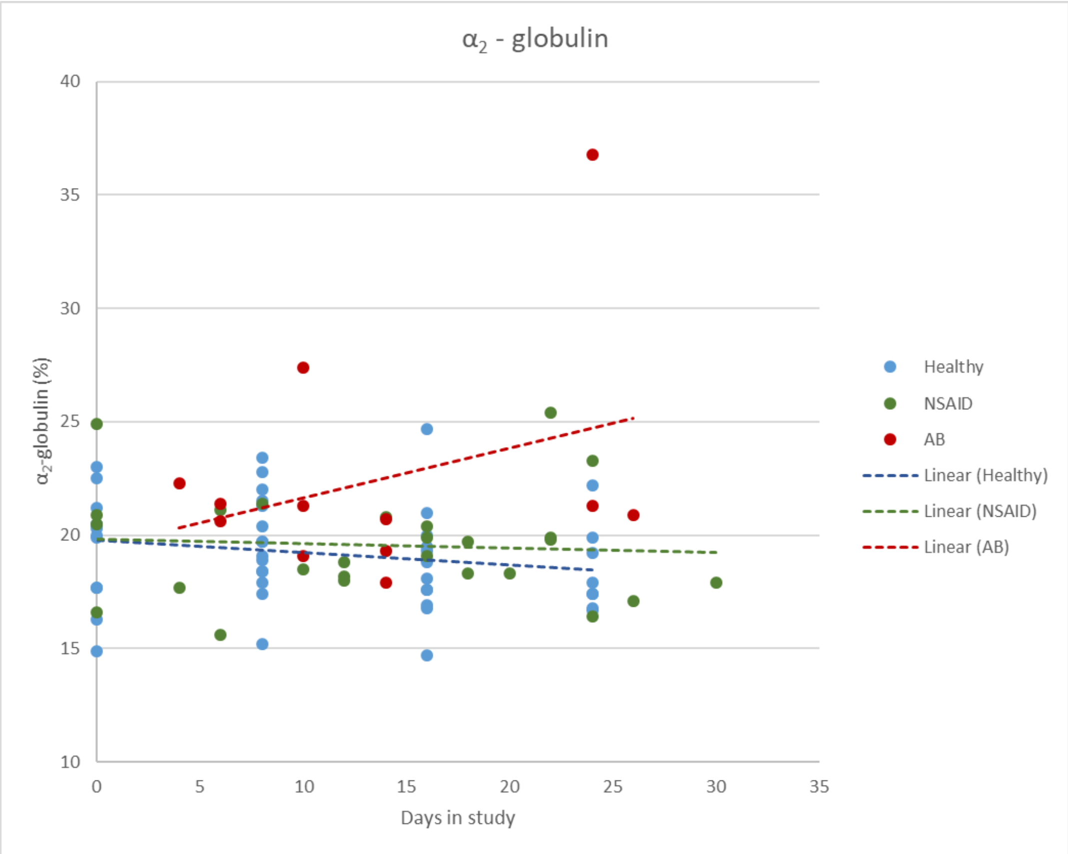

Figure 29. Scatter plots and linear regression line for α_2-globulin compared with days in study with different treatment regimens for bovine respiratory disease.
Blue: 47 observations from 17 healthy calves from the days they received a VAS score ≤ 1.5.
Green: 26 observations from 19 calves that only had NSAID and on the day they received a VAS score between 2.5 and 4.9.
Red: 12 observations from 11 calves treated with antibiotic on the day they received a VAS score > 5.0.

In Figure 29 we saw that NSAID and healthy calves' α_2-globulin fractions started at the same point, but NSAID calves had slightly higher α_2-globulin fraction at the end of the study. They both had a slight decreased with days in study, whereas AB calves' α_2-globulin fractions increased and were higher than the other groups. There was a great variation between samples, and in general the scatter plots for NSAID and healthy calves were in the same range from 14-26% α_2-globulin.

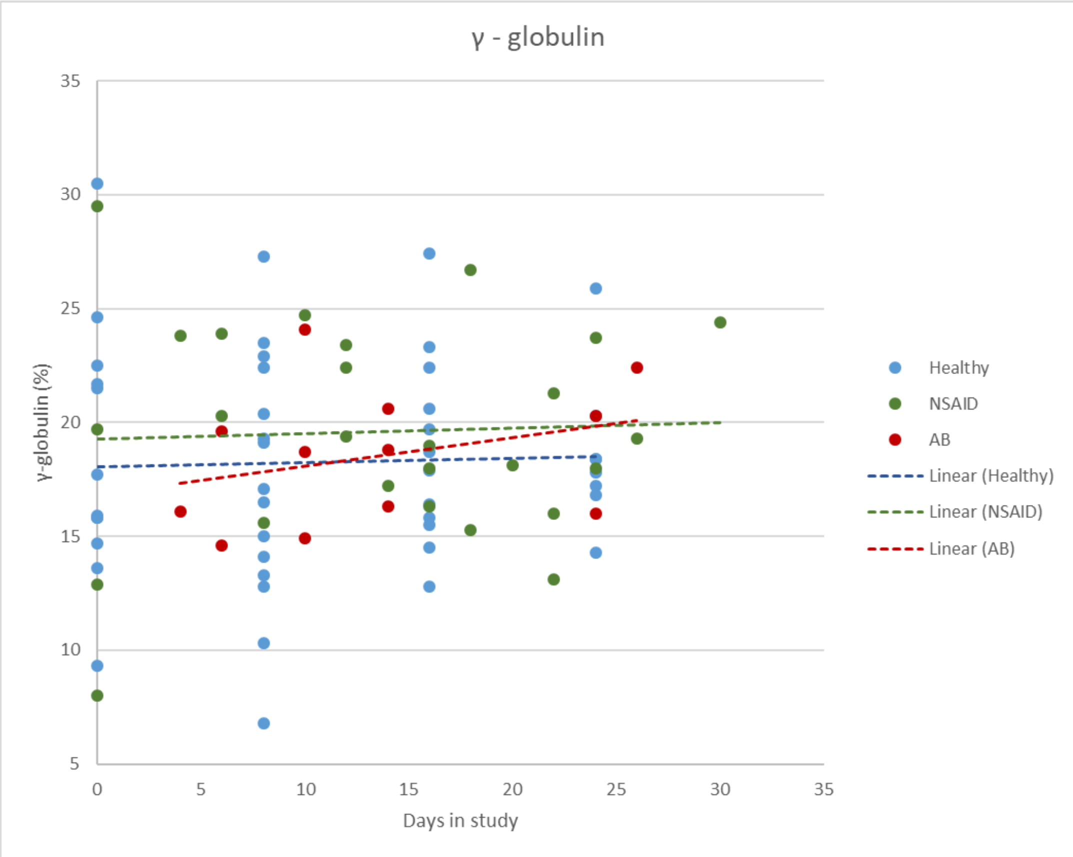

Figure 30. Scatter plots and linear regression line for γ-globulin compared with days in study with different treatment regimens for bovine respiratory disease.
Blue: 47 observations from 17 healthy calves from the days they received a VAS score ≤ 1.5.
Green: 26 observations from 19 calves that only had NSAID and on the day they received a VAS score between 2.5 and 4.9.
Red: 12 observations from 11 calves treated with antibiotic on the day they received a VAS score > 5.0.

As seen in Figure 30, healthy and NSAID calves' γ-globulin fractions were parallel, where NSAID calves' γ-globulin fractions were a little higher than healthy calves' γ-globulin fractions. AB calves' γ-globulin fractions crossed through the other two groups. Furthermore, there was a big variation between the calves' γ-globulin fractions, e.g. healthy calves' γ-globulin fractions on day eight in the study variated from 6.8% to 27.3% and NSAID calves' γ-globulin fractions varied from 8% to 29.5% on day zero.

3.2.2 Boxplot for healthy and NSAID calves

Boxplots were made to visualize the difference between healthy and NSAID calves. Table 3 presents the results from the t-test to compare if there were a significant difference between healthy calves and NSAID calves.

Table 3. Table of mean, standard deviation (SD) and significant level (P-value) for healthy calves and NSAID calves treated for bovine respiratory disease.
Healthy calves with observations from days the received a VAS score ≤ 1.5.
NSAID calves were calves treated with NSAID and observation from the days they received a VAS score between 2.5 and 4.9.
n: number of observations
α_2- and γ-globulin was in percentages from capillary electrophoresis.
Mature neutrophils and lymphocytes were from differential count per 100 cells counted
Activity was from SenseHub and temperature was in degrees Celsius

	Activity µ (±SD)	Temperature µ (±SD)	Mature neutrophils µ (±SD)	Lymphocytes µ (±SD)	α2-globulin µ (±SD)	γ-globulin µ (±SD)
Healthy calves	366.5 (87.2) n = 172	38.7 (0.4) n = 243	43.0 (16.2) n = 57	49.8 (16.5) n = 57	19.2 (2.3) n=47	18.3 (4.8) n=47
NSAID calves	307.8 (67.9) n = 25	39.1 (0.7) n = 27	47.1 (15.9) n = 27	46.4 (15.1) n = 27	19.6 (2.4) n=26	19.6 (4.8) n=26
P-value	< 0.001	0.03	0.28	0.35	0.55	0.25

Activity and rectal temperature. Figure 31 presents the boxplot for activity in the two groups (healthy and NSAID calves) based on observations from 15 healthy calves with a VAS score below 1.5 and 19 NSAID calves with a VAS score between 2.5 and 4.9. The boxplot and t-test showed that NSAID calves had a general lower activity (P < 0.001) compared to healthy calves (Table 3).

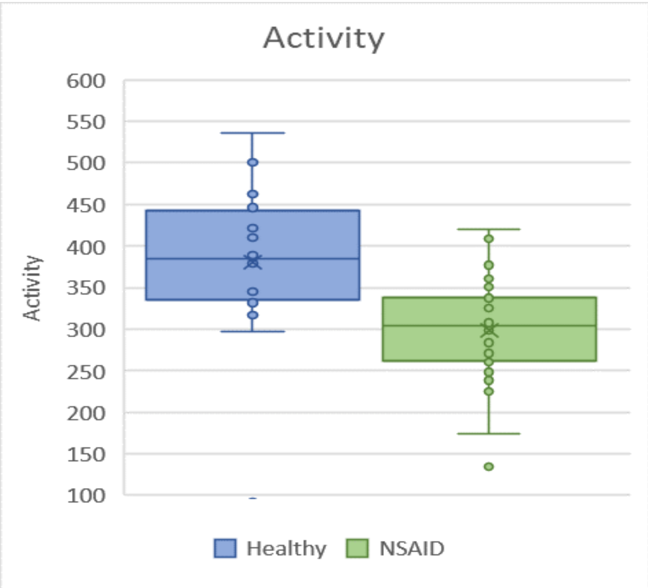

Figure 31. Boxplot for activity of healthy calves compared with NSAID calves treated for bovine respiratory disease.
Blue: 172 observations from 15 healthy calves from days the received a VAS score ≤ 1.5.
Green: 25 observations from 19 calves treated with NSAID and on the days they received a VAS score between 2.5 and 4.9.

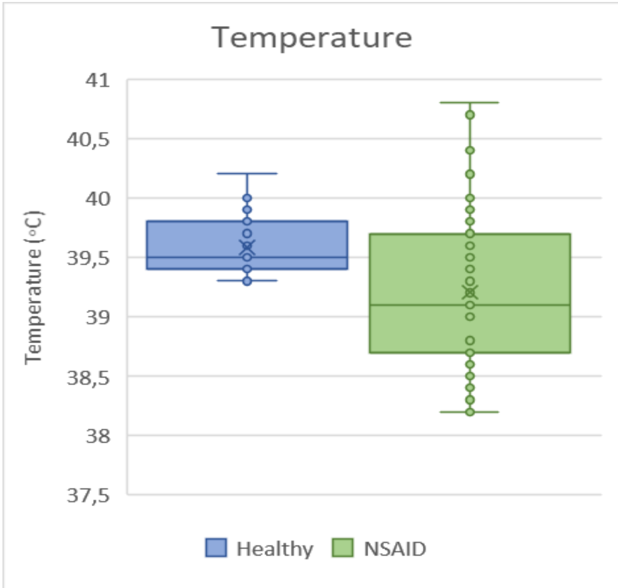

Figure 32. Boxplot for rectal temperature in degrees Celsius of healthy calves compared with NSAID calves treated for bovine respiratory disease.
Blue: 243 observations from 18 healthy calves from days the received a VAS score ≤ 1.5.
Green: 27 observations from 20 calves treated with NSAID and on the day they received a VAS score between 2.5 and 4.9.

Figure 32 presents the boxplot for rectal temperature from 18 healthy calves with a VAS score below 1.5 and 20 NSAID calves with a VAS score between 2.5 and 4.9. As shown in Figure 32, NSAID calves had a great variation between observations, but despite that NSAID calves had a higher temperature compared with healthy calves (P = 0.03) (Table 3).

Lymphocytes and mature neutrophils. Figure 33 and 34 present the number of lymphocytes and mature neutrophils from differential count for 18 healthy calves and 20 NSAID calves. As shown in Figure 33 and 34, there was a slight difference between healthy calves and NSAID calves. Both groups had a great variation between observations and did not differ from each other (P = 0.28 and P = 0.35) (Table 3).

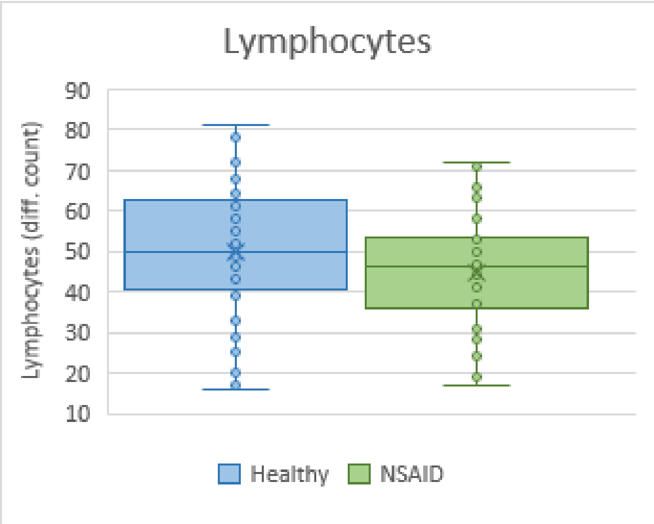

Figure 33. Boxplot for lymphocytes of healthy calves compared with NSAID calves treated for bovine respiratory disease.
Blue: 57 observations from 18 healthy calves from days they received a VAS score ≤ 1.5.
Green: 27 observations from 20 calves treated with NSAID and on the day they received a VAS score between 2.5 and 4.9.

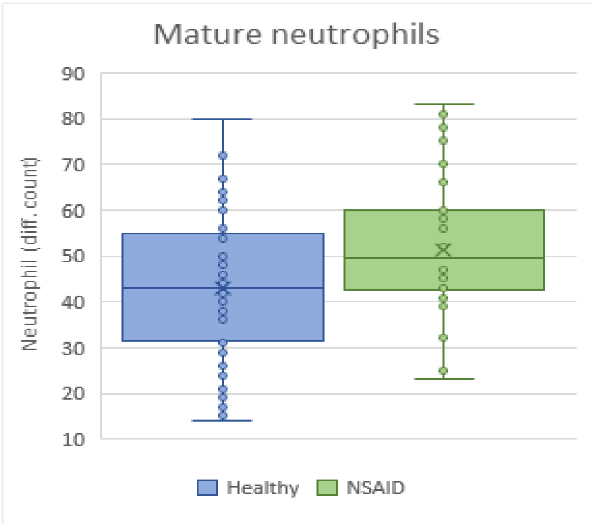

Figure 34. Boxplot for mature neutrophils of healthy calves compared with NSAID calves treated for bovine respiratory disease.
Blue: 57 observations from 18 healthy calves from days they received a VAS score ≤ 1.5.
Green: 27 observations from 20 calves treated with NSAID and on the day they received VAS score between 2.5 and 4.9.

α_2- and γ-globulins. Figure 35 and 36 present α_2- and γ-globulin fractions from capillary electrophoreses from 17 healthy and 19 NSAID calves. As we saw in Figure 35 and 36, α_2- and γ-globulins were slightly visually higher for NSAID calves compared to healthy calves, but there was no significant difference for either of the groups (P = 0.55 and P = 0.25) (Table 3).

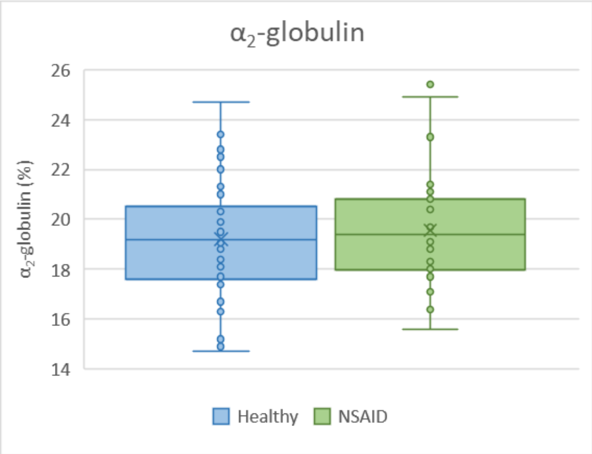

Figure 35. Boxplots from γ-globulins of healthy calves compared with NSAID calves treated for bovine respiratory disease.
Blue: 47 observations from 17 healthy calves from the days they received a VAS score ≤ 1.5.
Green: 26 observations from 19 calves that only had NSAID and on the day they received a VAS score between 2.5 and 4.9.

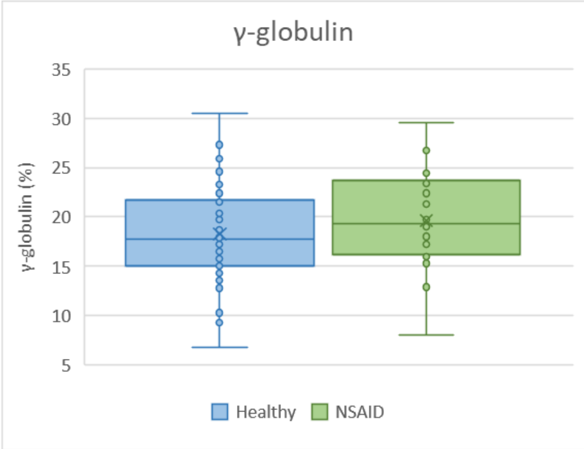

Figure 36. Boxplots from γ-globulins of healthy calves compared with NSAID calves treated for bovine respiratory disease.
Blue: 47 observations from 17 healthy calves from the days they received a VAS score ≤ 1.5.
Green: 26 observations from 19 calves that only had NSAID and on the day they received a VAS score between 2.5 and 4.9.

3.3 Course of NSAID treated calves and healthy calves

In this part of the results, we needed calves based on the criteria mentioned in section 2.4. Out of our 33 calves treated with NSAID, 15 calves were included. The 15 calves were only treated with NSAID, and had a disease course with samples from before, under and after the treatment with NSAID. The WDC corresponded to the following row color in Figure 9: white, green, green, white and white. Table 4 presents the P-values for every parameter from the repeated measure ANOVA made on the disease course from the 15 NSAID calves.

Table 5 was made to create an overview over the 15 calves' individual changes in parameters through the disease course and the NSAID treatment based on Figure 35 to 50.

Table 4. P-values for repeated measure ANOVA made on disease course from 15 NSAID calves with each individual calf as fixed effect.
α_2- and γ-globulin was in percentages from capillary electrophoresis.
Mature neutrophils and lymphocytes were from differential count per 100 cells counted
Activity was from SenseHub and temperature was in degrees Celsius

Parameters	P-value
Activity	0.06
Temperature	0.24
Mature neutrophils	0.14
Lymphocytes	0.11
Monocytes	0.71
Immature neutrophils	0.88
α_2-globulin	1.00
γ-globulin	0.53

Table 5. Overview over 15 calves change through bovine respiratory disease starting with being healthy and through treatment. Numbers of calves which decreased (↓), increases (↑) or where unchanged (→) from start (before illness) to 1st treatment with NSAID (sick, VAS score between 2.5 and 4.9), from 1st treatment with NSAID to 2nd treatment with NSAID, from 1st treatment with NSAIDS to 1st control and from start to 2nd control.
α_2- and γ-globulin was in percentages from capillary electrophoresis.
Mature neutrophils and lymphocytes were from differential count per 100 cells counted
Activity was from SenseHub and temperature was in degrees Celsius.

Increases/ decreases	Start → 1. treatment	1.treatment → 2. treatment	2. treatment → 1. control	1. treatment → 1. control	Start → 2. control
Activity	10/15 ↓ 3/15↑ 1/15→ 1/15 missing data	4/15↓ 11/15↑	4/15↓ 11/15↑	4/15↓ 10/15↑ 1/15→	6/15↓ 7/15↑ 1/15→ 1/15 missing data
Temperature	3/15↓ 11/15↑ 1/15→	11/15↓ 2/15↑ 1/15→	8/15↓ 5/15↑ 2/15→	10/15↓ 4/15↑ 1/15→	7/15↓ 6/15↑ 2/15→
Neutrophils	7/15↓ 8/15↑	8/15↓ 7/15↑	7/15↓ 7/15↑ 1/15→	11/15↓ 4/15↑	6/15↓ 3/15↑ 1/15→ 5/15 missing data
Lymphocytes	8/15↓ 7/15↑	7/15↓ 8/15↑	7/15↓ 8/15↑	4/15↓ 11/15↑	3/15↓ 6/15↑ 1/15→ 5/15 missing data
α_2-globulin	7/14↓ 3/14↑ 4/14→	6/14↓ 6/14↑ 2/14→	6/14↓ 3/14↑ 3/14→ 2/14 missing data	7/14↓ 2/14↑ 3/14→ 2/14 missing data	4/14↓ 2/14↑ 1/14→ 7/14 missing data
γ-globulin	9/14↓ 4/14↑ 1/14→	4/14↓ 5/14↑ 5/14→	7/14↓ 5/14↑ 0/14→ 2/14 missing data	5/14↓ 3/14↑ 4/14→ 2/14 missing data	5/14↓ 2/14↑ 0/14→ 7/14 missing data

Activity. In Figure 35 and 36 the activity for the 15 calves was presented. The calves acted quite different in their disease course (P = 0.06), but ten out of 15 decreased in activity when they got sick and 11 out of 15 had an increase in activity from first treatment to first control, see Table 4 and Table 5.

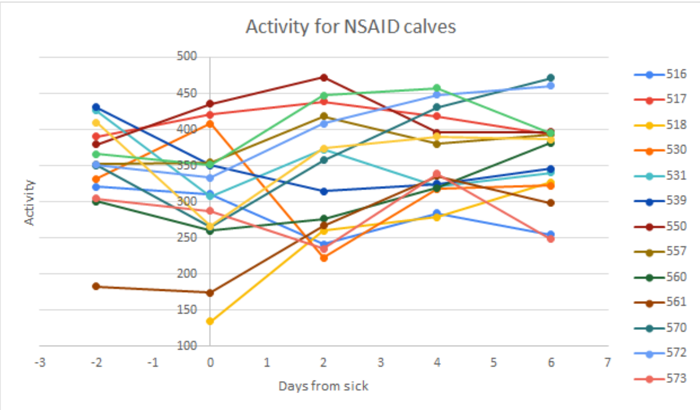

Figure 35. SenseHub activity data from 15 calves with a VAS score between 2.5 and 4.9 on day 0. The calves were treated with NSAID on day 0 and 2.
Data from 2 days before first day of treatment, from the days of treatments and controls day 4 and 6.
Calf #518 didn't have data from day -2.

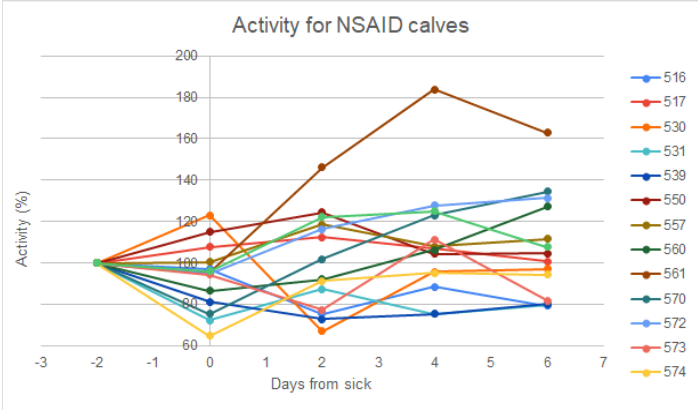

Figure 36. SenseHub activity data from 15 calves with a VAS score between 2.5 and 4.9 on day 0, where day -2 were set to 100 percent. The calves were treated with NSAID on day 0 and 2.
Data from 2 days before first day of treatment, from the days of treatments and controls from day 4 and 6.

To compare the activity from our 15 NSAID calves, we took the activity from nine healthy calves. The nine healthy calves, which had at least 10 observations with a VAS score ≤ 1.5, were present in Figure 37. Like the 15 NSAID calves, the healthy calves' activity was very individual with activity data from 200 to 600, and each calf differed through the study period.

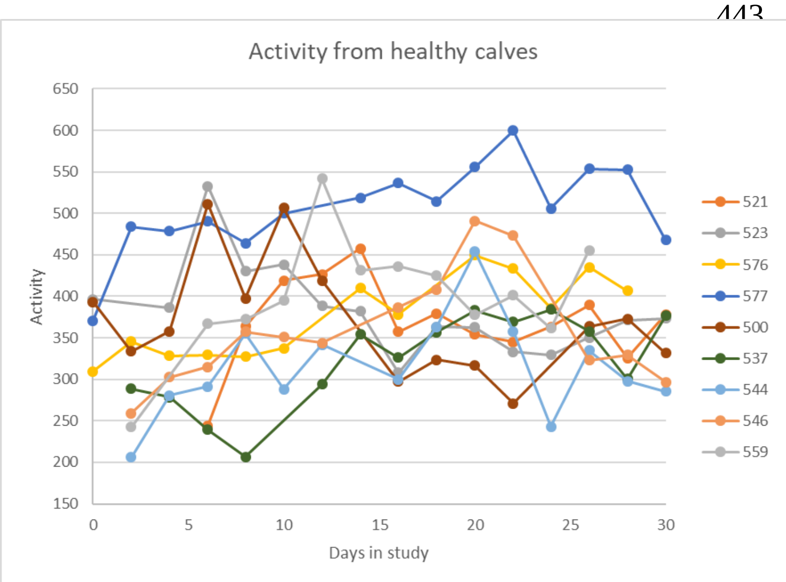

Figure 37. SenseHub activity from 9 healthy calves never treated in the study and 10 observations with a VAS score ≤ 1.5.

Rectal temperature. Figure 38 and 39 present the rectal temperature from the 15 NSAID calves. As shown in the two figures, the temperature varied between calves and there was no significant difference throughout the disease course (P = 0.24) (Table 4). Despite this, eleven out of 15 calves had an increase in temperature when they got sick and from first treatment to first control, we saw a drop in temperature in 11 of the 15 calves (Table 5). Eleven out of 15 claves almost returned to the starting temperature on day six (Figure 39).

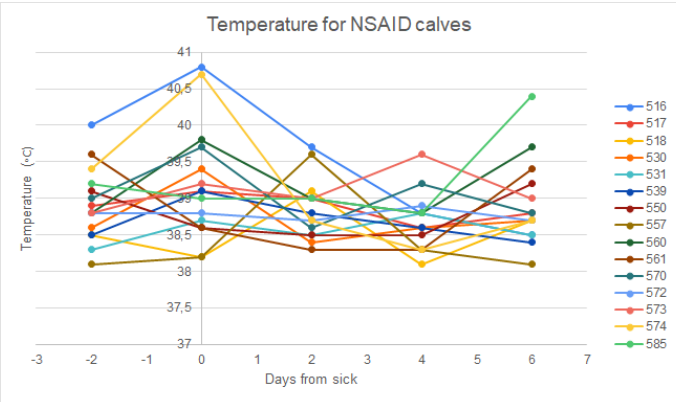

Figure 38. Temperature data from 15 calves with a VAS score between 2.5 and 4.9 on day 0. The calves were treated with NSAID on day 0 and 2.
Day -2 were two days before first treatment, day 0 were the day the calves were scored sick, day 2 were the day of second treatment and day 4 and 6 was the control.

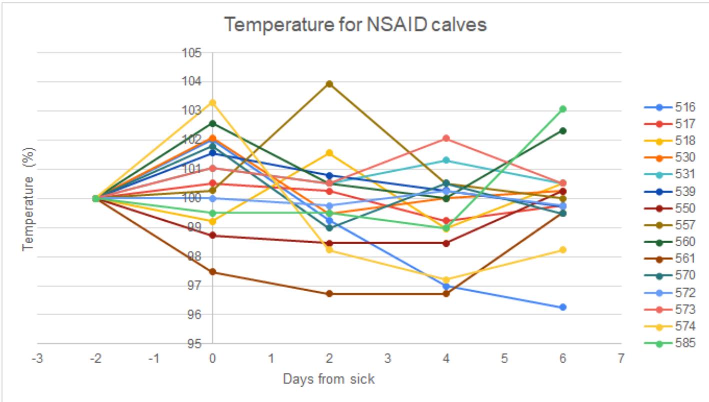

Figure 39. Temperature data from 15 calves with a VAS score between 2.5 and 4.9 on day 0, where day -2 were set to 100 percent. The calves were treated with NSAID on day 0 and 2.
Day -2 were two days before first treatment, day 0 were the day the calves were scored sick, day 2 were the day of second treatment and day 4 and 6 was the control.

To compare our 15 NSAID calves we made Figure 40 with temperature from 17 healthy calves that had 10 or more observations with a VAS score ≤ 1.5 and only from these days. As shown in Figure 40, the 17 healthy calves' temperature varied a lot like the 15 NSAID calves' temperature did. But, 93% (220/236) of the measured temperatures was between 38.0°C and 39.5°C, and only one out of the 17 healthy calves had a temperature above 40°C.

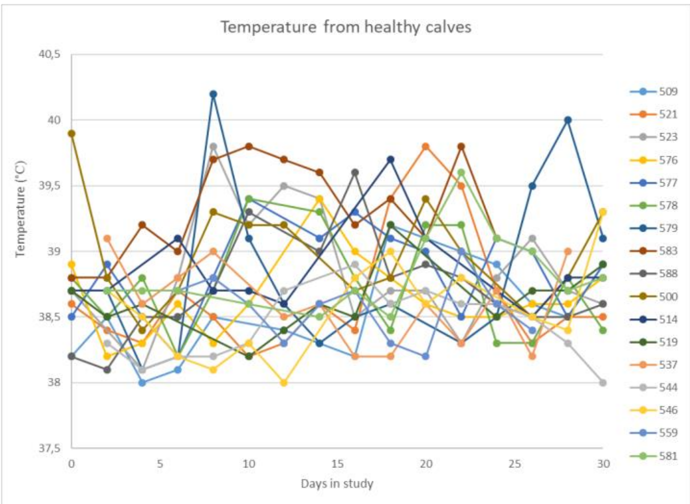

Figure 40. Temperature from 17 healthy calves never treated in the study and with a VAS score ≤ 1.5.

Mature neutrophils and lymphocytes. Figure 41 and 42 present the mature neutrophils for each of the 15 NSAID calves.

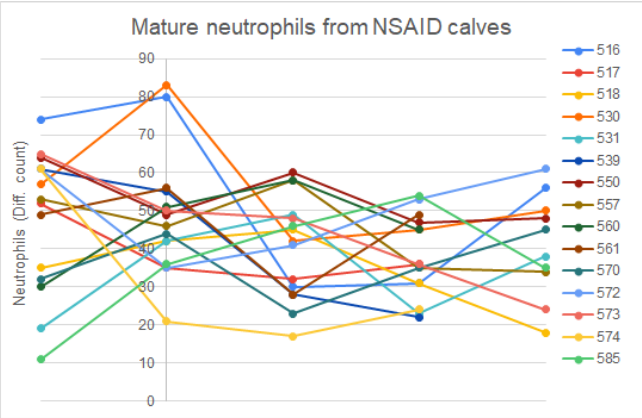

Figure 41. Differential count of mature neutrophils from 15 calves with a VAS score between 2.5 and 4.9 on day 0.
The calves were treated with NSAID on day 0 and 2.
1st point: Blood test from 2-8 days before illness.
2nd point: Ill (VAS score between 2.5 and 4.9), first NSAID treatment (day 0)
3rd point: Second NSAID treatment (day 2)
4th point: The control sample (day 4)
5th point: Control sample 6-20 days after illness.

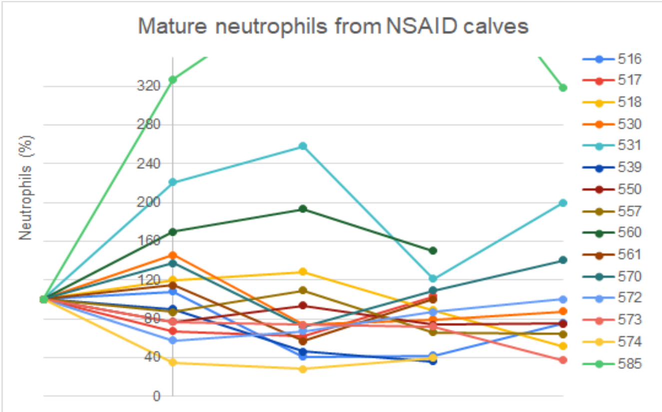

Figure 42. Mature neutrophils from 15 calves with a VAS score between 2.5 and 4.9 on day 0, where day -2 were set to 100 percent.
The calves were treated with NSAID on day 0 and 2.
1st point: Blood test from 2-8 days before illness.
2nd point: Ill (VAS score between 2.5 and 4.9), first NSAID treatment (day 0)
3rd point: Second NSAID treatment (day 2)
4th point: The control sample (day 4)
5th point: Control sample 6-20 days after illness.
Some of the line for calf #585 is not seen because it was so high (490 % on day 4).

In Figure 41 we saw the mature neutrophils were widely spread in our 15 calves. The amount of counted mature neutrophils varied from 15-75 per 100 counted cells before illness and almost the same range (25-83 per 100 counted cells) was seen on the day the calves were scored sick (VAS between 2.5 and 4.9). Almost the same number of calves increased and decreased until first control, though 11 out of 15 decreased from day zero to day four (Table 5).

Figure 43 and 44 present the differential count of lymphocytes for each of the 15 NSAID calves. In the same way as mature neutrophils, Figure 43 and Figure 44 showed that lymphocytes were widely spread. The first differential counts were between 24–73 lymphocytes per 100 counted cells and between 17-72 on the day they got a VAS score between 2.5 and 4.9. Approximately the same numbers of calves were increasing and decreasing in the disease course (P = 0,11), though 11 out of 15 increased from first treatment to first control, see Table 4 and Table 5.

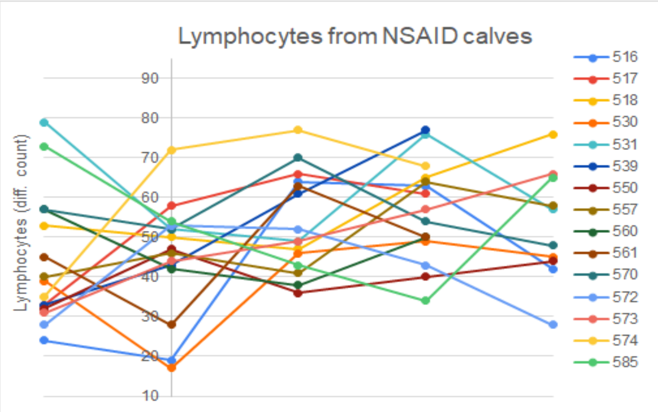

Figure 43. Differential count of lymphocytes from 15 calves with a VAS score between 2.5 and 4.9 on day 0. The calves were treated with NSAID on day 0 and 2.
1st point: Blood test from 2-8 days before illness.
2nd point: Ill (VAS score between 2.5 and 4.9), first NSAID treatment (day 0)
3rd point: Second NSAID treatment (day 2)
4th point: The control sample (day 4)
5th point: Control sample 6-20 days after illness.

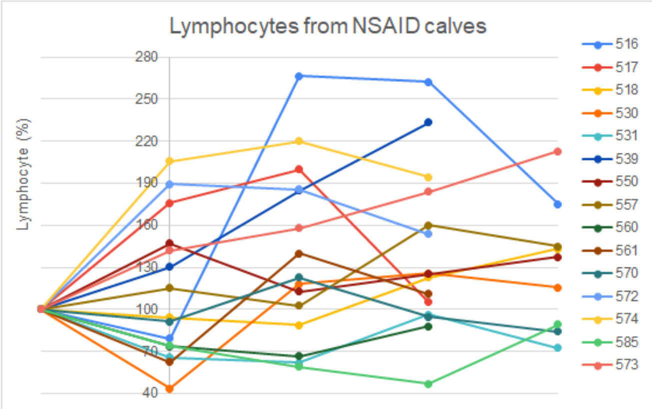

Figure 44. Lymphocytes *from 15 calves with a VAS score between 2.5 and 4.9 on day 0, where day -2 were set to 100 percent. The calves were treated with NSAID on day 0 and 2.*
1st point: Blood test from 2-8 days before illness.
2nd point: Ill (VAS score between 2.5 and 4.9), first NSAID treatment (day 0)
3rd point: Second NSAID treatment (day 2)
4th point: The control sample (day 4)
5th point: Control sample 6-20 days after illness.

Figure 45 and 46 present mature neutrophils and lymphocytes from nine healthy calves, which had ≤ 1.5 in VAS score on the four zero days: day zero, eight, 16 and 24. Both lymphocytes and mature neutrophils started close to the same starting point. Afterwards they both spread out, and there was a great variation between the healthy calves for both lymphocytes and neutrophils.

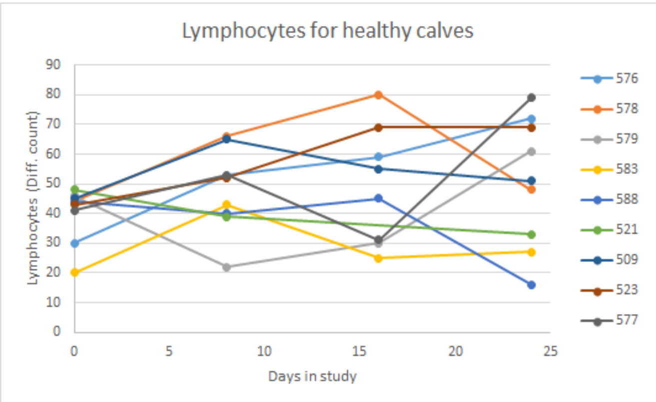

Figure 45. Differential count of lymphocytes from 9 healthy calves never treated in the study and with a VAS score ≤ 1.5.

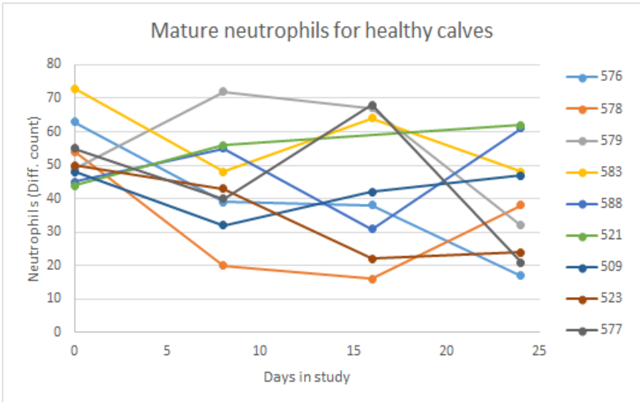

Figure 46. Differential count of mature neutrophils from 9 healthy calves never treated in the study and with a VAS score ≤ 1.5.

α_2- and γ-globulins. Figure 47 presents α_2-globulin from the capillary electrophoreses for 14 out of the 15 NSAID calves, due to samples missing for one calf. Figure 48 presents nine healthy calves that got scored with a VAS score ≤ 1.5 on day zero, eight, 16 and 24. Not all data were analyzed, therefore, some days were missing.

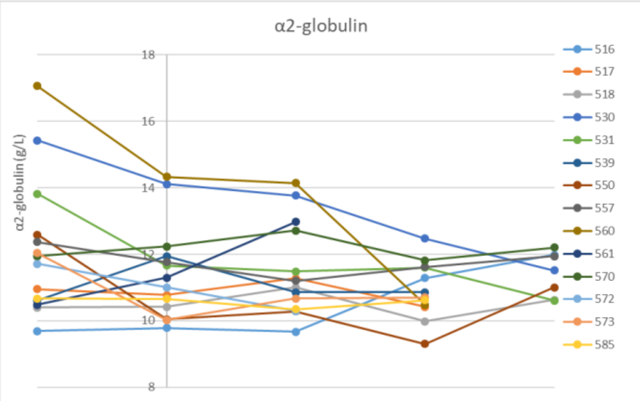

Figure 47. α_2-globulin fraction from 14 calves with a VAS score between 2.5 and 4.9 on day 0. The calves were treated with NSAID on day 0 and 2.
1st point: Blood test from 2-8 days before illness.
2nd point: Ill (VAS score between 2.5 and 4.9), first NSAID treatment (day 0)
3rd point: Second NSAID treatment (day 2)
4th point: The control sample (day 4)
5th point: Control sample 6-20 days after illness.

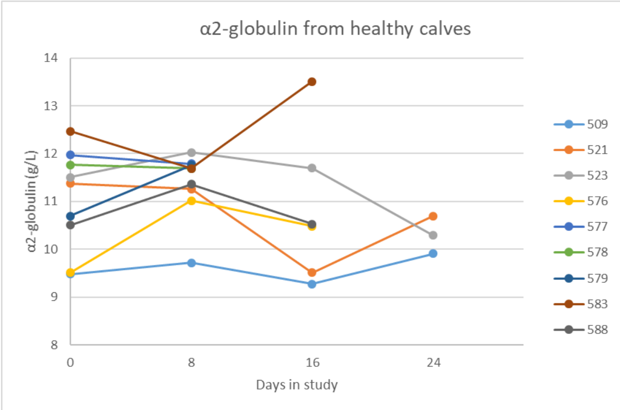

Figure 48. α_2-globulin fraction from 9 healthy calves never treated in the study and with a VAS score ≤ 1.5.

Figure 49 presents γ-globulins from the capillary electrophoreses for 14 out of the 15 NSAID calves, due to missing samples for one calf. Figure 50 presents nine healthy calves that got scored with a VAS score ≤ 1.5 on day zero, eight, 16 and 24. Not all data were analyzed, therefore some days were missing.

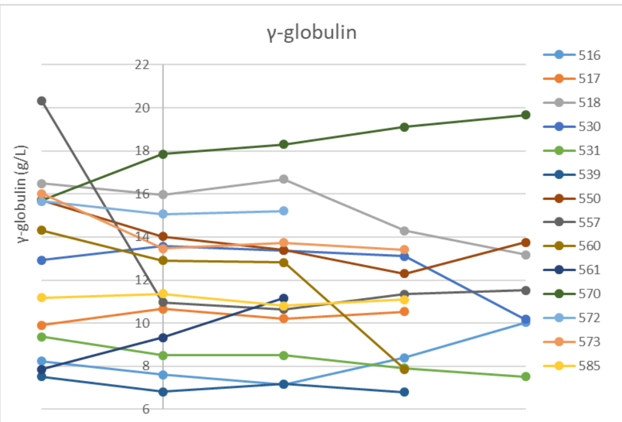

Figure 49. γ-globulin fraction from 14 calves with a VAS score between 2.5 and 4.9 on day 0. The calves were treated with NSAID on day 0 and 2.
1st point: Blood test from 2-8 days before illness.
2nd point: Ill (VAS score between 2.5 and 4.9), first NSAID treatment (day 0)
3rd point: Second NSAID treatment (day 2)
4th point: The control sample (day 4)
5th point: Control sample 6-20 days after illness.

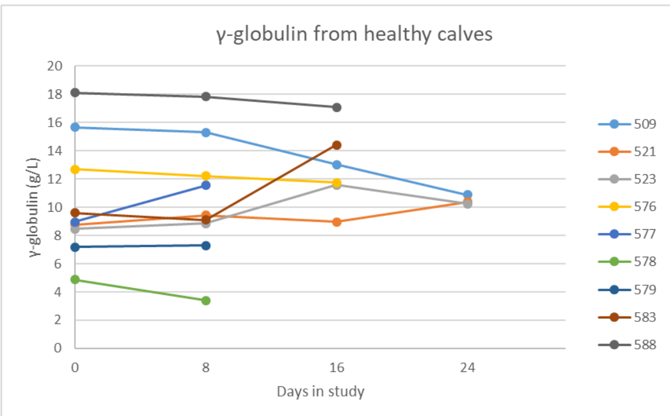

Figure 50. γ-globulin fraction from 9 healthy calves never treated in the study and with a VAS score ≤ 1.5.

When we looked at Figure 47 and 49, there were no clear tendency. Most of the calves' globulin levels did not vary much throughout the disease course (Table 5), if any changes at all the calves' globulin levels decreased a bit, but nothing were significant (P = 1.00 and P = 0.53), see table 4. There was on the other hand a difference in the level of globulins between the calves. The healthy calves' globulin levels were shown in Figure 48 and 50 did not change much either, but some of the healthy calves' globulin levels changed when we looked at α_2-globulin (Figure 48). However, due to missing data from the capillary electrophoreses it was not possible to see a tendency.

3.4 Correlation between α_2- and γ- globulins fractions in serum and our other parameters

The data consist of all 51 calves with different numbers of observations. In Figure 51, 53 and 55 we saw a correlation between α_2- globulin and activity ($P < 0.001$), rectal temperature ($P < 0.01$) and lymphocytes ($P = 0.04$), respectively. However, there was no correlation between α_2-globulin and mature neutrophils ($P = 0.15$), see Figure 57. There was no correlation between γ-globulin and the parameters, see Figure 52 ($P = 0.7$), Figure 54 ($P = 0.7$), Figure 56 ($P = 0.95$) and Figure 58 ($P = 0.97$).

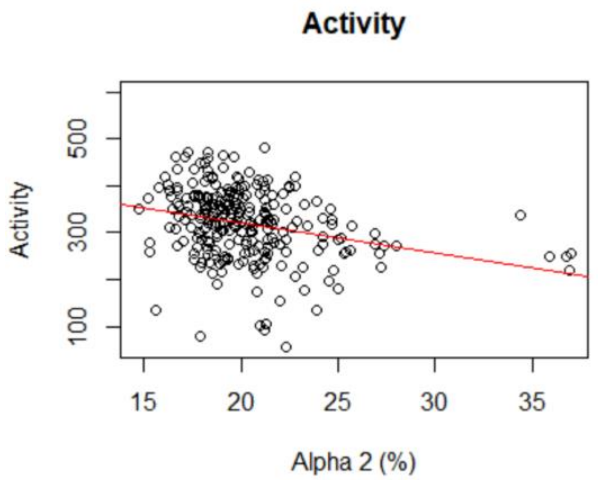

Figure 41. Scatter plots of activity data from SenseHub and the α_2-globulin fractions, with 257 observations from the 51 calves included in the study.
$R^2 = 0.08$, $P < 0.001$

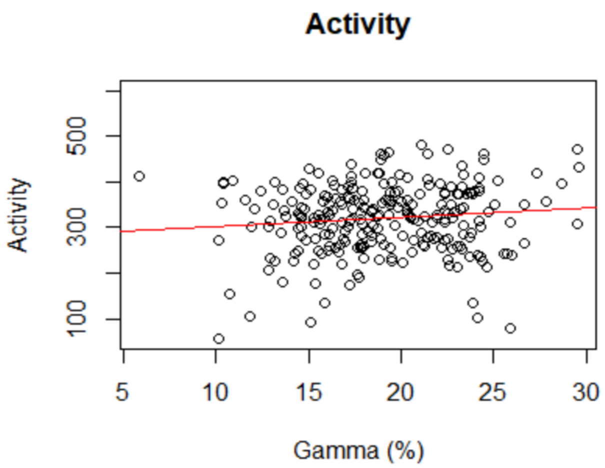

Figure 52. Scatter plots of activity data from SenseHub and the γ-globulin fractions, with 257 observations from the 51 calves included in the study.
$R^2 = 0.01$, $P = 0.7$

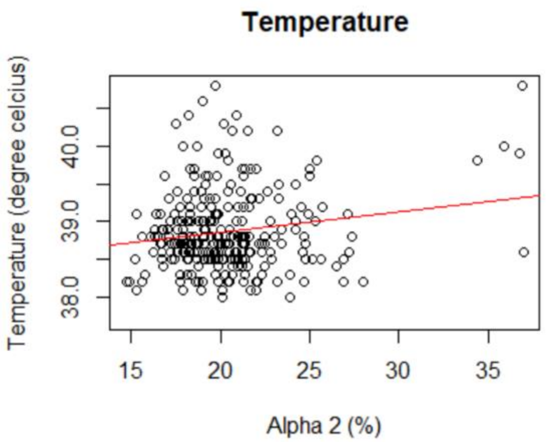

Figure 53. Scatter plots of rectal temperature data and the α2-globulins fractions, with 285 observations from the 51 calves included in the study.
$R^2 = 0.03, P < 0.01$

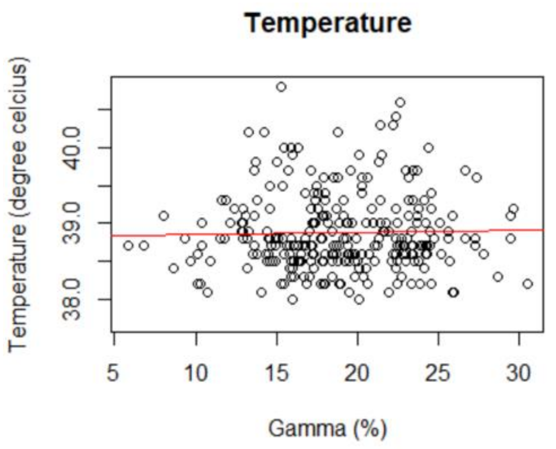

Figure 54. Scatter plots of rectal temperature data and the γ-globulins fractions, with 285 observations from the 51 calves included in the study.
$R^2 = 0.0006, P = 0.7$

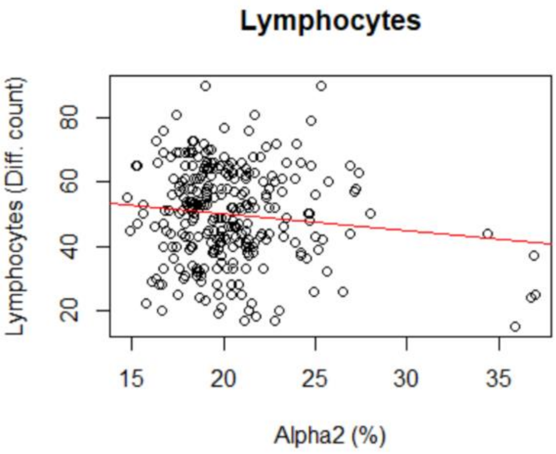

Figure 55. Scatter plots of the number of lymphocytes from the differential count and the α2-globulins fractions from capillary electrophorese on serum, with 282 observations from the 51 calves included in the study.
$R^2 = 0.01, P = 0.04$

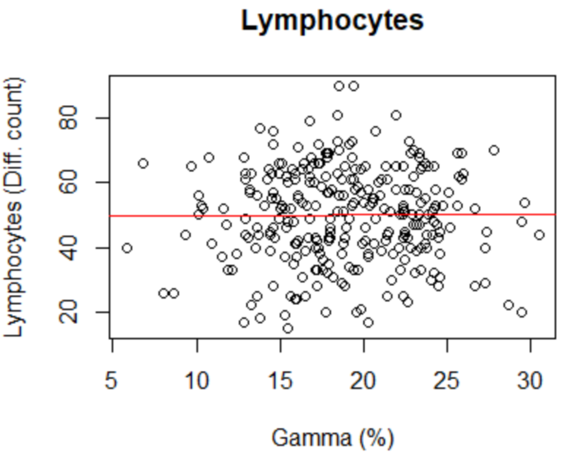

Figure 56. Scatter plots of the number of lymphocytes from the differential count and the γ-globulins fractions from capillary electrophoresis on serum, with 282 observations from the 51 calves included in the study.
$R^2 < 0.0001, P = 0.95$

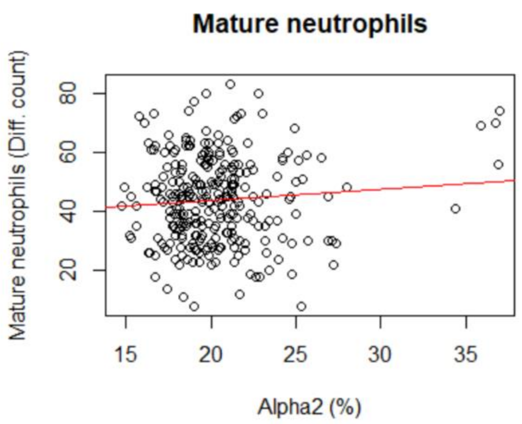

Figure 57 Scatter plots of the number of mature neutrophils from the differential count and the α2-globulins fractions from capillary electrophoresis on serum, with 282 observations from the 51 calves included in the study.
$R^2 = 0.01, P = 0.15.$

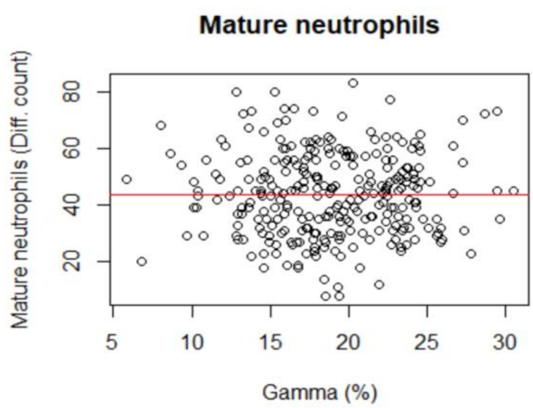

Figure 58 Scatter plots of the number of mature neutrophils from the differential count and the γ-globulins fractions from capillary electrophoresis on serum, with 282 observations from the 51 calves included in the study.
$R^2 < 0.001, P = 0.97$

4. DISCUSSION

4.1 Changes with age

In this study we looked at calves from 2.5 to eight weeks old. When we compared rumination and activity with age, we saw an increase. Rumination had a great increase, which probably was due to the development of the rumen (Warner et al., 1956). Therefore, we did not look further into rumination as a diagnostic marker for calves with BRD. Brun-Hansen et al. (2006) found that lymphocytes and neutrophils changed in the first months of life, especially in the first six to eight weeks. This corresponded to our findings, where lymphocytes increased with age and neutrophils decreased with age.

Our results showed a significant decrease in α_2-globulin compared age. This concurs with another study (Piccione et al., 2009) where they found a significant decrease in α_2-globulin fraction for calves from 15 to 30 days. Tóthová et al. (2015), on the other hand, did not find any significant difference in the α_2-globulin fraction for calves from 14 to 30 days. γ-globulin fraction did not have any correlation with age in our calves, which was also the case for Piccione et al. (2009) and Tóthová et al. (2015), who had no significant change in γ-globulin fraction from two weeks to 30 days. We found a significant increase in total protein with age, whereas Tóthová et al. (2015) found a decrease in calves from 14 to 30 days and Piccione et al. (2009) found no change in calves from 15 to 30 days. The different findings in total protein could be due to different colostrum quality between the calves and the studies but might also be because the studies examined calves at different ages. All three studies looked at calves at the point of time (two to four weeks), where the passive immunity would decrease and the active immunity should increase (Chase et al., 2008). Our study examined the calves until eight weeks, whereas the other studies (Piccone et al., 2009 and Tóthová et al., 2015) only examined calves until 30 days. This could explain why the other studies (Piccone et al., 2009 and Tóthová et al., 2015) saw either no difference or a decrease while we saw an increase in total protein.

4.2 Difference between healthy and sick calves

In this study we wanted to find calves at an early stage of BRD before the development of clinical signs of bacterial infection. Therefore, we compared NSAID calves with healthy calves to see if there was a change in rectal temperature, lymphocytes, neutrophils and activity as well as α_2- and γ-globulin fractions.

4.2.1 Activity and rectal temperature

We found that healthy calves had a higher activity level than NSAID calves, and there was a significant decrease in activity for NSAID calves which was in accordance with Hanzlicek el al. (2010). Hanzlicek el al. (2010) found that lying time, measured with an accelerometer on the distal limb, right after inoculation increased significantly. However, there was a great variation between calves in our study, and, therefore, a cut-off between healthy and NSAID calves was not set. When we compared NSAID calves with AB calves, we saw that AB calves were even lower in activity than NSAID calves. This showed that we possibly found our NSAID calves at an earlier state of the infection than we found our AB calves.

The rectal temperature started at the same level for NSAID and healthy calves, but in the end of the study there was a big difference in temperature between the two groups. Overall, there was a significant difference in temperature between NSAID and healthy calves, which also was found in other studies (Henzlicek et al., 2010; Toaff-Rosenstein and Tucker, 2018). The temperature limit is often set at 39.5°C between healthy and sick calves (Mahendran et al., 2017b; Toaff-Rosenstein and Tucker, 2018). In our study, seven percent of the healthy calves had a temperature above 39.5°C and 80% of NSAID calves had a temperature below 39.5°C. Toaff-Rosenstein and Tucker (2018) found that four percent of healthy calves had a temperature above 39.5°C and 14% of the sick calves had a temperature below 39.5°C. We found more sick calves below 39.5°C than Toaff-Rosenstein and Tucker (2018), which could indicate that we found our calves at an earlier stage of disease. Furthermore, we found that AB calves had higher temperature at all times compared to NSAID calves, which also could show that we found the NSAID calves at an earlier stage of disease than AB calves. Because of variation between calves and the relatively low temperature for NSAID calves it can be difficult to use temperature as a definitive measure for illness but instead only as a guidance.

4.2.2 Lymphocytes and neutrophils

When we looked at lymphocytes and mature neutrophils compared with days in study, we could see there was a difference between healthy and NSAID calves, but when we took the overall differential count for lymphocytes and neutrophils, there was only a slight visual difference and no significant difference. Another study (Buac et al., 2016) found a significant difference between healthy and sick calves for both lymphocytes and neutrophils. Fratric et al. (2011) also found a significant difference in lymphocytes between healthy and sick calves, but not for neutrophils. The different findings in significance level between studies, could be due to the relatively small sample size in the studies

(Fratric et al., 2011; Buac et al.,2016). Another reason for different significant findings, could be the change in the leukocytes after infection. Neutrophils and lymphocytes change a lot over the first 48 hours after infection, they range from higher to lower than normal, before getting back to a normal level three to four days after infection (Jones and Allison, 2007). Therefore, our blood samples could be drawn at a time with high, low or normal levels of neutrophils and lymphocytes, especially when we only took samples every second day. Furthermore, the level of lymphocytes and neutrophils change a lot compared with days in study (day zero in the study equals age 21 day ± four days). This affected that a healthy calf had a general lower level of lymphocytes at day zero in the study than a NSAID calf general had at day 30 in the study. The same applied for neutrophils, but opposite. Therefore, the mean of all calves in all ages could be hard to compare. Furthermore, there was a great difference in the density of cells when we looked at the blood smear. In some smears we only needed to count two to four light fields, and in other smears we should count several rows to find 100 cells.

4.2.3 α_2- and γ-globulin fractions

When we looked at α_2- and γ-globulin fractions compared with days in study, we saw a slight visual difference between healthy and NSAID calves. The values were diffusely distributed and there was no significant difference, especially γ-globulin had a great variation between observations. This could be due to the missing data from the capillary electrophoreses because of economic limitations, and, therefore, a smaller sample size. Furthermore, the level of APPs in the circulation increased rapidly within the first 24 to 48 hours (Gabay and Kushner, 1999; Peterson et al., 2004) and we were only at the farm every second day. Therefore, blood samples could be from different times in the increase of APPs. A study (Tóthová et al., 2012) did not find a difference in α_2-globulin either but found a significant difference in γ-globulin. This difference in γ-globulin between our studies could be because Tóthová et al. (2012) looked at calves from four to six-months-old after the calf's immune system works properly, whereas our calves' immune system was not fully developed yet (Chase et al., 2008). α_2-globulin did not increase, as expected (Kaneko, 1997; O'Connell et al., 2005). There is no clear reason for this. It could be because of the small sample size or because we found the calves at an early stage of disease and the APPs did not increase as much as earlier studies had shown.

4.3 NSAID calves

4.3.1 Individual calves

Out of the 51 calves in this study, 33 calves got treated at some point and 22 of them were treated with NSAID alone. To make sure we could follow the effect of NSAID treatment, we used calves with the WDC for the statistics. Only 15 calves had the WDC. We saw that calves were very individual with different starting points for both activity, rectal temperature, lymphocytes and mature neutrophils which made it difficult to compare and see an overall tendency. Therefore, we converted all our data, from the parameters mentioned previously, to percent so the first sample (the healthy sample) was converted to 100%, and the following days as percentage out from the first day. This still showed a great variation between calves and no significant differences through the disease course were found. We also compared the 15 NSAID calves with healthy calves, to see if the healthy calves had less variation. The healthy calves still variated a lot, both between the calves and within each calf's own samples. This could suggest that there in general was a great variation, which made it difficult to use the parameters as a clear sign of recovery.

Although we found that the calves were very individual in the various parameters, there were some tendency when we looked at how these parameters changed. Most calves had a decrease in activity when they became sick and an increase in activity after first treatment of NSAID. We saw the opposite tendency when we looked at the courses of rectal temperature, where most calves' temperature increased when they became sick and decreased after the first treatment. This could suggest that the NSAID treatment had an efficacy on calves found at an early stage of disease.

When we looked at lymphocytes and mature neutrophils, we saw that equal numbers of calves' lymphocytes and mature neutrophils increased and decreased when they became sick. However, if we looked at the courses for mature neutrophils from the day, they were scored sick (first treatment) to first control (four days after they were scored sick) there was a decrease in 11 out of 15 calves. For lymphocytes the opposite happened as 11 out of 15 calves had an increase in the number of lymphocytes from first treatment to first control sample. This could suggest that NSAID treatment had an effect, but with a slight delay regarding the leukocyte levels. Although, this can also be because of the normal changes in neutrophil and lymphocyte levels in blood after an infection (Jones and Allison, 2007) and the time between our blood samples.

When looking at α_2- and γ-globulin fractions for the course of each calf there was no clear tendency and the calves were very individual in their globulin levels. If anything, there was a slight decrease in the values for α_2-globulin fraction, this could be due to the change we found when comparing with age. Furthermore, it was difficult to compare with the healthy calves, because we did not have all our healthy calves' results.

4.3.2 NSAID treatment efficacy

When we looked at illness in our study, 65% (33/51) of the calves developed signs of BRD. This was almost the same as Mahendran et al. (2017a), who found that 61.9% of the calves got sick. Mahendran et al. (2017a) found that 25.7% of the calves recovered from treatment with NSAID (flunixin meglumine 2 mg/kg for three consecutive days) based on absence of fever (< 39.7°C) detected by TempVerified FeverTags. Our study used VAS score and not fever to determine illness and recovery and found that 37% (11/30) recovered after NSAID treatment (meloxicam twice with 48 hours between). The difference in recovered calves between the studies could be due to Mahendran et al. (2017a) using temperature whereas we used VAS score to find sick calves and measure recovery which might have an impact as we saw a lot (80%) of our NSAID calves' temperatures were lower than 39.5°C. Sixty-three percent (19/30) of our NSAID treated calves did not recover after NSAID treatment. Eleven out of 30 received more NSAID treatments and 27% (8/30) needed antibiotic treatment afterwards. One of the reasons for repeated treatments could be that we caught the disease too late since we only visit the farm every second day. Therefore, in some cases it was difficult to start treatment before the disease had progressed too much. This could also apply to the three calves which only got one antibiotic treatment and recovered from this without a previous NSAID treatment. It was difficult to know if a NSAID treatment could have been enough in these cases if we had caught them earlier in the disease course.

4.4 VAS score

In other studies WI score has been used to score calves for BRD (Mahendran et al., 2017b), but in this study we used the VAS score. The VAS score looks at the general well-being of the calves, including the clinical signs e.g. nasal discharge and heavy breathing. Activity, appetite and social interaction are clinical signs corresponding to the general well-being, and a change in these behaviors are some of the first signs of illness (Hixson et al., 2018). Whereas nasal discharge, cough and ocular discharge, which is used to score calves with WI (McGuirk and Peek, 2014), are clinical signs seen later in the disease course (Timisit et al., 2010). The advantage of using VAS to score the calves, was that we looked at their overall health, and not only the clinical signs for BRD. However, this did make the scoring with VAS score more subjective because there was no clear distinction between the scores, and the VAS score were continuous whereas the WI score was categoric. Furthermore, a study (Williamson and Hoggart, 2005) showed that repeated scores using VAS score can vary by as much as 20%. In addition, there were two observers (NC and HM) in this study, and there could be a difference between how they scored the calves. To minimize the biases, we tried to keep the observants blinded by not telling how the calf's in question was the last time, what the ear tag number of the calf they were looking at was etc. However, this was difficult. Sometimes the observant could remember which calves were treated a lot or last time, and, therefore, had this in mind when giving the score. Some of the calves got antibiotics after three times of NSAID treatment, even though they were not worse than a NSAID calf. Sometimes the score changed after it was given, e.g. if a calf had a high or low rectal temperature in the examination afterwards or had just slept and seemed sicker than they were. Some of the calves were feeble throughout the experiment, and, therefore, easy to remember and hard to score. For most of the study period we had around 50 calves that were given a VAS score every second day. More mistakes were made with 50 calves a day than in the beginning with fewer calves a day. A few calves got a treatment when they should not, and vice versa, due to wrong data handling and the fact that there were many calves to keep track of.

4.4.1 Parameters compared to VAS score

Even though VAS scores could be very subjective and have some biases, we saw some tendencies. The temperature increased with the VAS score, showing that there was a correlation between a sicker calf and a higher rectal temperature, but still with a variation. This could be because temperature decreased when calves receive NSAID. When looking at activity compared with the VAS score, we saw almost no change and the correlation was bad, with a great variation between calves. This could be because a calf that had been sick and two days later got a lower VAS score (after treatment), still had a low activity, whereas the temperature would decrease with treatment. When we looked at activity, we only took eight observations from the previous 48 hours to make an average and some calves either decreased or increased right before examination which resulted in the average not having time to change, but the VAS score had. There was a great variation between observations for both lymphocytes and neutrophils, though with a poor fitting. There was a correlation between a higher VAS score and a decrease in lymphocytes, and a higher VAS score and an increase in neutrophils. The correlation between VAS score and the changes in lymphocytes and neutrophils did indicate a change in differential count in our sick calves. As mentioned earlier we did not see a change in differential count when comparing lymphocytes and neutrophils in NSAID calves and healthy calves due to the very individual levels between calves. When looking at α_2-globulin fraction we saw a correlation with the VAS score. α_2-globulins increased with a higher VAS score, indicating that α_2-globulins increases with disease. This, as well, was not shown in our study due to great variation in globulin levels between calves and the relatively small sample size. When looking at γ-globulin fractions there was no correlation with VAS score and there was a great variation in values which could indicate no correlation between changes in γ-globulins and disease, which substantiates our findings in section 4.2.3.

4.5 Correlations with α_2- and γ-globulin fractions

α_2-globulin correlated with both activity, rectal temperature and lymphocytes but not mature neutrophils. For all the parameters there were a weak fitting. This indicated that the decrease in activity we saw when calves got sick was true, because the APPs in the α_2-globulin fraction will increase with inflammation. However, this did not correspond with any of the other findings for α_2-globulin in the study, where α_2-globulin did not have any clear tendency. The same could be applicable to temperature, where an increase in sick calves is a response to illness. γ-globulins had in general no correlation with any of the parameters. For lymphocytes and neutrophils, the correlation and fitting were so weak that the scatter plots for this study almost seemed like a coincidence. We did not find any significant changes in γ-globulins in any part of this study. This could be due to immunoglobulins having too small of a change or changed too late in the disease course.

4.6 Study design

The target population of this thesis was Danish pre-weaned calves in dairy herds. The herd we used in this study was chosen out of convenience, location and willingness to participate. The sample size was limited by time of the experiment and the number of calves born in the period of the trial, furthermore we included all heifers born in our study period. The small sample size probably affected our study results, e.g. α_2-globulins did not show very much and γ-globulins did not show anything at all. Furthermore, lymphocytes and mature neutrophils did not show very much, and the repeated measure ANOVA did not show any significant changes when calves received NSAID treatment. To make the sample more representative for Danish dairy herds we should have increased the sample size and used calves randomly selected from different herds, of all sizes, from all over Denmark and with data collection distributed in all seasons. We also had a few calves which received a treatment for BRD right before they went out of our trail, resulting in missing control samples. We could have considered that each calf receiving a treatment at the end of the study period was finished with control samples before it went out of the experiment. Then we had some calves with diarrhea and inflammation near one of the ear tags. These calves were noted in the datasheet, but we did not find any change in temperature or leukocytes connecting to this.

Calves in this study were only examined every second day resulting in an uncertainty to where in the disease course we saw them. It could have been more optimal to examine the calves every day or twice a day, especially looking at differential count, where we saw very individual courses between calves which depended on when the blood sample was drawn.

4.7 Calf management

Many different components can affect whether the calves get BRD. Stressors and risk factors can affect the calf's well-being, as well as failure of passive transfer.

The selected herd vaccinated the cows with Rotavec® Corona during the final period of pregnancy, to raise the antibody level in colostrum. It is important that calves get a sufficient amount of high-quality colostrum to make sure the calves have antibodies for the first two to four weeks (Chase et al., 2008; Godden, 2008). On the farm in our study they did not measure the IgG concentration in the colostrum before giving it to the calves, and, therefore, it was difficult to know anything about the quality of the colostrum the newborn calves received. To ensure the highest possible concentrations of IgG in colostrum cows should be milked within one to two hours after calving, and maximum six hours (Malantus et al., 2005). This can often be difficult with regular milking routines in the morning and afternoon. Values of total protein in serum below 52 g/L can indicate failure of passive transfer if the values were from blood samples taken within the first 2-3 days of the calf's life (Tyler et al., 1996).We saw that five out of our 51 calves had a total protein in serum below 52 g/L on the first day in the study, but we had 13 out of 51 calves with a total protein in serum below 52 g/L at some point in the study. The calves in our study were included at the age of 17 to 25 days and excluded 30 days later, which mean that we looked at the calves in a period of their life where the passive immunity from the cow decreases and the calves active immunity increases (Chase et al., 2008), leading to a certain vulnerability of the calves during this period. This could explain why we in the start of the study saw a decrease in total protein in some of the calves and then later in the study saw an increase. These calves with total protein values below 52 g/L were not treated more or less than other calves in the study.

Both moving and dehorning calves are considered as stressors which can increase the risk of BRD, especially when done in a young age (Windeyer et al., 2013). Calves in this study were moved from single pens to group pens and were dehorned in very different ages. They were between 3.5 weeks and five weeks old, right in the gap with the low immunity (Chase et al., 2008), and some calves were unlucky to be moved and dehorned on the same day. However, we did not see any clear connection between these factors and BRD. On the other hand, we saw that six calves (#650 - #569) placed in a group pen right next to a group of older calves all needed treatment, and four of the six calves needed treatment with antibiotics. There were only bars between the group pens, and Lago et al. (2006) found that a single solid barrier between calves was associated with decreased prevalence of respiratory disease compared to group pens with only bars between. Wet bedding is also associated with a greater risk of BRD (Lago et al., 2006), and even though the pens were topped with straw every second day in the selected herd, we often experienced wet bedding in the pens. Furthermore, there was only removed manure from the pens and cleaned with calcium hydroxide between calves, and cleaning is essential for a good environment for the calves (Windeyer et al., 2013).

4.8 Considerations

Management of the calves has a major impact on the calves' health, and it is important to remember that it is better to prevent the diseases than to treat it. BRD is a health problem all over the world, as well as in Danish calves (Jensen et al., 2018). There is no golden standard to diagnose the calves, and it is hard to find BRD at an early state where bacteria have not yet infected the calves and the use of antibiotics is not needed. The antibiotic consumption in cattle has decreased in the last decade measured in DAPD (defined animal daily dosage per 1000 animals per day), but for calves under 12 month it has increased with 39% (Statens Serums Institut, 2020). Especially treatment for respiratory diseases has increased and is the greatest category for antibiotics use in calves (Jensen et al., 2018; Staten Serums Institut, 2020). Our study suggests that NSAID could be used at an early stage, but further research is necessary to find the effect of NSAID treatment. To investigate the effect of NSAID treatment a trial could be made where calves, which received a VAS score between 2.5 and 5.0, were randomly divided into two groups. One group receiving NSAID treatment and the other group received no treatment. If calves got a VAS score above 5.0, they should receive antibiotics. The two groups then could be followed to see if there was a difference in recovery and need of antibiotics. Besides this, the VAS score has not earlier been used for detection of BRD in calves and should, therefore, be validated through further studies. Activity data from SenseHub was also not validated, and this could be done by looking at calves and comparing them to SenseHub Young Stock data. It is also necessary to make further investigations to make a cut-off between healthy and sick calves for activity. The SenseHub Young Stock is based on a health index, generated by the collected data about rumination and activity, from zero to 100 where 100 is healthy. When the health index gets below 85 the farmer gets an alarm on his/her computer or phone. To be sure if SenseHub Young Stock works in practice, it would therefore be necessary to investigate the health index and its precision in detection of sick calves.

This could help future diagnostics in calves at an early point, and hopefully, thereby, decrease the antibiotic use in dairy calves.

5. CONCLUSION

In this study we investigated the efficacy of NSAID treatment and early detection of BRD. Based on the results from the 51 calves, the t-test on healthy calves compared with NSAID calves, showed that there was a significant difference when looking at activity and rectal temperature. There was no significant difference when we looked at lymphocytes and mature neutrophils nor for α_2- and γ-globulins. When we summarized our results, they showed that activity and temperature could contribute to an early detection of BRD in calves, but it was not possible to use our differential count or the fractions from the electrophoreses to early detection.

When we determined the correlation between α_2- and γ-globulin fractions in serum compared with activity, rectal temperature, lymphocytes and mature neutrophils, we saw a clear correlation between α_2-globulin and activity, rectal temperature and lymphocytes but not with mature neutrophils. γ-globulin did not show any correlation with the parameters.

The VAS score proved to be a possible good way to examine the calves for BRD. The VAS score showed a correlation with both rectal temperature, lymphocytes, neutrophils, α_2-globulin and total protein.

When investigating the change in expression of BRD for calves treated with NSAID, we found that there were no significant changes in any of the parameters for each calf. Nevertheless, we saw that after treating with NSAID the activity for most of the calves increased and the rectal temperature decreased indicating some efficacy of the treatment. It seemed like NSAID treatment at an early stage could be a way to reduce antibiotic consumption in dairy herds. Thirty-seven percent of the calves recovered after receiving NSAID treatment. Though it was hard to know if the NSAID treated calves would have needed treatment to overcome the disease, had they not received NSAID.

Further research is needed to fully utilize the potential of NSAID as a treatment for BRD in calves, the same applies for SenseHub Young Stock ear tag and VAS score.

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