UNIVERSITY OF COPENHAGEN
FACULTY OR DEPARTMENT

Veterinary Master Thesis

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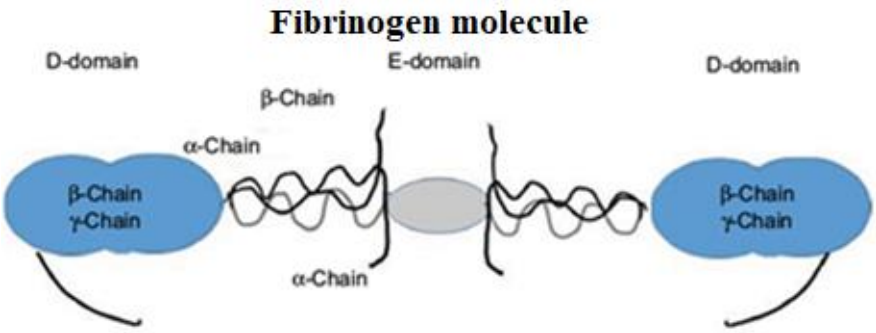

Plasma fibrinogen as a valuable tool in clinical decision-making?

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Preface

The Master thesis “Plasma fibrinogen as a valuable tool in clinical decision-making?” has been conducted to fulfil the requirements of veterinary medicine at the Faculty of Health and Medical Sciences at the University of Copenhagen.

The retrospective study was an idea pitched by Nynne Capion and Clara Büchner Marschner. The research questions of the thesis were formulated in collaboration with both supervisors. The data was drawn at the beginning of the study from VisuaLab Laboratory Information System Version:4,2,1,12,22,07a Site D by Claus Stjernegaard at Veterinary Diagnostic Laboratory. In consultation with both supervisors, the author was responsible for the processing and analysis of the data. Veterinarians, veterinarian nurses and people working in the veterinarian field with focus on inflammation in cattle will benefit from this Master thesis.

I hope you enjoy your reading.

Louise Spliid Knudsen

Copenhagen, 21st of June 2019

Plasma fibrinogen as a valuable tool in clinical decision-making?

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Summary

Reasons for performing study: Fibrinogen levels in blood has shown to be a valuable indicator of inflammation in cattle. However, the reference intervals for fibrinogen used as guidelines, are not reliable indicators on whether a cow is sick or healthy. It has been proposed that the fibrinogen level could be re-evaluated through new studies with specific selection of individuals, and thereby contribute as a reliable paraclinical parameter when declaring a cow sick or healthy.

Objectives: The aim of this study was to establish a clinical decision limit for fibrinogen in cattle and to evaluate fibrinogen levels and clinical records of cattle' admitted to the Large Animal Teaching Hospital. To study this, the following two research questions were made; First – Will inflammatory diseases in cattle elicit a fibrinogen response? and Second - Can a cut-off value of fibrinogen be established, which will distinguish between 95% of the healthy and 95% of the sick cattle?

Study design: A retrospective study.

Methods: A total of 1915 plasma fibrinogen samples collected from 27th of April 2010 until 5th of Marts 2019 were drawn from VisuaLab Laboratory Information System. A group of 134 healthy female cattle >1 year of age was formed of individuals who met the inclusion criteria. A group of 258 sick female cattle >1 year of age was formed of individuals who met the inclusion criteria. From the group of 258 sick cattle the most frequent diagnosis among these were selected and 6 new groups was formed. Twenty-four animals were allocated to the U+V (uterus and/or vagina disease). Twenty-three animals were allocated to the DD (digital dermatitis) group. Nineteen animals were allocated to the mastitis group. Sixty-eight animals were allocated to the group with multiple disease. Ten animals were allocated to the group with peritonitis. Nineteen animals were allocated to the group of pneumonia. Fourteen of 258 sick cattle had a blood sample taken twice, one when sick and one when healthy, these were used to evaluate the progress of fibrinogen. Data were not normal distributed and were analysed nonparametrically.

Results: There was a significant difference among plasma fibrinogen for the sick and healthy group of cattle (P=<0.0001). The area under the ROC-curve was 0.66 with a standard error of 0.03 at a confidence interval of 95%. Youden's index was highest at 0.27, suggesting a cut-off value of fibrinogen at 9.61g/L with a sensitivity at 77% and a specificity at 50%. There was a significant difference between the mean values of the 6 groups compared to the group of healthy cattle' means of plasma fibrinogen (P= <0.0001). There was no significant difference between the medians amongst the fourteen repeated blood samples (P= 0.14).

Conclusion: Fibrinogen is elevated in cattle with different diseases. The estimation of concentrations and the dynamics of changes in fibrinogen can be a valuable tool, supplementing the clinical assessment during treatment in determining whether a cow is sick or healthy. Fibrinogen values above 9.61 g/L can be and indicator of inflammatory conditions, but due to low specificity and sensitivity many patients risk being overlooked.

Keywords: Fibrinogen, cattle, blood, reference intervals, guidelines, cut-off

Introduction

In the time after an injury, trauma or infection of a tissue, a series of reactions are played out in the host to prevent ongoing tissue damage. In the host, processes that are necessary to return the host to normal function are activated and this haemostatic process is known as inflammation. There are early and immediate sets of reactions which are induced, and this is known as the acute phase response (APR). The acute phase protein (APP) fibrinogen is a part of this response and is produced in the liver, like many other APP's, and its primary function is to serve as substrate for thrombin in the formation of fibrin during haemostasis. Fibrinogen is essential in the coagulation cascade (Ceciliani, *et al*., 2012; Murata, *et al*., 2004; Smith, 2009).

The APR activates the release of mediators by resident and invading cells, such as interleukin-1 and tumor necrosis factor. This results in the initiation of systemic responses, including fever, leucocytosis, activation of complement and clotting systems, alterations in plasma concentration of trace minerals and changes in liver metabolism, including the production of APP's (Baumann, and Gauldie, 1994; Godson *et al*., 1995). The positive APP's are known to be valuable biomarkers that increase alongside inflammation, infection and trauma (Ceciliani *et al*., 2012; Cheryk, *et al.*, 1998; Conner *et al.,* 1988; Davalos & Akassoglou, 2012; Eckersall & Conner, 1988; Glenn, 1969; Hirvonen & Pyörälä, 1998; McSherry *et al*., 1970; Pfeffer *et al.*, 1993; Rubel *et al*., 2001; Sutton & Hobman, 1975). The main types of APP differ from species to species (Cray, 2012). There are specific characteristics among APP's, some increase (positive) or decrease (negative) during inflammation. All acute phase proteins are classified in three different groups (major, moderate and minor) depending on their level of increase during inflammation. Major APP's in cattle such as haptoglobin are highly sensitive and specific indicators of inflammation but cannot differentiate between different types of inflammation (Heegaard *et al*., 2000; Paulina & Tadeusz, 2011). Moderate acute phase proteins in cattle are serum amyloid A (SAA) and fibrinogen (Heegaard *et al*., 2000).

In the APR, fever is the first systemic response to evolve together with an increase in white blood cells (WBC). They both increase 6hours (h) after injury, but temperature often decrease again after 18 h and WBC decrease after 48 h. After 24 h the major acute phase protein haptoglobin increases and starts to decrease slowly again after 48 h. The moderate acute phase protein fibrinogen increases after 48 h and starts to decrease slowly again after 72 h. Haptoglobin and fibrinogen starts decreasing when the inflammation is decreasing or absent, otherwise they remain increased (figure 1) (Godson *et al*., 1995).

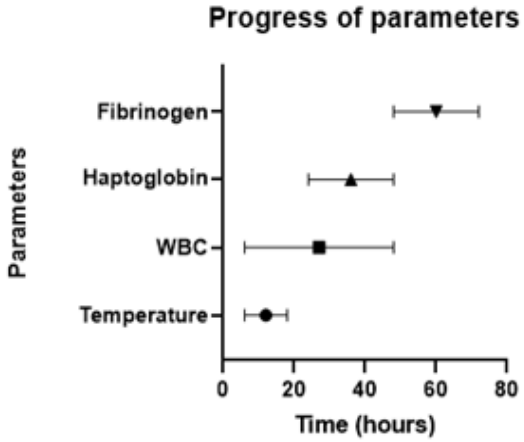

Figure 1. Progress in most cases for the different parameters of inflammation in cattle over time according to Godson *et al.,* 1995. Increasing at the minimum level and decreasing at the maximum level of hours.

Increase in fibrinogen in plasma is prolonged compared to other changes during inflammation (Paulina & Tadeusz, 2011), and is dependent on the magnitude of tissue involved (Gånheim *et al*., 2003).

Fibrinogen could be a diagnostic tool for the clinical decision-making in subclinical cases, where patients have unspecific clinical signs of illness (François, *et al.*, 1998) and also in conditions seen in peripartum dairy cows, including metritis (Schneider *et al.,* 2013) and endometritis (Krause *et al*., 2014).

Reference intervals (RI) are an integral component of clinical decision-making. RI contains possible values between and including an upper and lower limit from a specified proportion of values comprising 95% of a healthy reference population *(*Douglas J. Weiss, 2010; Friedrichs *et al*., 2012). RI is established by *de novo* determination guidelines (Friedrichs *et al*., 2012). When establishing RI by *de novo* the primary focus should be on sampling an appropriate reference sample group, preferably by a direct *a prior* method, where criteria for selection, exclusion and partitioning are planned before sampling. It is recommended that sample size is at least 120 reference individuals to assess the precision of the established reference limits. Establishing *de novo* RI is challenging, time consuming and expensive, which makes alternative methods, that are easier and cheaper yet less reliable, attractive to use instead (Friedrichs *et al*., 2012).

A decision limit is a predetermined threshold that distinguishes between 2 populations, e.g. those with and without a specific disease. They are defined by consensus and based on investigations of animals with and without a specific disease (Friedrichs *et al*., 2012). If clinical decision limits exist they should be used and reference intervals would be unnecessary (Douglas J. Weiss, 2010). To make the decision-making process consistent, established decision criteria

are essential. There are no recognized standard protocols for making decision limits in veterinary medicine, which makes the establishment of decision limits a local task (Douglas J. Weiss, 2010). In veterinary medicine the most commonly used establishment of decision limits are made from population-based reference intervals (Shine, 2008). Due to the variation of values in healthy animals from the population caused by geographical differences, the decision limits should only be used as guidelines (Friedrichs *et al*., 2012; Sacchini & Freeman, 2008).

Normal reference values of plasma fibrinogen are well documented in humans and dogs but are not well documented in other species such as cattle (McSherry *et al*., 1970). The normal values for cattle were found in studies from 1922 and 1925, where they recorded plasma fibrinogen levels from healthy cattle' and calves in various sex, ages and stages of gestation (McSherry *et al.*, 1970). Among these, the plasma fibrinogen levels were recorded for 113 healthy cattle' calves'. Some of the fibrinogen levels where very high which were likely due to unsuspected infections. These values found in the 1920ies align with the ones found in studies published later (Stormorken, 1957). The normal plasma fibrinogen level is 4.5-7.5 g/L (Howe, 1922; McSherry *et al*., 1970; Stormorken, 1957; Sutton & Hobman, 1975; Thomson, *et al.*, 1974). Some stages of clinical disease were found to not exceed this range of fibrinogen, but stayed in the "high normal", e.g. endometritis and metritis (Howe *et al.*, 1924; McSherry *et al.*, 1970). Newborn calves, until the first two weeks of life were found to not exceed the general reference limits used for healthy cattle and healthy calves aged 0 – 60 days were all at the lower end of the normal range of values of healthy adult cattle (Gentry *et al.*, 1994; Knowles *et al.*, 2000; Thornton *et al.*, 1972).

Veterinarians use plasma fibrinogen as a marker for inflammation in cattle but studies showed that veterinarians do not fully trust the reference intervals to be enough to declare their patient healthy (Hirvonen & Pyörälä, 1998). Veterinarians experience declaring patients healthy on behalf of their clinical examination even though the fibrinogen level is not within the normal range according to reference interval guidelines (Hirvonen & Pyörälä, 1998; Jawor, *et al.*, 2008). In one study from Hirvonen & Pyörälä, 1998, patients were sent home with a fibrinogen value of 11g/L. The veterinarians instead, looked at the increase and decrease of plasma fibrinogen level (Hirvonen & Pyörälä, 1998). Results of Hirvonen & Pyörälä, 1998, stated that cattle in the hospital with a diagnosis such as peritonitis had fibrinogen levels ranging from 5.4-31.3g/L, and cattle with a diagnosis such as pneumonia had fibrinogen levels ranging from 8.8-23g/L. On behalf of patient sent home with a fibrinogen level at 11g/L and the large range in fibrinogen in

different diseases, it is assumed in this study that patients with fibrinogen values above 22g/L are sick, and an overlap of sick and healthy cattle is found between fibrinogen level from 0g/L to a maximum of 22g/L.

As mentioned earlier the RI was made on behalf of studies from 1922 and 1925 and the production of cattle has evolved since then, along with increasing pressure on each cow in the production systems today. RI by *de novo* is, as mentioned earlier, challenging to establish, but if a new clinical decision limit was made on behalf of cattle in modern production instead of cattle from 1922 and 1925, fibrinogen might be a reliable paraclinical parameter, that veterinarians could fully trust and use in their clinical decision-making. A new clinical decision limit could be a valuable tool to tell when a cow is sick and when it is healthy, especially in cases with cattle that has unspecific clinical symptoms of illness (François *et al*., 1998; McSherry *et al*., 1970).

The aim of this study is to establish a clinical decision limit for fibrinogen in cattle. Another aim of the study is to evaluate fibrinogen levels and clinical records of cows admitted to the Large Animal Teaching Hospital. To study this, the following two research questions were made; First – Will inflammatory diseases in cattle elicit a fibrinogen response? and Second - Can a cut-off value of fibrinogen be established, which will distinguish between 95% of the healthy and 95% of the sick cattle?

Materials and methods

This Master's thesis is a retrospective study of cattle plasma fibrinogen samples from the Large Animal Teaching Hospital at Agrovej 8 in Taastrup. Primary literature from written, scientific sources of plasma fibrinogen has been reviewed. The articles used in the thesis where found online in databases such as pubmed, ovid, web of science and google scholar using the key words: Fibrinogen, plasma fibrinogen, reference intervals, cattle, blood, blood parameters, cows, inflammation, disease, retrospective, sick, healthy.

Sample collection

The study population was cattle admitted to the Large Animal Teaching Hospital at Agrovej 8 in Taastrup from 27th of April 2010 until 5th of Marts 2019. The first document of raw data contained 1915 individual blood samples with analysed plasma fibrinogen collected from the cattle. The study is retrospective and blood samples were conducted before the study was made in another context. The study was made from 1st of February 2019 until 21st of June 2019. Data

was collected from the file database called VetNetManagement TANG version 5.1 used at the hospital.

Blood analysis

Blood was drawn into citrate test tubes (BD Vacutainer ®, Na_3 Citrate, BD-Plymouth, UK) by educated personnel on the day the patient was admitted to the Large Animal Teaching Hospital. The sample was then analysed for fibrinogen using an ACL Top 500, Instrumentation Laboratory (ILS) at Veterinary Diagnostic Laboratory (VetLab) at Grønnegårdsvej 3, st. Building 1-61, 1870 Frederiksberg C.

Criteria for including individuals

Included animals was female heifers/cattle >1 year of age with a plasma fibrinogen level from 0g/L to a maximum of 22g/L. The minimum age of 1 year, was selected because it was assumed, that fibrinogen levels were different in young animals <1 year of age. Pregnancy and parturition was not taken into account in selecting individuals, because it was assumed that this would not affect the fibrinogen level (Gentry, *et al.,* 1979*)*. Sick and healthy individuals were selected on behalf of inclusion criteria listed in Table 1. Criteria were selected based on knowledge on inflammation parameters found in studies with focus on inflammation in cattle (Karreman, *et al.,* 2000). Criteria were found in patients files in their profiles from the database used at the hospital. A profile is in this context the patient's datasheet in TANG and a file is their documented course of treatment.

Table 1. Selection of sick and healthy individuals to be included in the study were on behalf of the listed inclusion criteria. A cow was listed sick if the temperature was above 39.6◦C or had one of the mentioned health issues. A cow was listed healthy if the temperature was below 39.5◦C and did not have any of the listed issues (Csilla *et al*, 2011; Karreman *et al*., 2000)

Inclusion criteria for sick and healthy individuals	
Sick individual	**Healthy individual**
Fever with temperature > 39.6◦C or at least one of following conditions: - Infectious disease - Abnormal blood parameters including WBC (neutrophils, leucocytes and basophiles) - Depressed - Pneumonia	No fever with temperature < 39.5◦C and none of following conditions: - Infectious disease - Depressed - Pneumonia - Mastitis (subclinical + clinical/moderate + severe) - Uterine discharge

- Mastitis (subclinical + clinical/moderate + severe) - Uterine discharge - Nasal and ocular discharge - Hoof problems (laminitis, sole ulcer, digital dermatitis and lameness)	- Nasal and ocular discharge

The group of 258 sick cattle was allocated to 6 different disease groups, which were the most frequent amongst the 258 sick cattle. Twenty-four animals were allocated to the U+V (uterus and/or vaginal disease) based on statements in the files addressing uterine discharge, vaginal discharge, vaginal injury or directly diagnosis involving uterus or vagina. Twenty-three animals were allocated to the DD (digital dermatitis) group on behalf of the mentioning of laminitis, sole ulcer and/or digital dermatitis in the file. Nineteen animals were allocated to the mastitis group on behalf of signs of clinical mastitis mentioned such as CMT>3, warm and/or painful and/or swollen udder. Sixty-eight animals were allocated to the group with multiple disease on behalf of more than one disease mentioned e.g. mastitis + peritonitis. Ten animals were allocated to the group with peritonitis on behalf of peritonitis mentioned in the file. Nineteen animals were allocated to the group of pneumonia on behalf of coughing, nasal and/or ocular discharge, raised vesicular respiration sound or bronchial respiration mentioned in the file. The remaining 95 animal profiles were left out, because they did not meet inclusion criteria, as mentioned above, for the 6 groups.

Data analysis

All data were analysed using Prism 8 (GraphPad Software, California, USA). Data were tested for normal distribution with Shapiro-Wilk test. None of the data were normal distributed. An F-test was made to test for equal variances. The data did not have equal variances. A two-tailed Mann Whitney test was performed on the groups of sick and healthy cattle to compare medians to test the hypothesis if fibrinogen would not elicit a response. A receiver operating characteristic curve (ROC-curve) was performed on the healthy and sick group of cattle to show sensitivity and specificity and to give an overview of the area under the ROC-curve. The sensitivity is the proportion of individuals with the disease that the test correctly identifies as positive. The specificity is the proportion of individuals without the disease that the test correctly identifies as negative (Dubensky & White, 1983). The area under the ROC-curve quantifies the overall ability of the test to discriminate between those individuals with disease and those without disease. A

truly useless test has an area of 0.5 and a perfect test has an area of 1.0. To find the optimal cut-off value, Youden's index was calculated in excel with the formula J=maximum {sensitivity(c) + specificity (c) -1}. The index ranges between 0 and 1, where values close to 1 indicates large effectiveness of a given biomarker in this case fibrinogen (Schisterman, *et al.*, 2005). The ROC-curve analysis and the calculation of Youden's index was performed to suggest a cut-off value of fibrinogen and to test the overall ability to distinguish between those individuals that were sick and those that were healthy and thereby answering the hypothesis if a cut-off value of fibrinogen would not distinguish between 95 % healthy vs. 95% sick cattle. To investigate fibrinogens process status of sick and healthy a Wilcoxon's matched-pairs signed rank test with a two-tailed P-value was performed on paired blood samples from the same patient when it was sick and when it was healthy to compare medians to see if there was a difference. A Kruskal-Wallis nonparametric test was performed followed by Dunn's Multiple Comparisons Test to make a multiple comparison of means between 6 diseases and a healthy group of cattle. The P= <0.05 were considered significant.

The following hypothesis were tested as aim of this study:

- **H_{01}**: Inflammatory diseases here defined as; infectious disease, abnormal blood parameters including WBC (neutrophils, leucocytes and basophiles), depressed, pneumonia, mastitis (subclinical + clinical/moderate + severe), uterine discharge (Karreman et al., 2000), nasal and ocular discharge, hoof problems (laminitis, sole ulcer, digital dermatitis and lameness (Csilla *et al.,* 2011)) in cattle will not elicit a fibrinogen response, therefore fibrinogen cannot be used as an indicator for inflammatory diseases.
- **H_{02}**: A cut off value of fibrinogen will not distinguish between 95% healthy vs. 95% sick, therefore a new fibrinogen value cannot be conducted for sick and healthy cattle.

Limitations

The Large Animal Teaching Hospital in Taastrup went from an electronic patient file system called VetVision to another file system called VetNetManagement TANG on the 15th May 2014. This gave some disturbance in the files. When the University used VetVision hand written files were used as a supplement for the program. From the beginning of the data set used in this master thesis from 27th April 2010 and to 15th May 2014 many files presented "not found" in the electronic file system, because they instead were handwritten files, placed in archives at the Large Animal Teaching Hospital in Taastrup. The files at the Large Animal Teaching Hospital in

Taastrup were often missing parameters such as temperature and clinical observations. This resulted in an exclusion of many files due to lack of information. Patients admitted to the hospital in the weekend received a problem oriented clinical exam upon arrival and a full clinical exam after the weekend. This resulted in missing values from the blood sampling date. The raw data sheet which was drawn from Veterinary Diagnostic Laboratory did not have file numbers on each patient, but only patient name. This made it difficult to locate the different patients in the file system since typing errors occurred and names like "?" and "1" resulted in non-existent patient names in the files. Some files were not able to be found, because the result of the blood sample was not connected to a file.

Results

Process of data selection

The first raw data contained 1915 blood samples from cattle of all ages, sexes and breeds. All patients with no sex listed where attempted looked up in the central husbandry register. All 371 male cattle blood samples were removed and 1544 blood samples from females remained. Then all 291 samples from female individuals under 1 year of age were removed. A total of 1253 samples from female individuals over 1 year of age remained. From these, 328 profiles were impossible to look up in the electronic patient file system VetNetManagement TANG, which left 925 files from samples of females over 1 year remaining. From those 925 files, 220 were removed due to lack of information or lack of files in the profile. Remaining were 705 female samples with existing profiles with files. From the 705 samples 313 were removed because they did not meet the inclusion criteria or because repeated samples of the same animal existed. Only 1 sample per patient was used in the study. Remaining were 258 samples from sick female individuals >1 year of age and 134 samples from healthy female individuals >1 year of age. An overview is given in Figure 2.

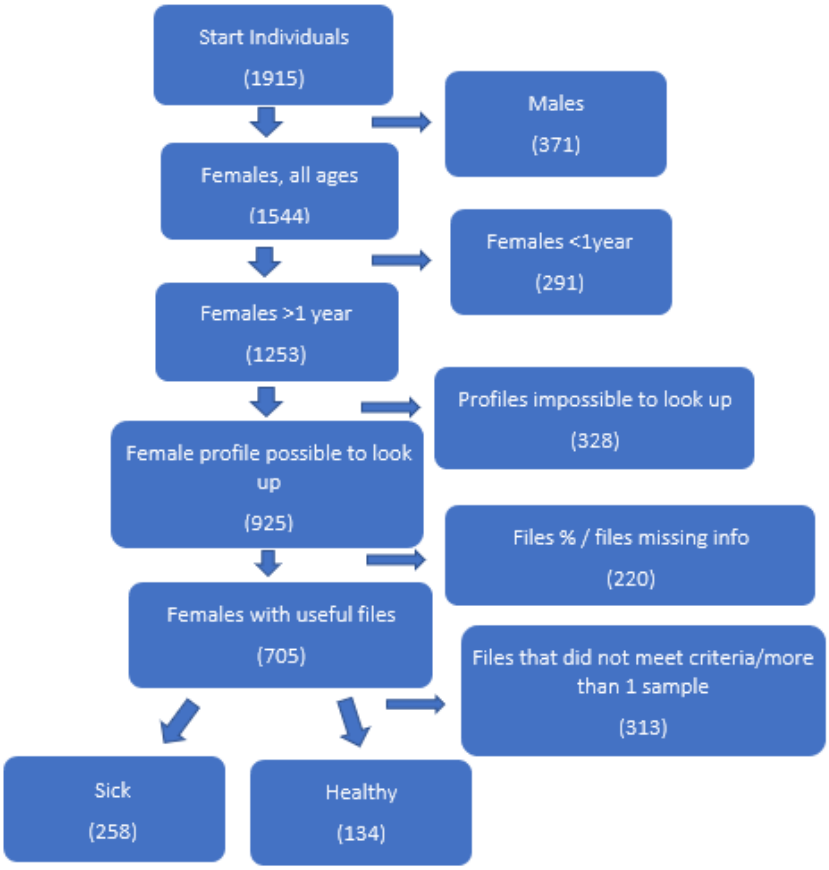

Figure 2. Flow diagram of the process of selecting samples on behalf of inclusion criteria stated in Table 1. Raw data consisted of 1915 blood samples from cattle analysed for plasma fibrinogen. Samples was discarded in the process as seen in the figure and the result was 258 samples from sick individuals and 134 samples from healthy individuals.

Overview of tested groups

All 134 healthy and 258 sick individuals are shown in a boxplot based on their fibrinogen level in g/L in Figure 3. The dotted line indicates maximum of the guideline reference interval at 7.5g/L. The mean value for healthy individuals was 10.43g/L and the mean for sick individuals was 12.95g/L. The minimum, maximum, 1st quartile (25%), median, 3rd quartile (75%), mean, standard deviation and standard error are listed in Table 2. The sample size in this study with the means of 10.43g/L and 12.95g/L with a standard deviation at 4 and a power at 80% would theoretical require a sample size at 40 individuals per group.

Table 2. Overview of the sick and healthy group are listed, minimum, maximum, 1st quartile (25%), median, 3rd quartile (75%), mean, standard deviation and standard error.

Descriptive statistics	Healthy group of cattle	Sick group of cattle
Number of values	134	258
Minimum	2.97	2.94
1st Quartile (25%)	6.96	9.73
Median	9.68	12.61
3rd Quartile (75%)	12.69	16.64
Maximum	20.89	21.90
Range	17.92	18.96
Mean	10.43	12.95
Standard deviation	3.72	4.55
Standard error of Mean	0.32	0.28

313

314 *Sick vs. healthy*

315 There was a significant difference among plasma fibrinogen for the sick and healthy group of
316 cattle (P<0.0001). The median of the sick group was 2.93 g/L higher than the healthy group
317 (Figure 3). It is noticable that almost 75% of the healthy group of cattle has fibrinogen values
318 above the maximum of the reference interval at 7.5g/L.

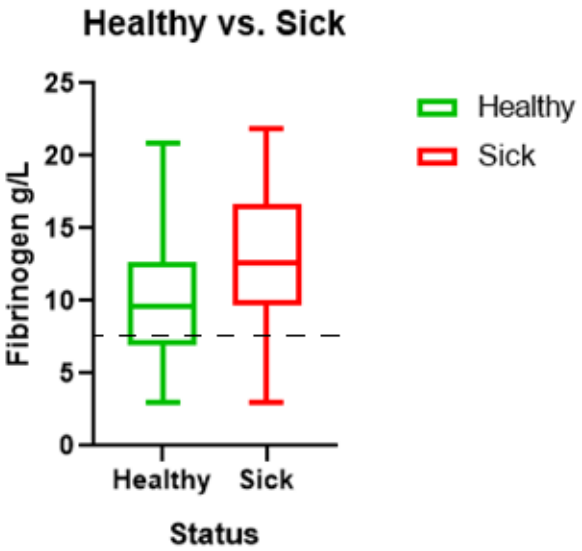

Figure 3. Boxplot of the group of 134 healthy fibrinogen samples and 258 sick fibrinogen samples. The median for the sick group was 12.95g/L and for the healthy group 10.43g/L. The 1st quartile (25%) and 3rd quartile (75%) is shown. The minimum and maximum value of plasma fibrinogen is shown for the two groups as well. The dotted line symbolises the maximum of the guideline reference interval of 7.5g/L.

The area under the ROC-curve was 0.66 with a standard error of 0.03 at a confidence interval of 95%. The ROC-curve area at 0.66 equals 66%, which states that there is overlapping of the distribution and a sick cow will have a more abnormal test result than 66% of the healthy cows (Figure 4). This indicates that the fibrinogen level will in 66% of the cases tell if a patient is sick or healthy. There is a significant difference between the area under ROC-curve = 0.5 and the acutal area at 0.66 (P value <0.0001). Youden's index was highest at 0.27 which suggested a cut-off value of fibrinogen at 9.61g/L with a sensitivity at 77% and a specificity at 50%.

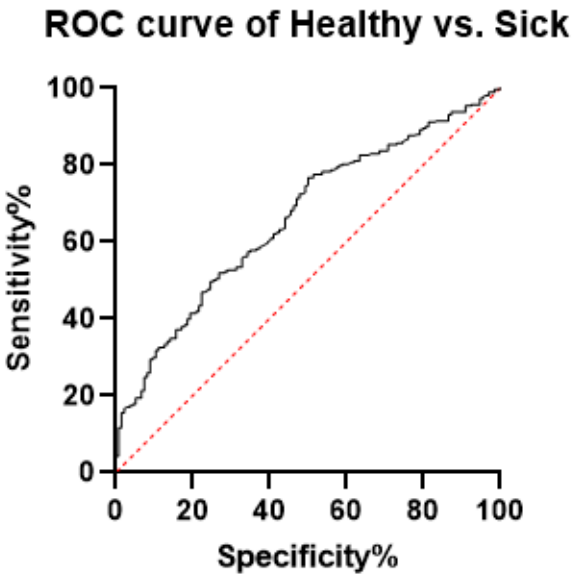

Figure 4. A receiver operating characteristic curve (ROC-curve) of the healthy and sick group of cattle. The ROC-curve is shown in %. The area under the ROC-curve was 0.66 with a standard error of 0.03 at a confidence interval of 95%. The P= <0.0001. The red dotted line symbolises an area of 0.5.

Comparison of paired samples

From fourteen cows, blood samples were collected at admittance, when the cows were sick and again at discharge from the hospital where the cows were declared healthy. Fibrinogen in the sample from the sick cow was 1.34g/L higher compared to the sample collected from the healthy

cow at discharge from the hospital. There was no significant difference between the medians (P = 0,14) (Figure 5).

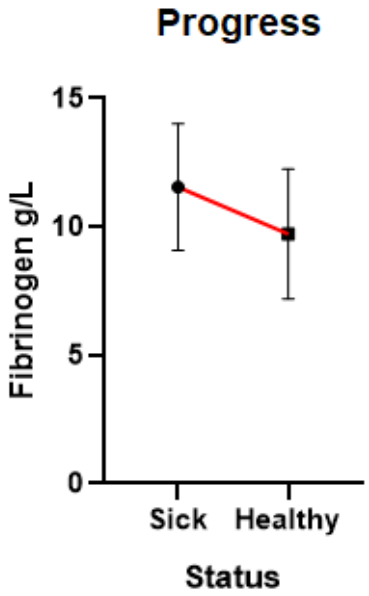

Figure 5. Wilcoxon's matched-pairs signed rank test with a two-tailed P-value, which was performed to compare parred medians and to look at fibrinogen progress from sick to healthy. The progress of plasma fibrinogen g/L from sick vs. healthy status of 14 cows is shown. 1 sample was drawn from a cow when it was healthy, and 1 sample was drawn from the same cow when it was

Fibrinogen levels in different diseases

There was a significant difference between the mean plasma fibrinogen (P= <0,0001) of the 6 disease groups compared to the group of healthy cattle (Figure 6).

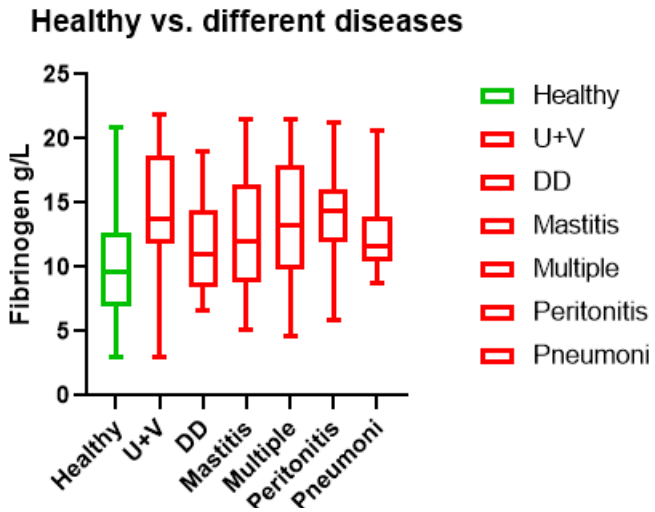

Figure 6. A Kruskal-Wallis nonparametric test was performed with a confidence interval of 95% to detect differences in the mean concentration of plasma fibrinogen g/L of healthy cattle and 6 different diseases. U+V is short for uterus and/or vagina disease. DD is short for digital dermatitis. Multiple is short for multiple disease. P =<0.0001, which indicated there was a significant difference between the mean values of the 6 groups compared to the group of healthy cattle.

Cattle with inflammation in relation to the reproductive tract, cattle with inflammation in multiple organs and cattle with peritonitis had significantly higher plasma fibrinogen compared to the other groups of diseases (table 3).

Table 3. Dunn's multiple comparisons test which was performed as part of the Kruskal-Wallis nonparametric two-tailed test. The test compares the mean fibrinogen level of the 6 groups of cattle with different diseases with the mean fibrinogen level of the control group of healthy cattle.

Groups	Mean rank difference	Significant?	Adjusted P Value
Healthy vs. U+V	-79.78	Yes	0.0002
Healthy vs. DD	-26.95	No	0.99
Healthy vs. Mastitis	-33.01	No	0.70
Healthy vs. Multiple	-58.91	Yes	<0.0001
Healthy vs. Peritonitis	-73.65	Yes	0.05
Healthy vs. Pneumonia	-49.80	No	0.11

Discussion

Fibrinogen is often compared to other acute phase proteins because they are long lasting hallmarks of inflammatory processes (Ceciliani *et al*., 2012; Cray, 2012). Increase in fibrinogen is prolonged compared to other acute phase proteins (figure 1). Fibrinogen is difficult to use in the clinic, because it increases after 48 h and begins to slowly decrease after 72 h, if inflammation is cleared (Godson *et al*., 1995; Paulina & Tadeusz, 2011).

The reference intervals used as guidelines today was conducted based on 113 healthy calves and cows in varies ages, but these were determined healthy without thorough examination (McSherry *et al.*, 1970). This is an unspecific collection of cattle due to the fact that newborn calves, until the first two weeks of life, did not exceed the general reference limits used for healthy cattle and healthy calves aged 0 – 60 days were all at the lower end of the normal range of values of healthy adult cattle (Gentry *et al*., 1994; Hirvonen & Pyörälä, 1998b; Knowles, *et al.*, 2000; Thornton *et al*., 1972).

In this study only, females were selected because the males admitted to the hospital often are under 1 year old. Age limit > 1 year old was selected due to the fact that plasma fibrinogen values are lower in young individuals (Gentry *et al*., 1994).

Pregnancy and parturition was not taken into account in selecting individuals because the

coagulation profile is stable during pregnancy and parturition in cattle in contrast to human females and the pregnant bitch (Gentry *et al*., 1979).

The criteria were determined on behalf of previous studies with cattle and inflammation. The healthy criteria were selected from knowledge of inflammation and on behalf of previous studies where criteria of healthy cattle was determined (Bagga, *et al.,* 2016; Karreman *et al*., 2000; Wilson, *et al.*, 1985). The criteria of sick individuals were selected based on knowledge on inflammation supported by previous studies where criteria of inflammation was determined (Bagga *et al*., 2016; Bake & Illek, 2006; Horadagoda, *et al.,* 1999; Karreman *et al*., 2000; McSherry *et al*., 1970). Compromises were made on behalf of the group of healthy individuals since lameness is a known diagnosis connected to the increase of fibrinogen but was not considered in the healthy group. This was because it is rarely mentioned in the files what the cause of the lameness is and how bad it is (Bagga *et al*., 2016; Jawor *et al.*, 2008). Fibrinogen is known to increase in connection to cortisol, which was not taken into consideration in this study, since the cattle are often examined and drawn blood from at arrival. This could mean that in some cases the increase in fibrinogen could be caused by the increase in cortisol (Fisher *et al*., 1997). Another compromise was made when the haematology of the healthy group was not a criterion, since almost every patient in the hospital has elevated blood values and was excluded as a criterion to determine when a patient was healthy. The criterion "depressed" is a very subjective assessment and therefore one could argue that since it is different students and employees assessing the patients from day to day different perspectives on whether a cow is depressed or not can vary a lot. Fever was a criterion and due to the previous studies of inflammation and time spectrum of fever at 48 h-72 h, the temperature of a patient was therefore evaluated in an interval from the blood sample was analysed and 72 h before the blood samples was analysed *(*Godson *et al*., 1995). The analysis of the blood sample in the laboratory was not always made on the same day the sample was collected which in this study gave some insecurity when comparing the blood sample to the file from the patients' profile. The criteria could be selected differently for studies in the future to ensure more credibility in e.g. when the sample was taken and analysed.

Out of 1915 blood samples 392 were used in this study. 548 samples had to be discarded because they were impossible to look up or had missing files or missing information. The study ended up with 258 sick and 134 healthy cattle, which is a larger sample collection compared to other retrospective studies (Dubensky & White, 1983; Hirsch & Townsend, 1982; McSherry *et*

al., 1970). For future research it would be valuable if a golden standard of what information should be in a file before it is saved in TANG was made. This would give a larger reservoir of files to use in studies.

The power of the test used in this study was not as high as it could have been due to data analysed. The more requirements for a test to be used, the stronger the test, and since the data were not normal distributed and variances were not alike, non-parametric tests with no or few requirements was used. The sample size should make up for the low power of the tests used in this study since the theoretical sample size required 40 individuals per group and this study had 258 and 134 respectively.

The plasma fibrinogen level reported in this study for healthy cattle did not agree with previous published fibrinogen levels, since the minimum was 2.97g/L and maximum was 20.89g/L for healthy cattle, also almost 75 % of the healthy cattle were above maximum reference interval at 7.5g/L. A previous study from McSherry et al., 1970 found a minimum at 3.2g/L and maximum at 5.8g/L and the results were on behalf of 76 heifers pregnant and non-pregnant. The difference in that study and this one is the selection of patients. Their healthy individuals were selected without a clinical examination and the only criteria used was that they seemed clinical normal. In this study different criteria were made to declare a patient healthy on behalf of the clinical examination and the total of healthy individuals was 134. Their maximum value was set at 8g/L where this study's maximum was set to 22g/L. In their study the healthy 76 heifers did not exceed their maximum level at 8g/L but in this study, there were healthy cattle with a level at 20.89g/L. This could be due to the change in production pressure, since the production and pressure on each cow has become greater since 1970. The prolonged decrease of fibrinogen could also have an impact on the differences in results because the cow could have been sick prior to the blood sample and thereby the fibrinogen level could be higher than usual due to the prolonged decrease. The sample size has almost twice as many individuals in this study in theirs which might have an impact on the spread of individuals. The minimum and maximum and mean is not in agreement with a study by Stormorken, 1957 either. The study was with a sample size of 10 non-pregnant healthy animals and they found them all be in the reference interval of 4.5-7.5g/L with a mean value of 5.6g/L. The mean value of healthy cattle in this study was 10.43g/L which is above maximum reference interval guideline as well as their mean value. The criteria of their study are not mentioned in detail besides that the individuals are healthy and not pregnant. The age, sex and why they are selected as healthy is not mentioned.

The plasma fibrinogen level reported in this study for sick cattle did not agree with previous published fibrinogen levels, since the minimum was 2.94g/L and maximum was 21.9g/L. Comparing these results with a previous study by Sutton & Hobman, 1975 56.8 % of their 716 sick indivuals, which corresponds to 407 sick individuals, where within reference interval guidelines, which they believed to be 2-7g/L. In this study the group of sick cattle was not divided into a group over and under a fibrinogen level at 8g/L why it cannot be compared. However 1st quartile (25%) of the group of sick cattle in this study was 9.73g/L which is above the maximum at 7g/L of their study. The reason for this big difference could be that fibriongen is prolonged and dependent on tissue involvement. From the files in Taastrup one cannot tell when the disease began in the patient and so the disease could have been there for a week and if multiple organs are involved the fibriongen level would rise which could give an unclear picture of the fibriongen level compared to the clinical examination when the patient arrives at the hospital. In their study, 143 sick individuals where above 8g/L with a mean at 12.4g/L which is lower than the mean of this study which where at 12.95g/L. The difference between this study and theirs could be the selection of individuals, because all their patients in their study were all suspected of having disease and the blood samples was collected over a period of 20 months, where the samples collected in this study was 9 years. They did not think that age was a factor that could give a difference which is why their group of individuals were mixed in age. Age is a factor in this study as previous mentioned. They do not mention the selection criteria further so sex, race, pregnancy status and which suspected disease they might have included is unknown and could differ from this study. The means of the study and this one was not that different and maybe the difference at 0.55g/L could be due to the sample size difference, since this study' sample size has 115 more sick individuals than theirs the spread is larger. Another cause of difference in means could be that the 143 sick individuals of their study had a minimum at 8g/L and the ones in this had a minimum at 2.94g/L which also makes the spread larger. They mention that patients with liver disease had a lower fibrinogen level which they did not consider, but this was not considered in this study either.

There was a significant difference ($P<0.0001$) between sick and healthy cattle' fibrinogen level, which indicated that inflammatory diseases, defined earlier, elicit a fibrinogen response, which is in agreement with previous studies (Ceciliani *et al*., 2012; Cheryk *et al.,* 1998; Conner *et al.,* 1988; Davalos & Akassoglou, 2012; Eckersall & Conner, 1988; Glenn, 1969; Hirvonen & Pyörälä, 1998; McSherry *et al*., 1970; Sutton & Hobman, 1975). H_{01} is therefore rejected.

The ROC-curve analysis evaluates the accuracy of a diagnostic test. The benefit is that it includes all threshold values for the test to be evaluated (Hirvonen & Pyörälä, 1998; Vida, 1993). In the ROC-curve analysis fibrinogen was found to be a poor indicator of sickness and health in cattle due to an area under the curve at 0.66. Fibrinogen will according to this analysis in 66% of the cases tell if a cow is sick or healthy. Even though there was a significant difference ($P<0.0001$) between ROC-curve area of 0.5 and the actual area at 0.66, the actual area is still close to 0.5 which is compared to flipping a coin to tell if a patient is sick or healthy (Hirvonen & Pyörälä, 1998). The optimal cut-off value was calculated to 9.61g/L with a sensitivity at 77% and a specificity at 50%. This indicates that 77% of the patients will test positive when sick but 23% sick patients will be overlooked when using fibrinogen to distinguish between sick and healthy, because they will test false negative (sick). Furthermore, a specificity at 50 %, indicates that 50% will test negative when healthy, but another 50% will be overlooked, because they will test false positive (healthy). The test due to the low sensitivity and low specificity will end up overlooking a lot of patients. The H_{02} is accepted, because this cut-off value will not distinguish between 95 % healthy vs. 95 % sick. A perfect test would have a 100% specificity and a 100% sensitivity, but this is rarely the case. If a veterinarian wanted to be sure that cattle with values above the cut-off value at 9.61g/L had inflammatory conditions a high specificity would be required. Comparing the results of ROC-curve area and cut-off value to the previous study of Hirvonen & Pyörälä, 1998 their area under the ROC-curve was 0.92 with a standard error of 0.04 and a suggested cut-off value at 11g/L. Their area showed a far more useful test than the one in this study. This could be because their patients were selected on behalf of internal disorders which were referred to the hospital for surgery. Their study was not a retrospective study and they divided the cattle' referred for surgery in groups of diagnosis at their arrival after clinical examination, this way of conducting a sample group compared to a retrospective study makes it easier to find patient that meets the specific inclusion criteria and all details looked for, can be conducted at the clinical examination. In this retrospective study it was in many cases difficult to find everything needed to divide the patients in the groups and therefore many was left out of the study and the inclusion criteria were more varied than the ones in their study, which makes their study seem much more specific. Another study made by Nazifi *et al.,* 2009 found an area under the ROC-curve at 0.87 with a standard error of 0.04 with a suggested cut-off value at 3.8g/L. In their study they focused on traumatic reticuloperitonitis (TRP) in cattle and it was a prospective study. They selected patients with TRP as one of the differential diagnoses among referred cattle

to the hospital. The patients they ended up using in the trial had their diagnosis of TRP confirmed by surgical intervention, slaughter or by a thorough clinical and laboratory follow-up. Comparing their study to this one the criteria can be mentioned again. In their study they were able to make sure that their patient in fact had the disease they were looking to investigate. In this study, which was retrospective, the selection of patients was limited as mentioned earlier. There was a study made with focus on TRP as well from Jafarzadeh, *et al.,* 2004 which suggested 3 cut-off values; 7.66g/L with a sensitivity at 55% and a specificity at 95 %, 6.91g/L with a sensitivity at 77% and a specificity at 76% and 6.22g/L with a sensitivity at 96% and a specificity at 60%. They concluded that due to the results, fibrinogen alone to select patients with TRP had low usefulness. Their study was a prospective study and they only took blood samples from cattle with gastrointestinal disease, which again in the inclusion criteria of selecting patients already are more specific, than the ones in this study. Also, to be mentioned again the advantages of prospective instead of retrospective. Even though there are many patients to choose amongst in this retrospective study many had been excluded and the ones used are not selected as specifically as the ones in their prospective study.

There was no significant difference among blood samples collected from the same cow when it was sick and healthy ($P<0.14$), however the mean value was still 1.34g/L higher in the patient when it was sick than when it was healthy. Previous studies also found a decrease in fibrinogen connected to the reduction in inflammation, and none of them describes if the decrease is significant or not *(*Conner *et al.,* 1988; Godson *et al.,* 1995; Hirvonen & Pyörälä, 1998; Jawor *et al*., 2008). It was indicated in a previous study that the decrease in fibrinogen can be used to distinguish between sick and healthy along with clinical examination, however in this study there was no significant difference in blood samples from the cow when it was sick and when it was healthy and only a small decrease in fibrinogen level (figure 5) (Hirvonen & Pyörälä, 1998). The sample size of 14 individuals could be the reason for the non-significance, but as stated previous the criteria for sick and healthy because of the ROC-curve are not as accurate as they could be.

There was a significant difference among the 6 different groups of cattle with disease and the healthy group. This indicated that depending on the disease, different fibrinogen levels can be found. Future studies with larger groups of cattle could result in possible cut-off values of fibrinogen for specific diseases. It was found that fibrinogen levels were consistently elevated in diseases like pericarditis and peritonitis (McSherry *et al*., 1970), yet another study reported that fibrinogen is a nonspecific indicator in connection to peritonitis, because the presence of

increased fibrinogen in the case of peritonitis is not always present (Hirsch & Townsend, 1982). Fibrinogen in cattle has been shown to be useful in differentiation of reticuloperitonitis from other gastrointestinal disorders with an area under the ROC-curve at 0.92 with a standard error of 0.04 and a suggested cut-off value at 11g/L (Hirvonen & Pyörälä, 1998). Studies of other acute phase proteins have shown to be useful in differentiating between pericarditis and other cardiac disorders (Nazifi, *et al.*, 2009).

Results from Dunns's multiple comparisons test showed that cows with inflammation in relation to the reproductive tract, cattle with inflammation in multiple organs and cattle with peritonitis had significantly higher plasma fibrinogen compared to other groups of diseases. Studies of the acute phase protein haptoglobin, has shown that haptoglobin may be used as an early predictor of developing metritis, endometritis and purulent vaginal discharge (Dubuc, *et al.*, 2010; Huzzey *et al*., 2009). Fibrinogen could be used as an early predictor as well if further studies were made, because it cannot be used according to this study, because the ROC-curve analysis showed low sensitivity and specificity, the sick and healthy group of cattle are not a valuable foundation to make these statements on. Multiple diseases could have increased in fibrinogen due to the fact that fibrinogen is dependent on the magnitude of tissue involvement (Gånheim *et al*., 2003).

Conclusion

It cannot be concluded that the decrease can distinguish between sick nor healthy and nor can it conclude that a cow is sick nor healthy judged by the fibrinogen level alone, because even though there was a significant difference between the sick and healthy group (figure 3), the ROC-curve analysis showed low sensitivity and specificity.

Fibrinogen is elevated in cattle with different diseases. The estimation of concentrations and the dynamics of changes in fibrinogen can be a valuable tool, supplementing the clinical assessment during treatment in determining whether a cow is sick or healthy.

Fibrinogen values above 9.61g/L can be an indicator of inflammatory diseases, but due to low specificity and sensitivity many patients risk being overlooked.

In the future new studies could be made prospective to ensure more specific inclusion criteria and thereby selecting sick and healthy cattle more precisely.

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