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# Acute phase response of sole ulcer, white line disease and digital dermatitis in dairy cows

Hertta Pirkkalainen [a,*], Isto Talvio [b], Minna Kujala-Wirth [a], Timo Soveri [a], Toomas Orro [b]

[a] Department of Production Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, Paroninkuja 20, Saarentaus, 04920 Finland
[b] Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Science, Kreutzwaldi 62, Tartu, 51006 Estonia

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ABSTRACT

Hoof disorders cause lameness and welfare problems for dairy cattle. Acute phase proteins, including serum amyloid A and haptoglobin, with increased rectal temperature and interleukin-6 concentrations, are markers of acute phase response. This study assessed the inflammatory response of cows with either sole ulcer, white line disease or digital dermatitis compared to healthy cows. Another aim was to monitor the inflammatory response changes over time after diagnosis (at hoof trimming, seven and 14 days later) in cows with different hoof disorders.

Serum amyloid A concentration in cows with sole ulcer was significantly higher compared with the control group (cows with no hoof lesions) within the two-week study period. Interleukin-6 and rectal temperature declined from day zero to day seven in the sole ulcer group. These results suggest that sole ulcers initiate a long lasting systemic inflammatory response in dairy cows.

## 1. Introduction

Sole ulcer (SU), white line disease (WLD) and digital dermatitis (DD) are common conditions that cause lameness and welfare problems to dairy cattle (Amory et al., 2008; Amstel & Shearer, 2006; Evans et al., 2016). These hoof disorders also cause substantial economic losses due to decreased fertility (Charfeddine & Pérez-Cabal, 2017; Omontese et al., 2020) reduced longevity (Charfeddine & Pérez-Cabal, 2017; Randall et al., 2016) and lower milk yield (Amory et al., 2008; Cha et al., 2010; Charfeddine & Pérez-Cabal, 2017).

SU and WLD are both hoof disorders categorized as claw horn disruption lesions (CHDL) (Hoblet & Weiss, 2001). SU begins to develop when increased compression of soft tissues of the sole start to hinder growth of these tissues and necrosis is initiated (Ossent & Lisher, 1998). Decreased digital cushion thickness and low body condition score are tightly correlated with the pathogenesis of the SU because these allow the pedal bone to compress the sole (Green et al., 2014; Griffiths et al., 2020; Newsome et al., 2017; Ossent & Lisher, 1998). Also, structural weakness of the collagen layer in the dermis can lead to sinkage of the pedal bone and increased compression (Mülling & Lischer, 2002). White line is the part where the coronary wall and sole are connected. Numerous lesions can occur due to structural weaknesses (Mülling & Lischer, 2002). Phase of lactation and number of lactations can indirectly predispose cattle to sole ulcers and white line disease (Bicalho et al., 2009; Newsome et al., 2017; Tarlton et al., 2002).

Digital dermatitis (DD) is a contagious bacterial hoof disease of cattle and has been found worldwide (Evans et al., 2016; Orsel et al., 2017). There are multiple stages of the disease (Biemans et al., 2017; Dopfer et al., 2012) of which M1, M2 and M4.1 lesions are considered active, and M3 and M4 lesions inactive (Biemans et al., 2017).

Acute phase response (APR) is an inflammatory response of the host following a tissue injury (Kushner, 1982). APR acts through pro-inflammatory cytokines such as interleukine-6 (IL-6) and acute phase proteins (APPs), restricting microbial growth and maintaining homeostasis (Murata et al., 2004). During the acute phase response APPs prevent the humoral and cell-mediated immune system from causing unnecessary damage to the host organism (Gatt et al., 1998; Rossbacher et al., 1999). Elevated bovine APPs such as serum amyloid A (SAA) and haptoglobin (Hp), have been detected in the venous blood of cattle suffering from various diseases, including lower respiratory disease (Prohl et al., 2015), mastitis (Suojala et al., 2008; Thomas et al., 2018) and hoof disorders (Bagga et al., 2016; Ilievska et al., 2019; Kontturi et al., 2020). Other parameters have also been investigated; Almeida et al. (2008) found that lame cows had a tendency for higher serum

* Corresponding author.
*E-mail address:* hertta.pirkkalainen@helsinki.fi (H. Pirkkalainen).

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cortisol levels, and a higher cortisol:dehydroepiandrosterone ratio, which is used as an indicator of inflammation.

Disorders of the locomotor system are usually painful, with damaged tissues and impaired homeostasis, which are all characteristics of inflammation, possibly associated with APR (Tóthová et al., 2011). In human medicine there is evidence, that certain cytokines like IL-6 are involved in the initiation and persistence of pathologic pain (Zhang & An, 2007). Tadich et al. (2013) concluded in their study, that sensitivity to pain increased with higher locomotion scores and that nociceptive pressure and Hp were sensitive measures of pain from lameness. Blackie et al. (2013) showed that cows with SU have altered gait and a higher locomotion score compared to cows with other hoof lesions like WLD, DD, heel horn erosion or no hoof lesions. This indicates that SU is one of the most painful disorders affecting cows' hooves.

Many different hoof disorders can trigger APR (Bagga et al., 2016; Ilievska et al., 2019; Jawor et al., 2008; Kontturi et al., 2020). Tóthová et al. (2011) reported that concentrations of Hp, SAA and fibrinogen increased significantly in heifers with hoof disorders and SAA and Hp were significantly increased in cattle affected by SU (Ilievska et al., 2019). Tóthová et al. (2011) and Jacobsen et al. (2004) reported that there was a wide range of variation in APP results among different individuals and suggested that the variation within APP results was most likely to be caused by a wide variety of different pathologies. Elevated rectal temperature has been found in cattle and reindeer and is associated with APR after exposure to endotoxins (Carroll et al., 2009; Orro et al., 2004).

Ilievska et al. (2019) reported that SU and acute laminitis generated higher SAA and Hp concentrations than heel horn erosion, DD or white line separation. Comparable results were documented by Kujala et al. (2010): SAA was significantly greater in cows affected by SU and white line abscesses compared to healthy control group. Because concentrations of APPs reflect the severity of inflammatory processes (Jawor et al., 2008) we considered that SU might cause more soft tissue degeneration and inflammation than other hoof pathologies. As shown, there are some studies concerning APR and hoof disorders. However, there are relatively few studies focused on the dynamics of APPs over a longer period after trimming. There also seems to be a paucity of studies using a control group of healthy animals over a longer time frame.

The aim of our study was to investigate systemic inflammatory response (APR) in cows with SU, WLD and DD compared with healthy cows during 14 days after hoof trimming. Serum concentrations of SAA, Hp and IL-6 were used as APR markers. Rectal temperature was also measured to exclude other pathologies.

## 2. Materials and methods

### 2.1. Background

This study was conducted in one Estonian commercial dairy farm, which also provides research and teaching facilities for the Estonian University of Life Sciences. Veterinary services for this farm are provided by veterinarians of the Veterinary Faculty of the Estonian University of Life Sciences. Ethical permission was issued by the Ethical Committee for Animal Experiments in the Estonian Ministry of Rural Affairs (no. 115). The dairy herd was divided and housed in two separate buildings on the farm premises. Young stock and dry cows were in their own building and milking cows were placed in separate facilities. The building for milking cows was divided into facilities for cows milked using an automated milking system (AMS) and cows milked in a milking parlour. In total there were approximately 60 cows in both milking systems. Overall conditions for cattle were comparable among pens. Cows were fed total mixed ratio (TMR) ad libitum, cubicles had rubber mattresses and all solid floors were covered with rubber mats. The routine hoof trimming regime was changed during the study period from twice a year to three times a year. Routine hoof trimming was performed by a trained professional hoof trimmer. Lame cows were treated by veterinarians within a few days of detection of lameness.

### 2.2. Study group and controls

Data for this study were gathered in 2016–2018. Animals with SU, WLD or active DD were detected either during routine hoof trimming or while performing hoof trimming on lame cows. 50 heifers and cows with hoof disorders and 29 healthy controls were included in the study. If a hoof disorder was recorded for the cow during the same lactation period, only the first case was included. The same cow was included in the study for a second time if the hoof disorders were recorded during different lactations (14 animals were included for a second time as different cases).

The lesions were categorized into three classes, based on the severity of lesions. A grading system from 1 to 3 was created for SU and WLD lesions. All grades were marked, but only levels 2 and 3 were taken into the study and calculations. The grades are explained below. One cow could have another mild lesion emanating from the same disease category in another of the legs. If the cow had multiple different lesions at the same time (for example DD and SU), it was categorized as Multi ($n = 5$).

A SU1 appeared as a small, dry spot on the sole area. All <0.5 cm Ø dry lesions were scored as SU1. A lesion that was over 0.5 cm Ø and necrotic, was scored as SU2. This equates with mild lesion in the Nordic Claw Atlas (Bergsten et al., 2020). A lesion that was over 2 cm Ø and necrotic was scored as SU3. This equates with severe lesion in the Nordic Claw Atlas (Bergsten et al., 2020). SU2 and SU3 were selected for inclusion in the study population. A WLD1 was a small dot or dark line in the white line. WLD2 had a larger opening (>2 cm) in the white line. This equates with mild lesion in the Nordic Claw Atlas (Bergsten et al., 2020). WLD3 had a larger opening in the white line, which reached the corium or included a visible abscess. This equates with severe lesion in the Nordic Claw Atlas (Bergsten et al., 2020). WLD2 and WLD3 were admitted into the study population. DD1 was any inactive DD lesion (M3, M4) (Biemans et al., 2017); DD2 was an M4.1 lesion and DD3 was an M2 lesion. DD2 and DD3 were included in the study population.

The lesions were diagnosed either by the project veterinarian, or other veterinarians of Estonian University of Life Sciences. On all occasions, the project veterinarian was contacted, and the inclusion of the animal and treatment plan were discussed. A visual examination was performed by one of the study veterinarians for every cow in the SU, WLD, DD and control groups before enrolling them in the study. The aim of the visual examination was to detect ocular, nasal or vaginal discharges and signs of lethargy. Health records were also looked at, to detect animals that were being treated with any medication. Cows with detectable concurrent pathologies were not taken into the study. Cows with hoof disorders were not accepted as control animals.

In addition to hoof trimming, most of the animals with SU, WLD or active DD were treated on admission (d0). Animals with DD ($n = 6$) were treated with local antibiotic spray (Pederipra spray, Hipra, Girona, Spain) containing tetracycline. Antibiotic spray proved to be ineffective, and after a month the treatment protocol was changed. Salicylic acid (Jørgen Kruuse A/S, Langeskov, Denmark) was used as active DD treatment until the end of the study ($n = 33$). Animals with WLD were randomly assigned either to the corrective hoof trimming group or to the corrective hoof trimming, shoe and NSAID group. The number of WLD cases appeared to be low, and therefore all the WLD cases after the first hoof trimming ($n = 7$) received one injection of a nonsteroidal anti-inflammatory drug (NSAID), carprofen at a dose 1.4 mg/kg (Rimadyl Bovis vet 50 mg/ml, Zoetis, Parsippany, USA), and a shoe (Demotec Easy Bloc, Demotec Demel e.K., Nidderau, Germany) on the healthy claw. Only three cows were treated solely with corrective hoof trimming. Animals with SU were randomly assigned to either the corrective hoof trimming group or to the corrective hoof trimming, shoe and NSAID group. Seven cases were treated with corrective hoof trimming and eight cows received one injection of a nonsteroidal anti-inflammatory drug

(NSAID), carprofen at a dose 1.4 mg/kg (Rimadyl Bovis vet 50 mg/ml, Zoetis, Parsippany, USA), and a shoe (Demotec Easy Bloc, Demotec Demel e.K., Nidderau, Germany) on the healthy claw. There were only five cows with multiple different severe lesions and therefore the Multi group was left out of calculations.

Blood sampling and measurement of rectal temperatures were performed on all study animals at similar intervals. These procedures were done on days 0 (d0), 7 (d7) and 14 (d14), d0 being the day of hoof trimming, diagnosis, and admission into the study. There could be +/- one day difference in the sampling on d7 and d14. Blood samples were taken after hoof trimming and diagnose, but before administration of NSAIDs. Rectal temperatures of study group animals and control animals were measured using Microlife VT 1831 thermometers (Microlife AG Swiss Corporation, Widnau, Swizerland).

### 2.3. Analysis of blood samples

Blood samples ($n$ = 279) were taken into vacutainer serum tubes using coccygeal vein puncture. In total, samples from control animals ($n$ = 87) and samples from cows with SU, WLD and DD ($n$ = 192) were taken within the study period. Serum was separated by centrifugation and frozen at -20°C until further analysis. SAA, Hp and IL-6 concentrations were measured from these samples. The haemolysis of samples was visually estimated in the following categories before analysis: No haemolysis ($n$ = 250), mild haemolysis ($n$ = 25), moderate haemolysis ($n$ = 1) and severe haemolysis ($n$ = 3). SAA and Hp analyses were performed in the Institute of Veterinary Medicine of the Estonian University of Life Sciences. Analyses determining the IL-6 concentrations were performed at the Clinical Research Laboratory of the Faculty of Veterinary Medicine of the University of Helsinki.

In order to determine SAA concentrations in serum samples, a commercial sandwich enzyme linked immunosorbent assay (ELISA) kit (Phase Serum Amyloid A Assay (SAA) – Multispecies, Tridelta Development Ltd., Maynooth, Co. Kildare, Ireland) was used. Analysis, calibrator, and dilution protocols designed for bovine species were used according to the manufacturer's instructions. Initial dilutions of 1:500 of the samples were used. A calibration curve, with a high value of 300 mg/l, was made for analysing the serum samples. If the results of the samples exceeded the measuring range, the samples were reanalysed using a dilution of 1:2000. The lower detection limit of the analytical procedure was 0.3 mg/l and intra- and inter-assay CV% were <12%.

Serum haptoglobin analyses were performed according to the method suggested by Makimura & Suzuki (1982) with a modification (Alsemgeest et al., 1994) using TMB (0.06 mg/ml) as a substrate instead of o-dianisidine. To create a standard curve, a pooled and lyophilized bovine acute phase serum aliquot was used (Orro et al., 2008). The calibration of a standard curve was achieved by using bovine serum sample of known Hp concentration, which was provided by the European Commission Concerted Action Project (number QLK5-CT-1999–0153). The calibration curve ranged from 60 mg/l to 674 mg/l. Samples with higher results were re-assayed using a sample 1:5 dilution with 0.9% NaCl solution. The detection limit of the analytical procedure was 60 mg/l and intra- and inter-assay CV% were <10%.

IL-6 concentrations in serum samples were measured using an amplified luminescent proximity homogeneous assay (AlphaLISA) kit (AlphaLISA Bovine IL-6 Detection Kit, PerkinElmer Inc., Waltham, USA) and an EnSight Multimode Plate Reader (PerkinElmer Inc., Waltham, USA). Analyses were performed according to the manufacturer's instructions. The lower detection limit of the analytical procedure was 0.3 µg/l and intra- and inter-assay CV% were <16%.

### 2.4. Statistical analysis

To study the differences among inflammatory markers in diseased groups (SU, WLD and DD) and control cows during the study period, tree-level nested linear mixed regression models were used. The cow, study inclusion time of the cow (14 animals were included twice during different lactation periods) and sample time were included as random factors. To model correlation of repeated sampling (d0, d7 and d14), an isotropic spatial exponential covariance structure was utilized. SAA, Hp, Il-6 concentrations and rectal temperature were used as outcome variables in these four models. To achieve a normal distribution of outcome variables, logarithmic transformation of SAA and IL-6 and inverse square root transformations for Hp values were used (due to using inverse transformation higher values representing lower Hp concentrations – see Fig. 1). Interaction between study group (DD, WLD, SU and control) and study day (d0, d7 and d14) was included as a fixed factor in all models. For comparison of inflammatory markers, differences between controls and hoof disease groups on different study days and between d0 values with d7 and d14 values within study groups, appropriate contrasts were used. Bonferroni corrected $p$-values for pairwise comparisons were used to avoid type 1 errors. Days in milk at

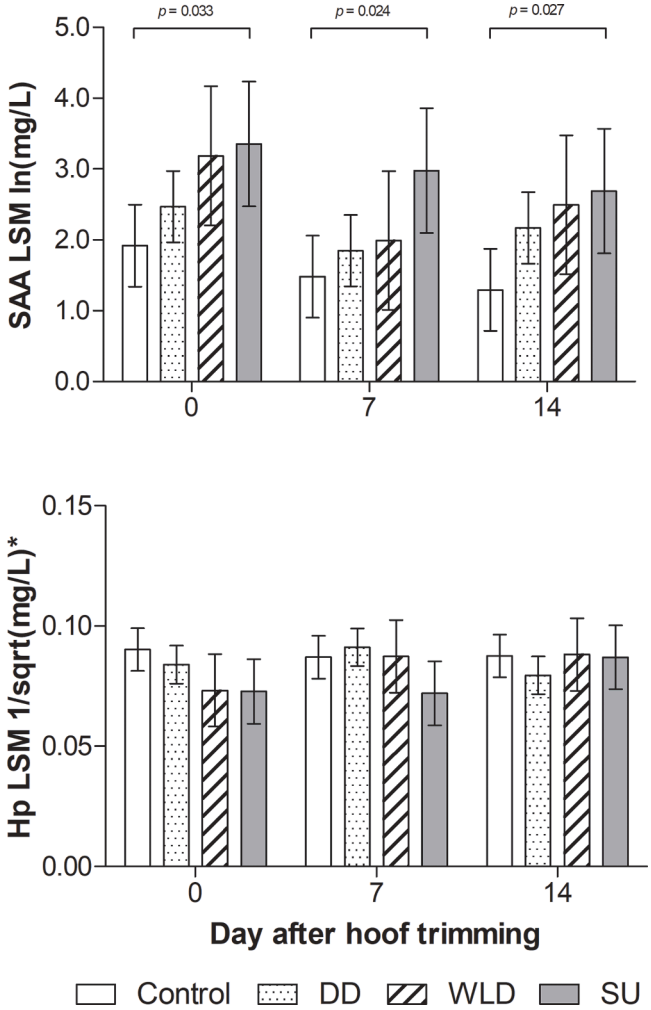


**Fig. 1.** Serum amyloid A and haptoglobin values in different hoof disorder groups within the two-week study period.
Least square means (LSM) and 95% confidence intervals of log transformed serum concentrations of serum amyloid A (SAA) and reciprocal square root* (1/sqrt) transformed haptoglobin (Hp) concentrations of cows in digital dermatitis (DD, $n$ = 39), white line disease (WLD, $n$ = 10), sole ulcer (SU, $n$ = 15) and control groups ($n$ = 29) on day 0, 7 and 14 after hoof trimming. $P$-values (Bonferroni corrected) indicate significant difference between columns connected with the line.
* This is an inverse transformation and higher values represent lower Hp concentrations.

d0 (negative value for heifers – days to parturition) as a covariate and a cow's lactation number group (heifer $n = 9$, 1–3 lactations $n = 54$, and over 4 lactations $n = 10$) as a three-level categorical variable were included in all models. Serum sample haemolysis (no or yes) was included in the model for Hp and IL-6 as a confounder (the change of coefficient over 15% after variable elimination). Initially the effect of treatment on the inflammatory markers separately in hoof disorder groups was evaluated with similar linear mixed models. Because there were no significant effects of treatment, this was not included in the final models.

Assumptions of models were controlled using normality and scatter plots of model residuals. Significance level was set at $p \leq 0.05$ and statistical analyses were performed using Stata 14.2 (StataCorp LP, Texas, USA). Microsoft Excel for Mac version 16.35 (Microsoft, Redmond, Washington, USA) was used for data management.

## 3. Results

### 3.1. Descriptive results

Our study consisted of 21 Estonian Red and 57 Estonian Holstein-Friesian cows and one Estonian Native Breed cow ($n = 79$). The mean number of lactations of all study cows on d0 was 2.1 (SD 1.7). The mean number of lactations was 4.4 (SD 1.8) in the SU group; 3.6 (SD 2.2) in the WLD group; 1.7 (SD 1.3) in the DD group and 1.5 (SD 1.2) in the control group. The mean 305-day lifetime average milk yield of study cows was 9015 kg (SD 1538 kg). The mean 305-day lifetime average milk yield in different groups was: 9855 kg (SD 838 kg) in the SU group; 8383 kg (SD 1580 kg) in the WLD group; 9358 kg (SD 1425 kg) in the DD group and 8596 kg (SD 1591 kg) in the control group.

The number of cows in different groups was: 15 in the SU group, 10 in the WLD group, 39 in the DD group and 29 in the control group. In total, four cows had SU2 and 11 cows SU3; three cows had WLD2 and 7 WLD3; 4 cows had SU2 and 11 cows SU3;12 cows had DD2 and 27 DD3.

### 3.2. SAA and Hp

Results for SAA and Hp are shown in Table 1 and Fig. 1. Results of linear mixed model for evaluating the differences of SAA concentrations (mg/l) after logarithmic transformation of diseased groups and control cows during the study period are shown in Table 2. SAA in the SU group was higher than in the control group on d0 (median (min-max); 54.9 mg/L, 2.0–192.4) ($p = 0.033$), d7 (32.7; 1.5–159.4 mg/L) ($p = 0.024$) and d14 (32.4 mg/L, 0.3–165.8) ($p = 0.027$). The median SAA levels in the control group were 22.4 mg/L (0.3–136.3) on d0, 16.4 mg/L (0.3–152.9) on d7 and 8.8 mg/L (0.3–40.2) on day 14 (Table 1, Fig. 1).

### 3.3. IL-6 and rectal temperature

Results of IL-6 and rectal temperature are shown in Table 1 and Fig. 2. IL-6 concentration was significantly lower ($p = 0.02$) on d7 (median (min-max); 13.4 μg/L, 0.3–28.7) than on d0 (15.8 μg/L, 0.3–27.2) in the SU group (Fig.2). The mean temperature was lower ($p = 0.01$) on d7 (mean (±SD);38.4 ± 0.31°C) compared with d0 (38.7 ± 0.19°C) in the SU group (Table 1, Fig. 2).

## 4. Discussion

### 4.1. Sole ulcer

SAA of the SU group was the only measured variable that consistently stayed elevated throughout the study period of 14 days when compared with that for control animals. Many other studies have also reported elevated SAA levels for SU cows (Ilievska et al., 2019; Kujala et al., 2010; Tóthová et al., 2011). At the last sampling, the statistical difference between the control group and the SU group was still significant. In the light of these findings, we suggest that SU can cause systemic APR, detectable by SAA. However, SAA concentration in the blood is proportional to tissue damage (Gatt et al., 1998; Murata et al., 2004) and this might explain why the SAA concentrations stayed elevated for as long as 14 days in our study. On admission and possibly even on d7, some of the SU lesions had necrotic tissue, which might have kept SAA concentrations elevated. Tóthová et al. (2011) proposed that mere pain may increase SAA concentration in the blood, and this is supported by research in human medicine (Zhang & An, 2007). Thomas et al. (2015) showed that the healing process of SU is slow, and our results support this and suggest that animals with SU may be affected by prolonged pain. Thomas et al. (2016) demonstrated that the response to treatment of cows with chronic CHDL was poor, regardless of the treatment administered.

Monitoring the healing progress of SU is laborious and sometimes nearly impossible using conventional means. In our study we are unable to estimate how long the cow might have been affected by SU on admission. A longitudinal study, focusing on the early detection and treatment of SU, would offer valuable information on the APR of SU lesions. If APPs could be used to monitor the healing process, we would be able to evaluate and adjust the treatment of cows better.

Unlike SAA, the blood Hp concentrations of SU were not significantly increased when compared with controls throughout the two-week period. Petersen et al. (2004) and Jacobsen et al. (2004) suggest that the synthesis of SAA and Hp are regulated in different ways. It seems that Hp is not a very sensitive marker for this type of hoof lesion. This might

**Table 1**
Serum concentrations of serum amyloid A (SAA), haptoglobin (Hp), interleukin (IL)-6 and rectal temperature of cows in digital dermatitis (DD, $n = 39$), white line disease (WLD, $n = 10$), sole ulcer (SU, $n = 15$) and control groups ($n = 29$) on day 0, 7 and 14 after hoof trimming.

| Measure | Group | Day 0 mean (±SD) median (min-max) | Day 7 mean (±SD) median (min-max) | Day 14 mean (±SD) median (min-max) |
|---|---|---|---|---|
| SAA (mg/L) | Control | 22.4 (33.4) | 16.4 (32.3) | 8.8 (10.9) |
| | | 9.4 (0.3–136.3) | 5.7 (0.3–152.9) | 4.5 (0.3–40.2) |
| | DD | 28.8 (40.9) | 23.9 (34.0) | 34.9 (55.2) |
| | | 12.1 (0.3–176.0) | 7.3 (0.3–140.7) | 11.3 (0.3–193.5) |
| | WLD | 49.9 (53.8) | 17.0 (20.8) | 26.5 (49.8) |
| | | 25.0 (1.4–153.0) | 9.4 (0.3–60.8) | 9.1 (3.8–165.9) |
| | SU | 54.9 (66.5)* | 32.7 (46.5)* | 32.4 (49.9)* |
| | | 23.4 (2.0–194.2) | 10.1 (1.5–159.4) | 10.1 (0.3–165.8) |
| Hp (mg/L) | Control | 148 (82) | 170 (131) | 151 (88) |
| | | 125 (60–711) | 124 (60–711) | 129 (60–458) |
| | DD | 243 (384) | 188 (183) | 310 (480) |
| | | 172 (60–2491) | 145 (60–1010) | 149 (60–2621) |
| | WLD | 239 (189) | 181 (173) | 189 (237) |
| | | 175 (81–607) | 132 (60–649) | 116 (60–852) |
| | SU | 277 (273) | 237 (205) | 211 (219) |
| | | 202 (60–1175) | 183 (72–920) | 108 (60–865) |
| IL-6 (μg/L) | Control | 16.5 (18.2) | 16.3 (12.1) | 16.3 (12.5) |
| | | 14.0 (1.4–101.5) | 14.2 (1–54.1) | 13.3 (1.6–55.1) |
| | DD | 12.4 (12.1) | 12.3 (13.0) | 11.8 (12.7) |
| | | 9.6 (0.4–59.7) | 9.5 (0.4–65) | 9.2 (0.3–58) |
| | WLD | 13.8 (15.3) | 13.2 (15.0) | 13.4 (14.2) |
| | | 5.1 (1.9–42.3) | 4.8 (1.8–41.7) | 5.7 (1.6–38.6) |
| | SU | 15.8 (7.8) | 13.4 (8.2) | 14.0 (8.4) |
| | | 18.2 (0.3–27.2) | 13.2 (0.3–28.7) | 13.7 (2.1–30.0) |
| Rectal temp. (°C) | Control | 38.5 (0.27) | 38.5 (0.24) | 38.5 (0.23) |
| | DD | 38.6 (0.24) | 38.5 (0.26) | 38.6 (0.22) |
| | WLD | 38.5 (0.36) | 38.4 (0.21) | 38.5 (0.21) |
| | SU | 38.7 (0.19) | 38.4 (0.31)# | 38.5 (0.22) |

* Significant difference compared to control group at the same study day evaluated by tree-level nested linear random model ($p < 0.05$, Bonferroni corrected for pairwise comparisons).

# Significant difference compared to 0 day in the same group at the same study day evaluated by tree-level nested linear random model ($p < 0.05$, Bonferroni corrected for pairwise comparisons).

**Tabel 2**
Results of linear mixed model for evaluating the differences of serum amyloid A (SAA) concentrations (mg/l) after logarithmic transformation of diseased groups and control cows during the study period.

| Variable | Coef. | ±SE | *p*-value | Wald*p*-value |
|---|---|---|---|---|
| **Sample day in control group:** | | | | 0.200 |
| day 0 | 0 | | | |
| day 7 | -0.436 | 0.307 | 0.156 | |
| day 14 | -0.626 | 0.361 | 0.083 | |
| **Hoof disorder in day 0:** | | | | 0.041 |
| control | 0 | | | |
| DD | 0.550 | 0.390 | 0.158 | |
| WLD | 1.273 | 0.592 | 0.032 | |
| SU | 1.432 | 0.564 | 0.011 | |
| **Day*hoof disorder:** | | | | 0.794 |
| day 0*control | 0 | | | |
| day 7*DD | -0.137 | 0.404 | 0.735 | |
| day 7*WLD | -0.654 | 0.609 | 0.283 | |
| day 7*SU | 0.059 | 0.531 | 0.912 | |
| day 14*DD | 0.337 | 0.478 | 0.480 | |
| day 14*WLD | -0.064 | 0.716 | 0.929 | |
| day 14*SU | 0.037 | 0.622 | 0.953 | |
| **Lactation number:** | | | | 0.011 |
| Heifer | 0 | | | |
| 1–3 lactations | 1.432 | 0.516 | 0.006 | |
| ≥ 4 lactations | 0.892 | 0.599 | 0.137 | |
| **Days in milk:** | -0.004 | 0.001 | <0.001 | |
| **Constant** | 1.303 | 0.489 | 0.008 | |

Tree-level nested mixed regression models were used. The cow, study inclusion time of the cow (14 animals were included twice during different lactation periods) and sample time were included as random factors and correlation between repeated samples were accounted using isotropic spatial exponential covariance structure. Data from cows with digital dermatitis (DD, $n = 39$), white line disease (WLD, $n = 10$), sole ulcer (SU, $n = 15$) and from cows in control group (control, $n = 29$) on day 0, 7 and 14 after hoof trimming was used (altogether 279 samples). For evaluating group differences contrasts were used and Bonferroni corrected *p*-values were calculated (results are in Fig. 1).

be due to the non-infectious origin of SU (Ossent & Lisher, 1998) for Hp is associated with bacterial infection and not with non-infectious conditions (Skinner et al., 1991).

IL-6 is one of the cytokines that initiate the release of APPs, especially SAA (Alsemgeest et al., 1994; Murata et al., 2004; Yoshioka et al., 2002). As evident in the results, the level of IL-6 decreased in the SU group from d0 to d7. The reason behind this might be that IL-6 appears to peak only for a short time (a few hours to a day) in blood (Hagiwara et al., 2001). In our study, IL-6 concentrations might have already dropped due to most pathologies existing for longer than a day. As shown by Thomas et al. (2016) SU lesions are slow to develop.

Regarding the IL-6 levels in the control group, the concentration was higher than in any other group throughout the study period, although none of these findings had $p < 0.05$. At the same time, the body temperature remained at a normal, similar level between the days. Studies suggest that diseased animals may have lower proinflammatory cytokine levels due to downregulation of cytokines production by peripheral blood mononuclear cells during DD (Zuerner et al., 2007) and after lipopolysaccharide challenge in cattle (Jacobsen et al., 2007). It can be concluded that this level is normal for the healthy cows, and they are ready for immunological challenges.

A decrease in rectal temperature was recorded only in the SU group from d0 to d7. However, the change in average body temperature was only 0.26°C. SAA concentrations suggest that SU animals had tissue damage and possibly pain on d7 and d14. However, changes in temperature and IL-6 might indicate how the clinical status of the animals with SU had improved between d0 and d14. NSAIDs act by inhibiting of the synthesis and release of prostaglandins and thus resulting in decreased body temperature, pain, and inflammation (Weissmann et al., 1987). The body temperature decreasing effect of NSAIDs used in our study does not last for more than 2–3 days. However, there could be

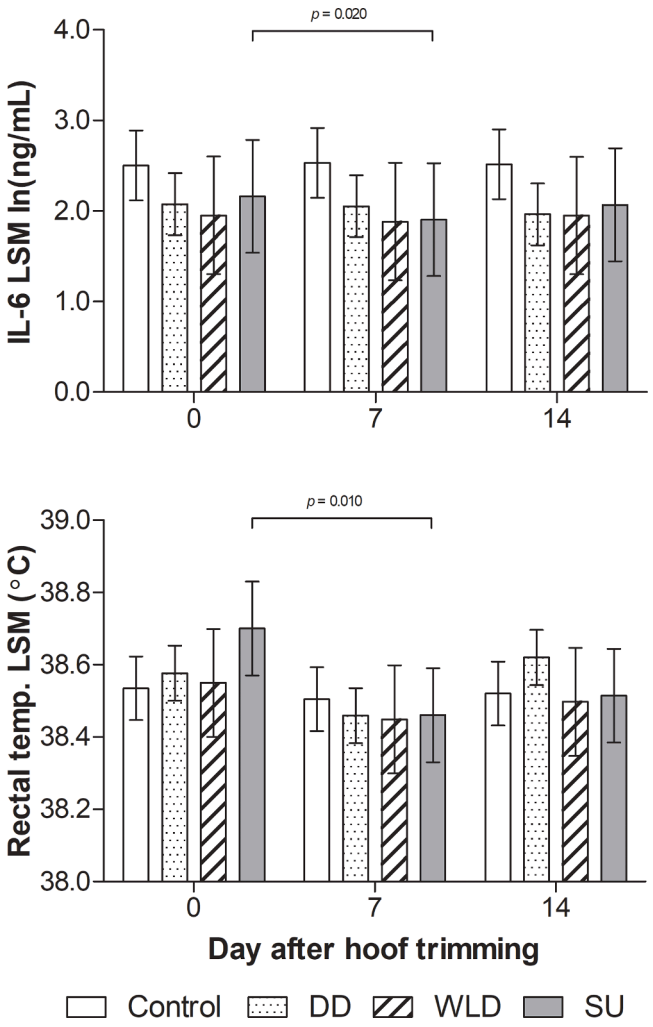


**Fig. 2.** Iinterleukin-6 and rectal temperature values in different hoof disorder groups within the two-week study period.
Least square means (LSM) and 95% confidence intervals of log transformed serum concentrations of interleukin (IL)-6 and rectal temperature of cows in digital dermatitis (DD, $n = 39$), white line disease (WLD, $n = 10$), sole ulcer (SU, $n = 15$) and control groups ($n = 29$) on day 0, 7 and 14 after hoof trimming. *P*-values (Bonferroni corrected) indicate significant difference between columns connected with the line.

prolonged effects on inflammation. We administered NSAIDs on d0 after diagnosis and sampling, and thus believe that the rectal temperatures on d7 or d14 were not affected by the use of NSAIDs.

### 4.2. *White line disease*

WLD is an umbrella term for various lesions affecting the white line (Mülling & Lischer, 2002). White line abscesses are considered more painful (Shearer & van Amstel, 2017) than smaller lesions. In our study seven of the WLD cases were found during regular hoof trimming and did not appear to have an abscess, even though some of them were categorised as WLD3. Three individuals were included into the study due to lameness and were diagnosed with an abscess. We placed all the cows with moderate to severe white line disorders in the same group due to low number of cases. However, the APR could have differed between the individuals and severities. It would be interesting to collect data over a longer period in order to locate those animals with more severe white line abscesses. Concentration of APPs could be higher in animals with more severe lesions than in individuals with milder lesions.

### 4.3. Digital dermatitis

Digital dermatitis is a local skin disease (Dopfer et al., 2012) and although it has been reported to elevate the APPs (Ilievska et al., 2019), there is little information available on the different lesion stages and their acute phase response.

DD had caused problems for our study farm for an extended period. Approximately 30% of our DD lesions were M4.1 class, meaning that they were reactivated chronic lesions and might not have caused a similar systemic response as new M2 lesions. Also, many of the M2 lesions seemed to be proliferative and thus of chronic origin (ICAR). Another interesting issue is the re-occurrence of active DD lesions, which are known to reappear in treated cows (Berry et al., 2010, 2012). After a month, 8% of lesions were re-treated and in total, 54% of lesions were re-treated with lincomycin during a 11-month study made by Berry et al. (2012). It is possible that some of the lesions in our study did not heal properly or might have even begun to reactivate and this might have affected the APR of DD lesions.

There is evidence of a systemic effect of DD as the disease has been shown to decrease milk yield (Gomez et al., 2015; Relun et al., 2013). Additionally, Relun et al. (2013) showed that the milk yield was reduced to a greater extent in cows with more severe DD lesions (M2). Only a few studies have investigated the proinflammatory cytokines and local inflammatory response at cell level (Scholey et al., 2013; Zuerner et al., 2007). Scholey et al. (2013) studied the host pathogenic pathways in cows with DD. The skin biopsies of lesions had increased expression of mRNA for α-2-macroglobulin-like 1, keratin 6A and IL-1β, but reduced expression of most other keratin and keratin-associated genes. There was little evidence of local immune reactions to the bacterial infection present in DD lesions. An earlier study of Zuerner et al. (2007) suggested that the innate immune and wound repair functions of bovine macrophages exposed to treponeme cellular components were impaired, and thus enabled bacteria to resist elimination and induce lesion formation. Evans et al. (2014) also showed the upregulation of genes encoding several inflammatory mediators, such as tumour necrosis factor alpha in fibroblasts, but not in keratinocytes.

It would be important to study APR further in lesions of different chronicity, and for a longer period in order to understand the systemic reaction related to these hoof disorders and to understand the healing process.

### 4.4. General

We took the first blood samples a few moments after hoof trimming, but before administration of NSAIDs. Although hoof trimming causes stress to the animals, also control animals were subjected to the stress of hoof trimming on d0 and therefore we consider the stress to have been similar in the two study groups. As we took samples on d7 and d14, the animals were not subjected to similar stress as on the day of admission. Because stress levels were different on d0 when compared with d7 and d14, we must take this into account when interpreting APP concentrations (Lomborg et al., 2008; Murata et al., 2004) and rectal temperature results. Because of this aspect of the study setting, comparison of the results between sampling days must be made with caution. We performed a visual examination for all groups to exclude any concurrent systemic disease on admission, but we could not completely exclude all possible underlying pathologies, which might have increased APPs in the blood. However, we suggest that effects of underlying pathologies had an equal effect on the control group and hoof disorder groups.

This study had several shortcomings. The sample size for dairy cows with SU ($n = 15$) and WLD ($n = 10$) was relatively small, which could predispose our study to error. We included DIM of the cows in the statistical analysis because blood APP concentrations increase at calving (Alsemgeest et al., 1994). As mentioned by Pohl et al. (2015) APP levels increase at calving because of the trauma experienced. Though calving increases APP concentration in blood, the level also decreases gradually after labour. By including DIM in the statistical model, we were able to balance the effect of this gradual change of APPs. In addition, APP concentrations are lower in multiparous cows than in primiparous cows (Pohl et al., 2015). The range of parities of animals admitted to our study was also quite wide. Both phase of lactation (Bicalho et al., 2009) and the number of lactations (Charfeddine & Pérez-Cabal, 2017; Knott et al., 2007; Räber et al., 2004) along with parturition (Knott et al., 2007) itself, affect the susceptibility of cows to hoof disorders. By adding DIM and parity into statistical models, we hoped to eliminate the effect of imbalance of groups to our results. However, we cannot totally exclude, that on some occasion it may still have influenced the results. Probably this may have reduced the hoof lesion groups APP concentration differences from control animals e.g., in case of SU and WLD where cows were considerably older than control cows.

The treatment differed between SU2 and SU3 cows, and between WLD2 and WLD3 cows. We did categorize these animals into two different subgroups, but the numbers of animals were low, and we did not establish any statistically significant difference between the different treatment groups. Differences based on the treatment and healing process would merit further research. However, as Thomas et al. (2016) suggested, the healing of chronic lesions appears not to be affected by the treatment.

## 5. Conclusions

We studied acute phase response of cows with different hoof lesions. Our study showed that serum amyloid A, a marker of inflammation, was still elevated two weeks after treatment of sole ulcers. This indicates that we have a chronic hoof disorder that heals slowly. We ought to focus on early detection, good management, and proper pain medication of cows with sole ulcers.

## Ethical statement

This research had an ethical permission issued by the Ethical Committee for Animal Experiments in the Estonian Ministry of Rural Affairs (no. 115).

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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