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PII: S0165-2427(24)00024-2

DOI: https://doi.org/10.1016/j.vetimm.2024.110738

Reference: VETIMM110738

To appear in: *Veterinary Immunology and Immunopathology*

Received date: 22 May 2023
Revised date: 20 February 2024
Accepted date: 21 February 2024

Please cite this article as: Nazaré Storms, Geoffroy de la Rebière, Thierry Franck, Ange Mouithys Mickalad, Charlotte Sandersen, Justine Ceusters and Didier Serteyn, Neutrophil extracellular traps and active myeloperoxidase concentrate in lamellar tissue of equids with naturally occurring laminitis, *Veterinary Immunology and Immunopathology,* (2024) doi:https://doi.org/10.1016/j.vetimm.2024.110738

Neutrophil extracellular traps and active myeloperoxidase concentrate in lamellar tissue of equids with naturally occurring laminitis

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Abstract

Laminitis is a pathology of the equine digit ultimately leading to a failure of the dermo-epidermal interface. Neutrophil activation is recognized as a major factor in SIRS-associated laminitis and has recently been described in induced endocrinopathic laminitis evidenced by the presence of myeloperoxidase (MPO). Neutrophil extracellular traps (NET) are released with neutrophil activation. This study aimed to investigate the presence and activity of MPO and NET in the lamellar tissue of equids presented with naturally occurring laminitis.

Samples of lamellar tissue of five horses and five donkeys presented with laminitis, as well as eight control horses without laminitis, were collected. Lamellar tissue extracts were submitted to ELISA and specific immuno-extraction followed by enzymatic detection (SIEFED) assays to confirm the presence and activity of both MPO and NET. Lamellar sections were also immunohistopathologically stained for MPO and NET.

Analysis of lamellar tissue extracts revealed that laminitis cases had significantly higher levels of total MPO concentration, MPO activity, and NET-bound MPO activity in comparison to control horses. Moreover, a strong correlation was identified between the activity of NET-bound MPO and the total MPO activity, which suggests that MPO activity ~~predominantly~~ partly originates from NET-bound MPO. Immunohistochemical staining showed that MPO and NET labelling in laminitis cases was moderate to marked, primarily in the epidermis and in inflammatory infiltrates containing neutrophils, while labelling in control horses was minimal. This article constitutes the first ~~confirmation~~ indication of the presence and activity of NET-bound MPO in the lamellar tissue of horses and donkeys with naturally occurring laminitis. Targeting these substances may provide new treatment possibilities for this debilitating disease.

Keywords: Horse – Laminitis – Neutrophil extracellular traps (NET) – Myeloperoxidase (MPO) – Polymorphonuclear cell

Abbreviations list:

BCS: body condition score

BSA: bovine serum albumin

BWE: black walnut extract

citH3: citrullinated histone H3

CNS: cresty neck score

EHV-1: equine herpes virus 1

EMS: equine metabolic syndrome

HE: hematoxylin-eosin

IV: intravenous

MPO: myeloperoxidase

msMSCs: muscle-derived mesenchymal stem cells

NET: neutrophil extracellular traps

PAD4: peptidyl arginine deiminase 4

PBS: phosphate buffer saline

pEHC: prolonged euglycemic hyperinsulinemic clamp

PEL: primary epidermal lamellae

PPID: pars pituitary intermedia dysfunction

SEL: secondary epidermal lamellae

SIEFED: specific immuno-extraction followed by enzymatic detection

Declarations of interest: none

Funding source: This work was supported by Lamistem a project of the University of Liège.

1. Introduction

Laminitis is a common and severe disease of the digit of horses, characterized by a structural failure of the dermal–epidermal interface, causing destabilization and ultimately displacement of the distal phalanx. This leads to severe lameness and dramatic pain, sometimes requiring euthanasia (Menzies-Gow et al., 2010; Katz and Bailey, 2012; Leise, 2018). Laminitis results

from local consequences of an excessive systemic inflammatory reaction with decreased blood flow and inflammation in the foot, endothelial/vascular dysfunctions, degradation of the extracellular matrix, and metabolic disturbances in keratinocytes (Peroni et al., 2005; Loftus et al., 2006; Treiber et al., 2009; Holbrook et al., 2012; Serteyn, 2014).

The activation of polymorphonuclear neutrophils in the initial stages of experimental laminitis is well described (Lunn and Hurley, 2009; Leise, 2018). Our group specifically demonstrated the presence of both myeloperoxidase (MPO) and elastase, 2 major proteins from the alpha granules of neutrophils, in the bloodstream, skin and lamellar tissue of horses with black walnut extract (BWE) induced laminitis (Riggs et al., 2007; de la Rebière de Pouyade et al., 2010). MPO is a hemic enzyme, released from activated neutrophils during uncontrolled and excessive inflammation or cell death (Riggs et al., 2007; Belknap, 2010). MPO has a dual peroxidase and chlorination activity responsible for the direct or indirect synthesis of many oxidizing species that participate in host defense mechanisms. However, their derived products can induce chlorination, nitration, and oxidation of protein residues which results in severe cell and tissue damage (Klebanoff, 2005; Valadez-Cosmes et al., 2022). In addition, MPO can be taken up by endothelial cells, which can subsequently be damaged by its derived products (Mathy-Hartert et al., 1995; Benbarek et al., 2000; de la Rebière et al., 2008). Therefore, MPO and its derived oxidative species could play a role in establishing events leading to laminitis in a variety of ways.

In previous studies, a limited number of neutrophils has been observed in lamellar tissue of horses undergoing prolonged euglycemic hyperinsulinemic clamp (pEHC) laminitis induction, a model for endocrinopathic laminitis, and it was concluded that the extent and severity of inflammation are less important than would be expected in such form of laminitis (Asplin et

al., 2007; de Laat et al., 2011). However, the presence of MPO in lamellar tissue was recently demonstrated in cases using the same model (Storms et al., 2022). Even though neutrophils themselves were also only rarely visible, this supports a role for neutrophil activation in the early phase of the disease (Storms et al., 2022). The absence of neutrophils themselves might be explained by distant degranulation with secondary MPO infiltration, the transport of MPO through neutrophil extracellular vesicles or the formation of neutrophil extracellular traps (NET) from dying infiltrated neutrophils.

NETs are extracellular strands of decondensed (unwound) DNA in complex with histones and neutrophil granule proteins, such as MPO and elastase, which were expelled from neutrophils to ensnare and kill microbes (Sørensen and Borregaard, 2016; Delgado-Rizo et al., 2017). "NETosis" is the term commonly used to describe the sequence of cellular events leading up to the active release of NET (Yipp and Kubes, 2013; Li and Tablin, 2018). In these mechanisms, citrullination of histones by peptidyl arginine deiminase 4 (PAD4) is central for NET formation in vivo as well as neutrophil elastase as it cleaves histones during NET formation (Papayannopoulos et al., 2010; Li and Tablin, 2018). Different types of NETosis have been described: "Suicidal NETosis" which is dependent on reactive oxygen species for histone citrullination by PAD4 before releasing DNA as extracellular traps and death of the neutrophil as the nuclear and plasmatic membranes are lost in the process. In contrast, in "vital NETosis" the neutrophil survives as all NET components are released in the extracellular area by the fusion of vesicles containing NET components with the plasmatic membrane of the neutrophil (Yipp and Kubes, 2013; Delgado-Rizo et al., 2017). Although NETs have protective roles in the initial stages of sepsis, uncontrolled inflammatory responses with excessive NET formation have been found to induce thrombosis and multiple organ failure in murine sepsis models (Czaikoski et al., 2016; Li and Tablin, 2018). Indeed, cell-free DNA and histones present on NET, are activators of the coagulation system (Gould et al., 2015). Furthermore, in murine

models, endotoxemia activates platelets which then adhere to neutrophils in the reticuloendothelial system to induce NET formation that bind bacteria but mediate significant endothelial cell damage (Clark et al., 2007; Sørensen and Borregaard, 2016). This evidence highlights the pathophysiological role of NET in cases of sepsis and endotoxemia, both recognised causes of laminitis in the horse.

Interestingly, NET formation has also been confirmed during non-infectious endocrinological diseases. In the human patient, plasma NET parameters such as MPO-DNA complexes were higher in obese patients than in the control group and correlated with body mass index, body weight, waist and hip circumference, systolic blood pressure and glucometabolic parameters (D'Abbondanza et al., 2019). Furthermore, type II diabetes patients had increased NETosis compared to healthy controls (Carestia et al., 2016). In addition, NETosis from isolated neutrophils is increased by high levels of glucose in vitro (Menegazzo et al., 2015) and neutrophils from type 1 and type 2 diabetes patients and mice showed increased NET production (Wong et al., 2015). NET seems therefore implicated in processes similar to the ones encountered in endocrinopathic laminitis as well. Finally, evidence indicates that insulin regulates neutrophil function in diabetic people by increasing neutrophil chemotaxis and oxidative burst. A similar marked increase in neutrophil oxidative burst activity was shown in hyperinsulinemic obese horses (Holbrook et al., 2012).

To conclude, the presence of MPO has been demonstrated in varying experimental laminitis models but whether this fully represents naturally occurring laminitis, remains unclear. Additionally, neutrophil stimulation is also associated with NET release on which MPO molecules are bound. To our knowledge the presence of NET has never been studied in lamellar tissues of naturally occurring laminitis. Therefore, the objectives of this study were to investigate the presence and activity of MPO and NET-bound MPO in lamellar tissues of

naturally occurring laminitis. Whether the activity of MPO was associated with NET was also investigated. We hypothesized that active MPO would be present in the lamellar tissue of affected laminitis cases in greater amounts than in control horses and that its presence would correlate with active NET-bound MPO.

2. Materials and Methods

2.1 Animals

A total of 18 animals were included in this study. Ten equids (5 horses and 5 donkeys) presented at the Teaching Hospital of the University of Liège between June 2020 and October 2021 with naturally developed laminitis diagnosed based on clinical signs and radiographic examination. History, signalment, clinical examination findings, and radiographic findings were recorded for all cases and lamellar tissues were sampled with the consent of the owners.

Lamellar tissue samples of 8 horses from the slaughterhouse without any sign of acute laminitis or systemic inflammatory disease based on the clinical examination were used as controls. Signalment and clinical examination findings were collected for these cases.

2.2 Collection of lamellar tissue

Lamellar tissues were collected immediately after euthanasia. Laminitis horses were sedated with intravenous (IV) xylazine (0.6 mg/kg, Proxylaz 2%, Prodivet Pharmaceuticals, Eynatten, Belgium), then induced with a combination of ketamine (2.2 mg/kg, IV, Ketamidor 100 mg/mL, Ecuphar, Oostkamp, Belgium) and midazolam (0.06 mg/kg, IV, Midazolam 5 mg/mL, Mylan, Hoeilaart, Belgium). Finally, pentobarbital was administered (100 mg/kg, IV, Euthanimal, Kela Veterinarian, Sint-Niklaas, Belgium). Control horses were euthanized at the slaughterhouse using a penetrating captive bolt gun. The hard, keratinized part of the hoof wall was removed using an electrical rasp until the tissue was thin enough to allow sharp dissection of a full-

thickness specimen. A 1 cm broad segment of mid-dorsal lamellar tissue was collected by tracing two parallel vertical lines from the coronary band to the toe with a scalpel. These were connected with horizontal cuts at the proximal and distal parts. The tissue was then elevated and carefully detached from the third phalanx with a scalpel. Two of these segments were prepared for each horse. The tissues were placed in 4% formaldehyde for 24h and then transferred to 70% ethanol for 48h before paraffin embedding. In addition, some extra lamellar tissues were collected and placed in a dry Eppendorf container and stored at -80°C until used for the extraction protocol to determine the presence and activity of MPO and NET-bound MPO. The delay between the euthanasia and the sample storage was 20 minutes.

2.3 Chemicals and reagents

Table 1 provides a list of chemicals and reagents and their manufacturers used in this article.

The purified equine neutrophil MPO was obtained as previously described (Franck et al., 2005) with the following characteristics: 70.4 U/mg as specific activity and 3.38 mg/mL as protein concentration.

2.4 Extraction of MPO and NET in lamellar tissue

2.4.1 Extraction of lamellar tissue

The biopsy of the lamellar tissue was frozen in liquid nitrogen and cold-grounded with an analytical grinder (A11 basic, IKA®, Staufen, Germany). The crumbled tissue was transferred in a thick glass tube with 2.5 ml of 20 mmol/l cold PBS buffer (pH 7.4) and crushed with a homogeniser (T25 Ultra-Turax, IKA®, Staufen, Germany). The homogenate was transferred with 1 ml PBS buffer in a 10 ml glass potter homogenizer to finalize the tissue extraction. The homogenate was centrifuged at 14,000 g for 15 min at 4°C. The supernatants were aliquoted and frozen at -80°C. The final extraction volume was 3.5 ml. The final supernatants were used

for the total protein assay and all the immunological assays performed in this study (ELISA, SIEFED MPO and SIEFD NET).

2.4.2 ELISA for measurement of total MPO concentration

MPO concentration was measured with a specific sandwich ELISA as described by Franck et al. (2005) in biological fluid and used in lamellar tissue by Riggs et al. (2007). Briefly, rabbit anti-MPO IgG antibody was coated onto 96-well microtiter plates. The sample was then added to the microplate and incubated overnight at 4°C. After washing with PBS solution containing 0.1% Tween 20, the secondary guinea pig anti-MPO IgG conjugated with alkaline phosphatase was added. After washing the ready-for-use substrate solution (paranitrophenyl phosphate, D-Tek, Mons, Belgium) was added to detect the alkaline phosphatase activity. Finally, the absorbance (405 nm) was read with the Multiskan Ascent plate reader (Fisher Scientific, Merelbeke, Belgium). Control (blank) and dilutions of the samples (20 x) were done with the dilution buffer, and each sample was run in duplicate. The absorbance value was directly proportional to the concentration of MPO in the samples calculated via a calibration curve performed with purified equine MPO ranging from 2 to 140 ng/ml. The total MPO was referred to the total protein concentration measured by a Coomassie protein assay reagent (Fischer Scientific, Merelbeke, Belgium) in the supernatant of lamellar tissue extracts and expressed in ng total MPO/mg proteins.

2.4.3 Measurement of MPO activity by SIEFED assay:

Immediately before performing the specific immuno-extraction followed by enzymatic detection (SIEFED) assay, the supernatants obtained after the extraction of the biopsies were thawed and diluted 1:2 in a dilution buffer prepared with 20 mmol/l PBS solution (pH 7.4) containing bovine serum albumin (BSA, 5 g/l) and 0.1% Tween 20. The measurement of the peroxidase activity of MPO was performed by SIEFED assays as described on biological fluids

by Franck et al. (2006) and on lamellar tissue by Riggs et al. (2007). This technique uses an immobilized primary antibody (polyclonal rabbit anti-MPO IgG antibody) coated onto the microplate wells. The sample was added to the wells and incubated for 2 hours at 37°C in darkness to allow the capture of MPO by the antibodies. After removing the sample and three washings with 20 mM phosphate buffered saline (PBS) containing 0.1% Tween 20, the substrate (H_2O_2) and co-substrates (nitrite and Amplex Red) were added to reveal the peroxidase activity of MPO as evidenced by the oxidation of Amplex Red into its fluorescent adduct resorufin. Fluorescence was measured at the excitation and emission wavelengths of 544 and 590 nm respectively, for 30 min at 37°C with the fluorescent plate reader (Fluoroskan Ascent, Fisher, Merelbeke, Belgium). The fluorescence value was directly proportional to the quantity of active MPO in the sample. MPO concentrations were calculated in reference of a calibration curve carried out with purified equine MPO ranged from 2 to 140 ng/ml. The active MPO was referred to the total protein concentration and expressed in ng active MPO/mg proteins.

2.4.4 Measurement of active NET-bound MPO (SIEFED)

The NET released by the neutrophils was captured by anti-histone H3 (citrulline R2 + R8 + R17; anti-H3Cit) antibodies as performed in Franck et al. (2021). Then the presence of active MPO bound to the NET was detected in the same way as the SIEFED assay. This technique used an immobilized primary rabbit anti-H3Cit antibody (0.5 µg/mL) diluted with 20 mM PBS buffer coated onto a transparent 96-well microplate to capture NET. After removal of the coating solution, the plates were incubated (150 min, 22°C) with blocking buffer (PBS buffer with 5 g/L of BSA) and washed four times with PBS buffer with 0.1% Tween 20. The plates were then dried for 3 hours at 22°C and conserved in a dry atmosphere in a hermetic bag at 4°C until use. The samples were loaded into the wells of the anti-H3Cit coated microplate in

duplicate and incubated for 2 hours at 37°C. Then, the supernatants were removed, and the wells were washed four times with a PBS solution containing 0.1% Tween 20 before active MPO was measured. For the revelation of the peroxidase activity of MPO bound to NET, sodium nitrite and Amplex Red solution were added as described above (SIEFED) and fluorescence was measured over 30 min with the fluorescent plate reader (Fluoroskan Ascent, Fisher, Merelbeke, Belgium). To evaluate the level of active MPO bound to the NET, a calibration curve ranging from 2 to 140 ng/ml was performed parallelly with purified equine MPO but using wells coated with polyclonal rabbit anti-MPO IgG antibody. NET-bound MPO was referred to the total protein concentration and expressed in ng active NET-bound MPO/mg proteins.

2.5 Histology

Histological sections were prepared at 4 µm thickness and mounted on a glass slide following the standard technique. For each case, one slide was stained using a standard haematoxylin-eosin colouration as a reference for the anatomic analysis, a second slide was used for MPO immunohistochemical staining, a third slide was used for NET immunohistochemical staining and a final slide for the negative control.

2.5.1 Preparation for immunohistochemical staining for MPO and NET

Histological sections were deparaffinized and rehydrated following standard protocol: Slides were incubated overnight at 60°C, followed by successive baths in xylene (2 x 5min), ethanol 100% (2 x 2min), 95% (1 x 1min), 70% (1 x 2min) and PBS (2 x 3min). Next, an antigen retrieval protocol was performed consisting of 20 min incubation in a solution of 10 mM sodium citrate (pH = 6 and temperature 90<x< 96°C). Slides were then rinsed for 2 min with distilled water and afterwards with PBS for 2 min. The tissue was surrounded with a hydrophobic barrier

pen (PAP pen, Abcam, Cambridge, United Kingdom) and the hydrogen peroxide block (IHC kit) was applied for 10 min. The slides were then rinsed twice again with PBS for 2 min.

2.5.2 Immunohistochemical staining for MPO and NET

Immunostaining of MPO was performed as described by Storms et al. (2022). Briefly, after the addition of a protein block solution (IHC kit) and washing the slides with PBS (1 x 3min), the primary anti-MPO antibody (rabbit antibody obtained against purified equine MPO) diluted 1:1000 in dilution buffer (20 mM PBS pH 7.4 + 0.5% BSA and 0.1% Tween 20) was applied for 1h at 37°C in a humid chamber. For immunostaining of NET, a primary rabbit anti-H3Cit antibody diluted 1:200 in dilution buffer (20mM PBS pH 7.4 + 0.5% BSA and 0.1% Tween 20) was added instead of the primary anti-MPO antibody. Negative control sections were prepared by adding only the dilution buffer without the primary antibody. Then, after another washing with PBS (3 x 3min), the secondary anti-rabbit antibody produced in the goat and conjugated with biotin (IHC kit) was added for 15 min at 22°C. Slides were washed again with PBS (3 x 3min) and the streptavidin-peroxidase solution (IHC kit) was added for 15 min at 22°C. Finally, after rinsing with PBS (3 x 3min), the chromogen diaminobenzidine/substrate solution (IHC kit) was added and the appearance of MPO labelling (brown colouration) was monitored. The slides were then placed in water (3 x 3min), and counterstained with hematoxylin solution for 45 s. Slides of control horses and laminitis cases were prepared in parallel and stained simultaneously.

2.5.3 Histological assessment of the immunohistochemical staining

The sections were assessed using light microscopy (Zeiss Axioskop, Zaventem, Belgium), and all photographs were obtained using the same light intensity and shutter speed. Histological assessment was performed blindly by a well-trained PhD student and a researcher with previous

experience in assessing histologic sections of hoof lamellae. Inflammatory cell infiltration was assessed using a semi-quantitative scoring (0: none, 1: rare single inflammatory cells, 2: localized small amounts of inflammatory cell infiltrations, 3: large areas of diffuse inflammatory cell infiltration). In addition, the intensity of the immunohistochemical staining for MPO and NET was evaluated in a semi-quantitative manner (0: none, 1: mild, localized brown staining, 2: mild, diffuse brown staining, 3: moderate brown staining, 4: intense brown staining) by 10 researchers not involved in the study.

2.6 Statistical analysis

Statistical analyses were performed with commercially available software (Medcalc 20.218, Ostend, Belgium). All clinicopathological parameters and extraction results were normally distributed according to the Kolmogorov-Smirnov test and expressed as mean + standard deviation. An unpaired t-test was performed to compare differences between the values of the different variables (age, body condition score (BCS), cresty neck score (CNS), MPO slide score, NET slide score, control slide score, total MPO concentration, active MPO and active NET-bound MPO concentrations). Linear regressions were performed on the extraction results to investigate correlations between the different parameters (total MPO, active MPO, active NET-bound MPO concentrations). Statistical significance was set at $p<0.05$.

3. Results

3.1 Signalment and history

Table 2 summarizes the details about the history and signalment of each case.

3.1.1 Laminitis cases

The mean age of the 10 laminitis cases was 12.9 + 5.8 years old, with eight females and two geldings. There were three warmblood-type horses, one paint horse, one fjord pony and five donkeys. The mean weight was 438.5 + 124.5 kg with a mean BCS of 7.1 + 1.7/9 and a mean CNS of 3.2 + 1.0/5. The mean weight of the donkeys and the pony was 410.83 + 149.48 kg and the mean weight of the horses was 480.00 + 73.94 kg.

3.1.2 Control horses

The mean age of the eight control horses was 21.6 + 5.0 years old, with 6 females and two geldings. There were 6 warmblood-type horses, one Haflinger and one pony. Their mean BCS was 5.9 + 1.0/9 and their mean CNS was 1.5 + 0.8/5. Control horses were significantly older ($p=0.004$), and had a significantly lower CNS ($p=0.001$). Their BCS was not significantly different from laminitis cases ($p=0.08$).

3.2 Clinical examination and radiographic findings

The clinical parameters and radiographic findings for each case are shown in table 3 and table 4, respectively.

All but one laminitis cases presented with tachycardia (mean: 77.6 + 24.8 bpm) and tachypnoea (mean: 42.4 + 28.2 rpm). Only one laminitis case had fever (mean 37.9 + 1.3°C). All laminitis cases had increased digital pulses. Other clinical signs related to laminitis included a palpable depression at the coronary band (9/10 cases), growth lines (7/10 cases), palpable increased hoof temperature (6/10 cases), visibly thickened white line (6/7 cases), convex sole (4/9 cases) and a painful response to palpation with a hoof tester (4/5 cases). Palpation with the hoof tester, observation of a thickened white line and convex sole observation was not possible in some severe cases as the animals were too painful to allow lifting of the foot long enough for adequate observation/testing of these parameters.

Most of the laminitis cases presented with an abnormal posture (8/9 cases) including saw-horse stance (7/8 cases), lying down more frequently (2/8) and one horse was recumbent. All of the non-recumbent cases presented a stilted gait with a variable degree of reluctance to move. Horse 3 was also ataxic due to its EHV-1. The mean Obel grade of lameness was 3.4 + 0.7/5. None of the control horses presented with the clinical signs related to laminitis discussed here. Furthermore, they did not present any fever (mean temperature: 37.5 + 0.6°C), indicating probable absence of severe systemic disease. Heart rate and respiratory rate were not assessed as they were not considered representative for their normal condition given the stressful environment the animals were in.

Radiographs were performed in 9/10 laminitis cases. The most common radiographic finding was capsular rotation (8/9 cases) followed by sinking of the third phalanx (7/9 cases) and phalangeal rotation (4/9 cases). Furthermore, some horses presented a convex sole (4/9), osteitis of the third phalanx (3/9), air opacities (3/9) and lipping (2/9). No radiographs were taken of the feet of the control horses.

3.3 MPO and NET extraction from lamellar tissue

Tissue samples for extractions were available for all cases except two laminitis horses. In all cases, the presence of MPO and its activity was confirmed as well as NET-bound MPO activity. Individual values for each case can be found in table 5. The mean values for the total MPO concentration, MPO activity and NET-bound MPO activity corrected to the total protein concentration were 101.9 + 53.1 ng/mg, 3.5 + 1.5 ng/mg and 1.9 + 0.9 ng/mg in laminitis cases, whereas they were 29.0 + 9.2 ng/mg, 2.1 + 0.6 ng/mg and 1.2 + 0.3 ng/mg in control horses (Figure 1). The total MPO concentration ($p=0.002$), the MPO activity ($p=0.028$) and the NET-bound MPO activity ($p=0.045$) were significantly higher in laminitis cases compared to control horses. The ratio of NET-bound MPO activity and total MPO activity was 0.56 + 0.17 in

laminitis cases and 0.58 + 0.05 in control horses. The ratio of NET-bound MPO activity and total MPO activity did not seem altered by the laminitis pathology.

When combining laminitis cases and control horses, linear regression results showed significant, strong, positive correlation between active MPO and total MPO ($p=0.0007$, $R=0.76$, $n=16$), NET-bound MPO activity and total MPO concentration ($p=0.0007$, $R=0.76$, $n=16$) and the strongest positive correlation was found between the activity of NET-bound MPO and the total MPO activity ($p<0.0001$, $R=0.86$, $n=16$). When only control horses were considered, a significant strong correlation was found between active MPO and total MPO ($p=0.049$, $R=0.71$, $n=8$), NET-bound MPO activity and total MPO concentration ($p=0.041$, $R=0.73$, $n=8$) and a significant, very strong correlation existed between NET-bound MPO activity and total MPO activity ($p<0.001$, $R=0.98$, $n=8$). For laminitis horses a significant, strong correlation existed between NET-bound MPO activity and total MPO activity ($p<0.023$, $R=0.78$, $n=8$, Figure 2). There were no significant correlations between active MPO and total MPO ($p=0.092$, $R=0.62$, $n=8$), NET-bound MPO activity and total MPO concentration ($p=0.068$, $R=0.67$, $n=8$).

3.4 Histopathology and immunohistochemical staining for MPO and NET

3.4.1 General anatomical observations

All control horses presented normal lamellar anatomy (Figure 3A). In contrast, all laminitis cases presented general characteristics of laminitis (Figure 4A) like narrow and elongated secondary epidermal lamellae (SEL), an acute angle between the primary epidermal lamellae (PEL) and the SEL and rounded and centrally located nuclei. Furthermore, SEL tips were tapered in all cases except for one donkey. In 5/10 cases (3 horses and 2 donkeys) tissue destruction was too severe to adequately identify the different anatomic structures (Figure 5A). In these cases, the dermal and epidermal tissues were completely separated.

The presence of inflammatory cell infiltration was observed in 7/10 cases and was usually localized in the dermal tissue of the more destroyed areas. Neutrophils accounted for the majority of observed cells in all 7 cases and were present in varying degrees ranging from one rare neutrophil (5/7 cases) to severe neutrophil infiltration (2/7 cases, Figure 6A). The mean neutrophil infiltration score was 1.1 + 1.1 and individual scores per case can be found in table 5. Red blood cells were observed in 8/10 cases and were located in the dermal tissue mainly around blood vessels and in areas of severe lamellar destruction.

3.4.2 MPO and NET immunohistochemical staining

All laminitis cases had brown colouration indicating the presence of MPO and NET. Differences were observed between control horses and laminitis cases when comparing slides stained for the MPO and NET. In control horses, only some mild brown colouration was observed on MPO and NET stained slides (Figure 3C, 3D), whereas in laminitis cases moderate to intense MPO and NET labelling was observed (Figure 4C, 4D, 5C, 5D, 6B, 7). The mean MPO and NET scores were significantly greater for laminitis cases than for control horses (3.1 + 0.4 and 3.7 + 0.4 ($p<0.001$) versus 1.7 + 0.5 and 1.4 + 0.3 ($p<0.001$), respectively). There was no significant difference in mean scores of the negative control slides between laminitis cases and control horses (0.1 + 0.1 for both, $p=0.604$). Individual scores per case can be found in table 5.

In laminitis cases, MPO and NET labelling were mainly present in the cytoplasm of keratinocytes composing the secondary epidermal lamellae (Figure 4D, 5C, 5D, 7). In selected cases, the lamellar basal cells were labelled but less intensely than the other keratinocytes composing the epidermal lamellae (Figure 7). Neutrophils appeared brown on MPO immunohistochemical staining (Figure 6B). NET labelling was mainly observed around the neutrophils and some of the neutrophils appeared brown on the NET stained slides (Figure 6B).

No marked differences were observed in brown colouration localization and intensity between horses and donkeys. In all cases, the absence of brown staining in negative control slides confirmed the absence of non-specific binding of the secondary antibody (3B, 4B, 5B).

4. Discussion

This article is the first to report a clinical study indicating both the presence and the activity of MPO and NET-bound MPO in the lamellar tissue of laminitis cases. Furthermore, for the first time, the results of experimental models (Riggs et al., 2007; Storms et al., 2022) are confirmed in cases of naturally occurring laminitis of varying aetiologies, and this both for horses and donkeys. Our hypothesis that the intense MPO labelling could be related to the presence of NET in end-stage laminitis cases was confirmed.

All histologic sections of the laminitis cases presented the well-described histological features of laminitis (Asplin et al., 2007; de Laat et al., 2011; McGowan and Patterson-Kane, 2017; Stokes et al., 2020). This, in addition to our clinical parameters and radiographical findings, confirmed the well-installed laminitis pathology. The cases in our study were euthanized due to persistent, severe, uncontrolled laminitis. Consequently, the degree of anatomic abnormalities was severe with difficult identification of the anatomic structures in some cases. Whereas experimental models usually only investigate the early stages of laminitis, this study of naturally developed laminitis focussed on the changes observed in the end-stage cases. Indeed, all included cases presented naturally developed laminitis severe enough to require euthanasia.

All cases presented marked MPO and NET labelling confirming the presence of a marked inflammatory response. In contrast to the study of Storms et al. (2022), where MPO labelling mainly occurred in dermal tissue, it was noted in our study that the labelling for MPO and NET

mainly concerned the epidermal tissue. As in our clinical study, the cases had a more advanced degree of laminitis than the cases in the experimental study using the pEHC model, it can be hypothesized that MPO and NET depositions start near the blood vessels of the dermal tissues and then migrate and/or expand towards the epidermal tissues as the disease progresses and symptoms become more severe.

Our results show a high correlation between MPO activity and NET-bound MPO activity, indicating that a part of the MPO activity was due to NET-bound MPO. This supports our hypothesis and provides a potential explanation for the absence of visible neutrophils noted on histological slides in previous studies (de la Rebière de Pouyade et al., 2010; Storms et al., 2022). Interestingly, some neutrophil infiltration was noted on histopathological sections in our study, mainly in the most severe cases. Even though it has been demonstrated that MPO, a degranulation product of neutrophils, was present in the initial stages of laminitis (Riggs et al., 2007, Storms et al., 2022), it cannot be excluded, that the presence of neutrophils in our severe cases could be a consequence related to the damage caused by the laminitis pathology, rather than the cause of it. A combination of both these factors likely contributes to their presence. Neutrophils stained positive for MPO labelling as neutrophilic granules contain MPO. However, they did not consistently stain positive for NET. Similar observations have been made in lung sputa from human patients with chronic obstructive pulmonary disease using confocal laser microscopy and electron microscopy (Obermayer et al., 2014) and in a study using anti-citH3 antibodies for immunohistochemistry of lung tissue in COVID-19 patients (Obermayer et al., 2021). This can be explained by the fact that an anti-citH3 antibody was used and that histone citrullination occurs during the process of NET formation. NET-negative neutrophils did not yet present citrullinated histones and were therefore not in NETosis. In contrast, NET-labelled neutrophils indicate activated neutrophils in the process of NETosis. The NET labelling

around the neutrophils reveals the NET expelled from the neutrophil. Interestingly, a study using human neutrophils confirmed that MPO is necessary for NETosis to occur, as pharmacologic inhibition of MPO decreased NETosis (Metzler et al., 2011). However, in a murine model, this effect was not reproduced, indicating a potential species difference (Akong-Moore et al., 2012). Elastase also seems to be an essential player in the process of NETosis and this substance has been identified in increased amounts in plasma, skin and lamellar tissue of horses with induced laminitis (de la Rebière de Pouyade et al., 2010; Papayannopoulos et al., 2010).

Despite the different aetiologies, the marked presence and activity of MPO and NET-bound MPO were noted in all laminitis cases, therefore supporting a role for neutrophil activation in the pathophysiology of laminitis regardless of the aetiology. The majority of our cases were suspected to present "endocrinopathic laminitis" based on a suspicion of underlying EMS or PPID. In other cases, an underlying systemic inflammatory phenomenon was suspected, as in the horse presented with EHV-1 and renal insufficiency. Finally, a multifactorial aetiology was proposed, as in the case of the donkey with the clinical appearance of an EMS case, but presented with hepatic lipidosis and endotoxemia.

The main limitation of this study is the lack of a homogeneous control group. Samples of control horses were harvested at the slaughterhouse. While none of the horses showed any signs of chronic or acute laminitis on the clinical examination and no abnormalities were noted on the histopathologic sections, previous episodes of the disease or another inflammatory process could not be excluded with certainty. Medical history and/or detailed medical and paramedical information should ideally have been obtained to exclude any subclinical disease.

In addition, despite having a reasonable number of cases compared to experimental laminitis studies, the sample size remains limited for drawing solid statistically confirmed conclusions. For example, attempts were made to correlate the severity of the clinical signs to the severity of histologic abnormalities and amounts of MPO and NET-bound MPO activity, but the findings were variable. A larger sample size would likely provide more insightful results in this regard. Furthermore, even though the extraction analysis provides a quantitative evaluation, a quantitative assessment of the immunohistochemistry results rather than a semi-quantitative method could have provided more solid support for our findings. Finally, the omission of the primary antibody as a negative control for the immunohistochemistry does not demonstrate the specificity of our staining. It would have been ideal to also use pre-immune rabbit serum as an additional negative control as suggested by Hewitt et al. (2014), but this was unfortunately not available. However, the marked differences in MPO and NET labelling between control horses and laminitis cases in relationship with the quantitative results obtained by our immunological techniques (SIEFED and ELISA) performed on tissue extraction support the differences observed by the immunohistochmistry.

Future research could be helpful to elucidate the relation between the severity of the clinical signs and the degree of MPO and NET concentration in lamellar tissues. Correlating these values to MPO and NET concentrations in blood samples could be interesting to confirm that the local inflammation comes from systemic disease and could potentially provide a prognostic indicator in clinical cases. Finally, investigating substances inhibiting NET production and activity could be proposed as a new treatment perspective for laminitis cases. Indeed, Franck et al. (2021) showed that equine muscle-derived mesenchymal stem cells (mdMSCs) inhibit the activity of the free and the NET-bound MPO in an in vitro study, thereby confirming the potential of mdMSCs to lower the oxidant response of neutrophils. Other potential treatment

options include decreasing NETosis using PAD4 inhibition (Knight et al., 2015) or antiplatelet therapy (Caudrillier et al., 2012), enhancing the degradation of NETs by recombinant DNase (Czaikoski et al., 2016) and finally preventing histone-mediated cytotoxicity using nonanticoagulant heparin (Wildhagen et al., 2014) or by blocking citH3 (Li et al., 2014).

5. Conclusion

Our study provides evidence suggesting the presence and activity of both MPO and NET in end-stage laminitis cases. Therefore, these substances could be the target of future therapies for this debilitating condition by inhibiting their release and/or activity. However, further research is needed to determine the exact role of both these substances in the pathophysiology of laminitis.

Acknowledgements

The authors would like to thank the whole team of the Equine Hospital of the University of Liège for their help in taking care of these patients as well as the owners for their confidence in our hospital. Furthermore, the authors would like to thank Johan Dupierreux, Remy Harray, Jennifer Romainville and Cyrielle Bougeard for their excellent technical assistance.

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Tables

Table 1: Summary of chemicals and reagents.

Substance	Manufacturer	Address
Analytical-grade phosphate salts, sodium and potassium chloride, sodium hydroxide, sodium acetate, H_2O_2 (30%), Tween 20, Xylene, ethanol absolute, hematoxylin-eosin (HE) solution	Merck	VWR International, Leuven, Belgium

Bovine serum albumin fraction V	Roche Diagnostics	Mannheim, Germany
Sodium nitrite	Sigma-Aldrich	Bornem, Belgium
96-well microtiter plates (Combiplate 8 EB), the fluorogenic substrate, amplex red (10-acetyl-3,7-dihydroxyphenoxazine) (Invitrogen)	Fischer Scientific	Merelbeke, Belgium
Dulbecco's phosphate buffer saline	Lonza	Verviers, Belgium
Rabbit and guinea pig antibodies against equine MPO (Frank et al., 2021)	Biopti s	Vielsalm, Belgium
Rabbit polyclonal antibodies to citrullinated Histone H3 (citrulline R2 + R8 + R17) (Frank et al., 2021)	Abcam	Cambridge, United Kingdom
Mouse and rabbit antibody horseradish peroxidase/ diaminobenzidine (ABC) detection immunohistochemistry kit	Abcam	Cambridge, United Kingdom

Table 2: Summary of the history and signalment for each case. EMS: equine metabolic syndrome, PPID: pars pituitary intermedia dysfunction, BCS: body condition score, CNS: cresty neck score, G: gelding, F: female.

	History	Signalment					
Case		Breed	Gender	Age (years)	Weight (kg)	BCS (/9)	CNS (/5)
Horse 1	Severe laminitis not responding to anti-inflammatory medication for 3 weeks leading to secondary renal insufficiency	Paint horse	G	14	450	6	/
Horse 2	First laminitis episode, highly suspected of EMS and/or PPID (not tested)	Fjord	F	19	645	9	4
Horse 3	Herpes virus myeloencephalopathy with secondary laminitis	Warmblood	F	5	550	4	2
Horse 4	First laminitis episode, highly suspected of EMS (not tested)	Warmblood	G	16	530	8	3
Horse 5	Second laminitis episode with another episode one month prior causing severe weight loss	Warmblood	F	20	390	5	3

Donkey 1	Hepatic lipidosis, endotoxemia, first laminitis episode, suspected of EMS/PPID (not tested)	Donkey	F	17	400	9	3
Donkey 2	Second laminitis episode, first laminitis episode one year prior, suspected of EMS (not tested)	Donkey	F	12	500	8	3
Donkey 3	First laminitis episode, suspected of EMS (not tested)	Donkey	F	8	400	8	4
Donkey 4	Recurrent laminitis episodes causing severe weight loss, no EMS/PPID tests performed	Donkey	F	5	220	6	2
Donkey 5	Recurrent laminitis episodes, suspected of EMS (not tested)	Donkey	F	/	300	8	5
Control 1	/	Haflinger	F	21	/	6	2
Control 2	/	Warmblood	F	15	/	8	3
Control 3	/	Warmnlood	G	20	/	6	1
Control 4	/	Warmblood	F	20	/	5	1
Control 5	/	Warmblood	F	17	/	6	2
Control 6	/	Warmblood	F	25	/	5	1
Control 7	/	Warmblood	G	31	/	5	1
Control 8	/	Poney	F	24	/	6	1

Table 3: Summary of the clinical parameters for each case. Bpm: beats per minute, rpm: respirations per minute, FL: Forelimb, HL: Hindlimb, LF: left forefoot, RF: right forefoot.

	Clinical parameters											
Case	Heart rate	Respiratory	Rectal temper	Growth	Increased hoof	Increased	Depression	Convex	Visibly thickene	Positive response	Obel grade of	Posture

	(bpm)	rate (rpm)	ature (°C)	lines	temperature	digital pulse	coronary groove	sole hoof	d white line	to hoof tester	lamenes s (/4)	
Horse 1	84	36	38,3	No	FL, HL	FL > HL	FL, HL	FL	/	/	4	Often recumbent, saw-horse stance
Horse 2	60	16	38,1	FL, HL	No	FL > HL	FL, HL	No	FL	Fl, HL	4	Often recumbent, saw-horse stance
Horse 3	40	12	37,1	No	FL	FL > HL	FL (RF)	FL (RF)	No	FL (RF)	3	Saw-horse stance
Horse 4	70	40	37,6	FL, HL	No	FL > HL	FL (LF>RF)	FL > HL	/	No	3	Normal
Horse 5	68	36	36,5	FL	FL, HL	FL, HL	FL, HL	Perforation RH	FL, HL	FL, HL	4	/
Donkey 1	130	100	41,2	No	No	FL > HL	FL, HL	No	FL, HL	/	3	Saw-horse stance
Donkey 2	80	20	36,9	FL	FL	FL > HL	FL	No	FL	/	Recumbent	Permanently recumbent
Donkey 3	100	80	37,7	FL, HL	FL, HL	FL, HL	FL, HL	/	/	/	4	Saw-horse stance
Donkey 4	60	32	37,2	FL, HL	FL, HL	FL, HL	FL, HL	No	FL	FL, HL	2	Saw-horse stance
Donkey 5	84	52	38,0	FL	No	FL > HL	No	No	FL	/	4	Saw-horse stance
Control 1	/	/	38,3	No	No	No	No	No	No	/	No	No
Control 2	/	/	37,9	No	No	No	No	No	No	/	No	No
Control 3	/	/	36,7	No	No	No	No	No	No	/	No	No
Control 4	/	/	37,7	No	No	No	No	No	No	/	No	No
Control 5	/	/	37,2	No	No	No	No	No	No	/	No	No
Control 6	/	/	37,5	No	No	No	No	No	No	/	No	No
Control 7	/	/	37,8	No	No	No	No	No	No	/	No	No
Control 8	/	/	36,7	No	No	No	No	No	No	/	No	No

Table 4: Summary of the radiographic parameters for each case. P3: third phalanx.

	Radiographic parameters						
Case	Capsular rotation	Phalangeal rotation	Sinking P3	Convex sole	Lipping	Osteitis P3	Air opacity
Horse 1	Yes	Yes	Yes	Yes	No	No	No
Horse 2	Yes	No	Yes	No	Yes	No	Yes
Horse 3	No	No	Yes	Yes	No	No	No
Horse 4	Yes	No	Yes	Yes	No	No	No
Horse 5	Yes	Yes	Yes	Sole perforation	Yes	No	Yes
Donkey 1	Yes	No	No	No	No	Yes	No
Donkey 2	Yes	No	No	No	No	Yes	No
Donkey 3	/	/	/	/	/	/	/
Donkey 4	Yes	Yes	Yes	No	No	Yes	Yes
Donkey 5	Yes	Yes	Yes	No	No	No	No
Control 1	/	/	/	/	/	/	/
Control 2	/	/	/	/	/	/	/
Control 3	/	/	/	/	/	/	/
Control 4	/	/	/	/	/	/	/
Control 5	/	/	/	/	/	/	/
Control 6	/	/	/	/	/	/	/
Control 7	/	/	/	/	/	/	/
Control 8	/	/	/	/	/	/	/

Table 5: Histology and extraction results for each case. MPO: myeloperoxidase, NET: neutrophil extracellular trap, prot: total proteins.

	Histology	Immunohistochemsitry			Extraction analysis			
Case	Neutrophil inflammatory infiltrates	Negative control	MPO score	NET score	Total MPO/prot (ng/mg)	Active MPO/prot (ng/mg)	Actve NET/prot (ng/mg)	Active NET/activeMPO
Horse 1	1	0,1	3,4	4,0	68,60	1,25	0,80	0,64
Horse 2	0	0,2	2,9	2,9	34,44	2,60	1,19	0,46
Horse 3	0	0,1	3,6	3,9	67,12	2,15	0,93	0,43
Horse 4	1	0,1	2,4	3,4	/	/	/	/
Horse 5	1	0,0	3,1	4,0	/	/	/	/
Donkey 1	1	0,2	3,3	3,6	170,62	5,54	2,51	0,45
Donkey 2	1	0,0	2,5	3,5	138,29	5,64	2,78	0,49
Donkey 3	3	0,3	3,0	4,0	153,10	3,19	3,04	0,95
Donkey 4	3	0,2	3,6	3,7	136,10	3,55	1,62	0,46
Donkey 5	0	0,0	2,8	3,9	47,19	3,93	2,31	0,59
Control 1	0	0,0	2,0	1,8	28,72	3,07	1,55	0,51
Control 2	0	0,1	1,5	1,2	38,24	2,17	1,13	0,52
Control 3	0	0,0	1,3	1,5	18,14	1,34	0,84	0,63
Control 4	0	0,0	1,7	1,5	28,85	1,54	0,90	0,58
Control 5	0	0,0	1,4	1,2	22,61	2,10	1,18	0,56
Control 6	0	0,4	1,8	1,2	29,06	2,04	1,17	0,57

Control 7	0	0,2	1,5	1,0	20,70	1,77	1,12	0,63
Control 8	0	0,0	2,7	1,7	45,80	2,46	1,46	0,59

Figures

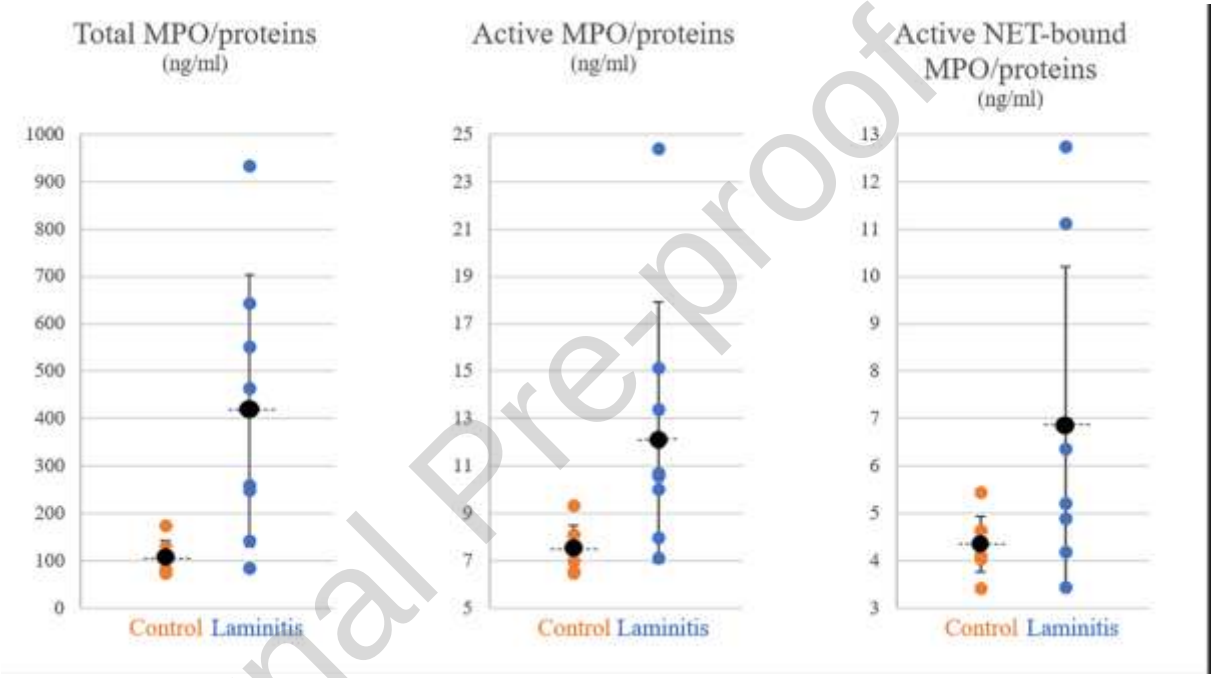


Figure 1 – Dot plots showing the total MPO, active MPO and active NET-bound MPO concentrations for control horses and laminitis cases. MPO: myeloperoxidase, NET: neutrophil extracellular traps, prot: total proteins.

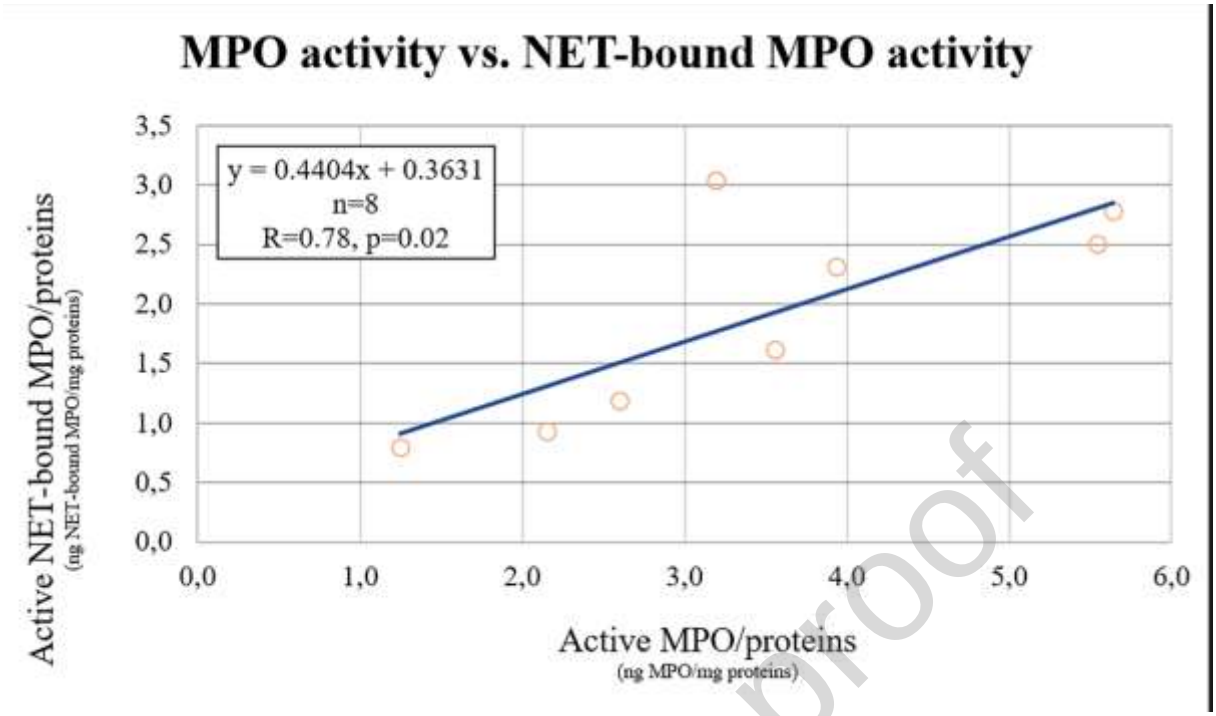
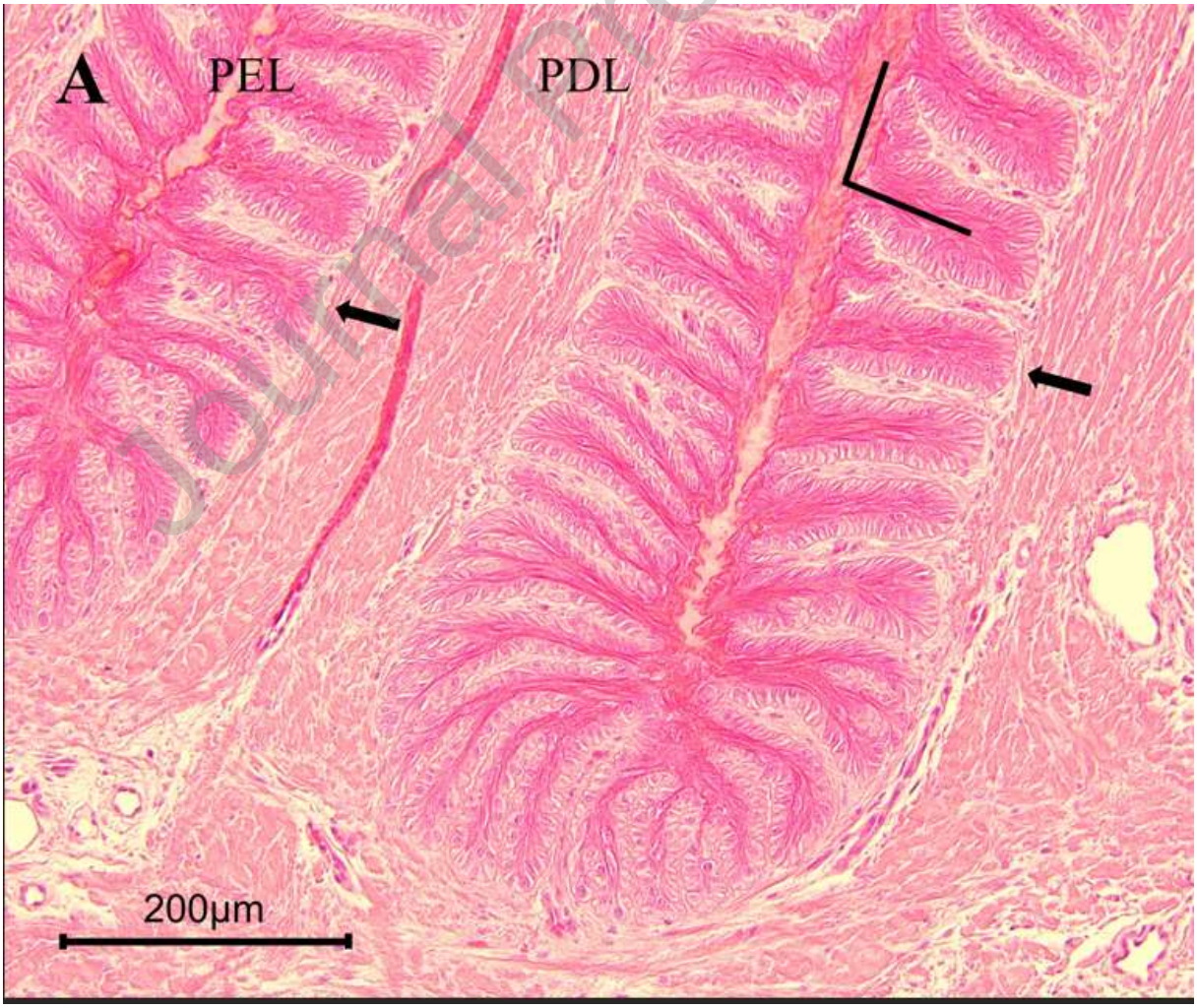
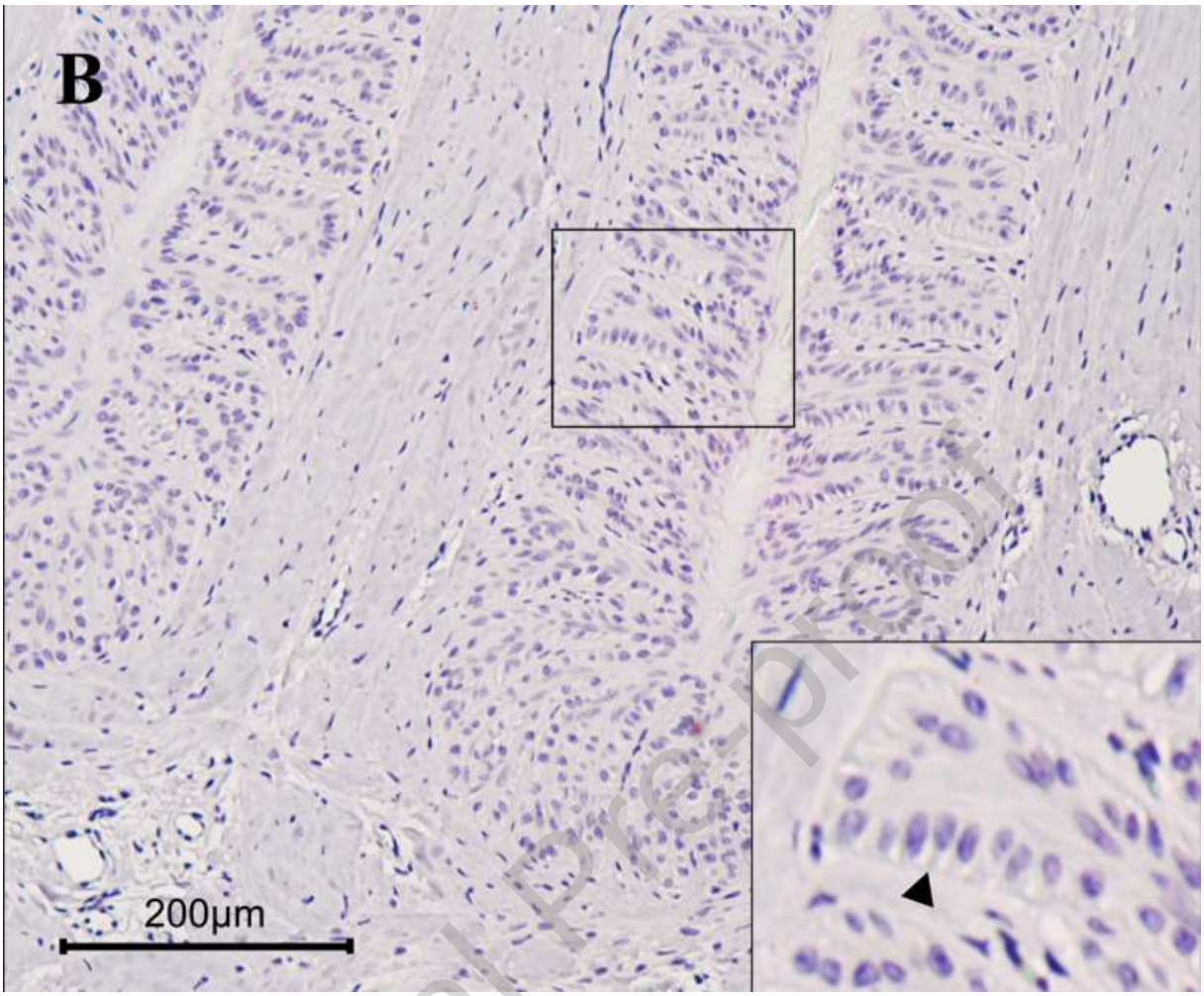
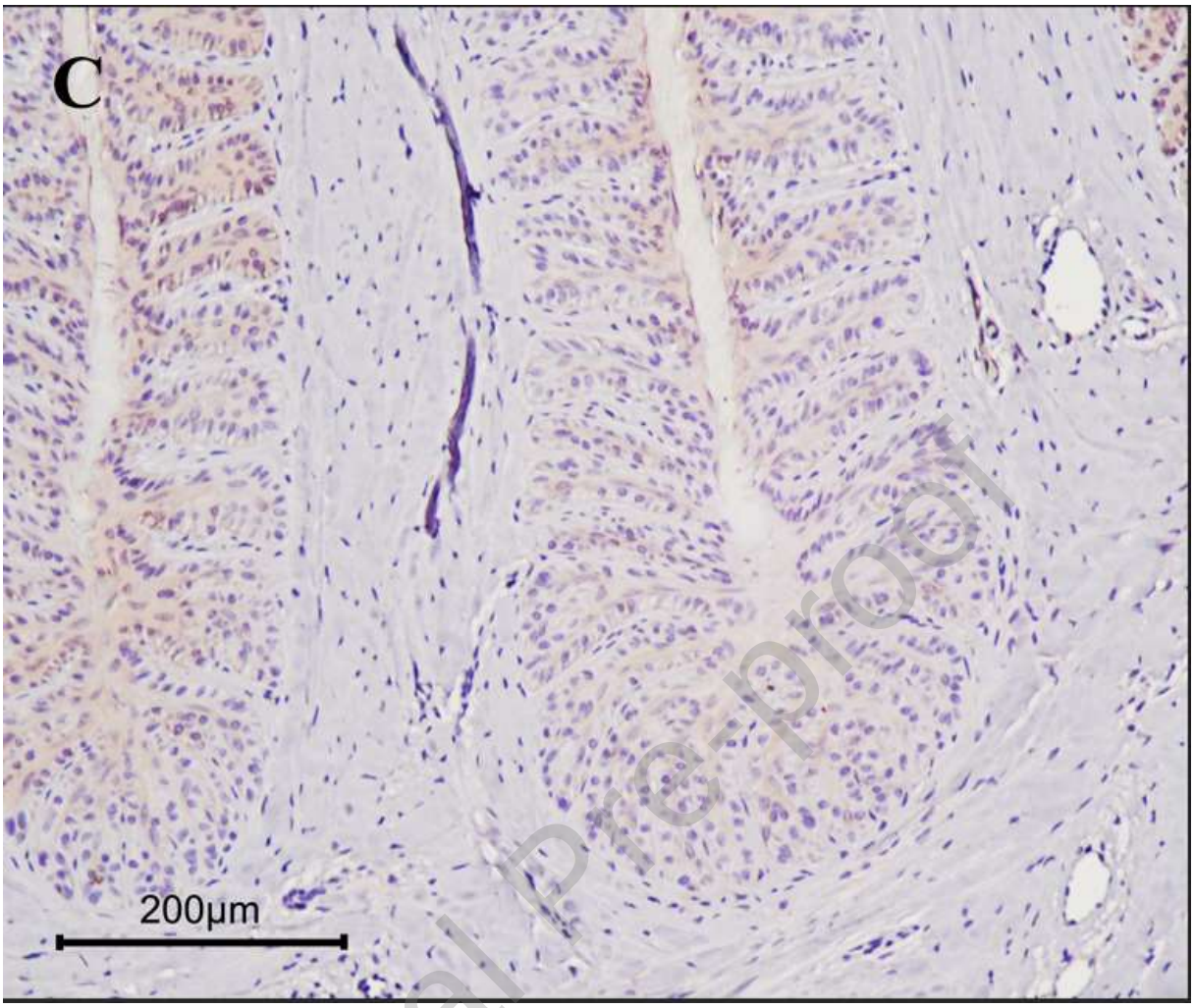


Figure 2– Correlation between MPO activity and NET-bound MPO activity.







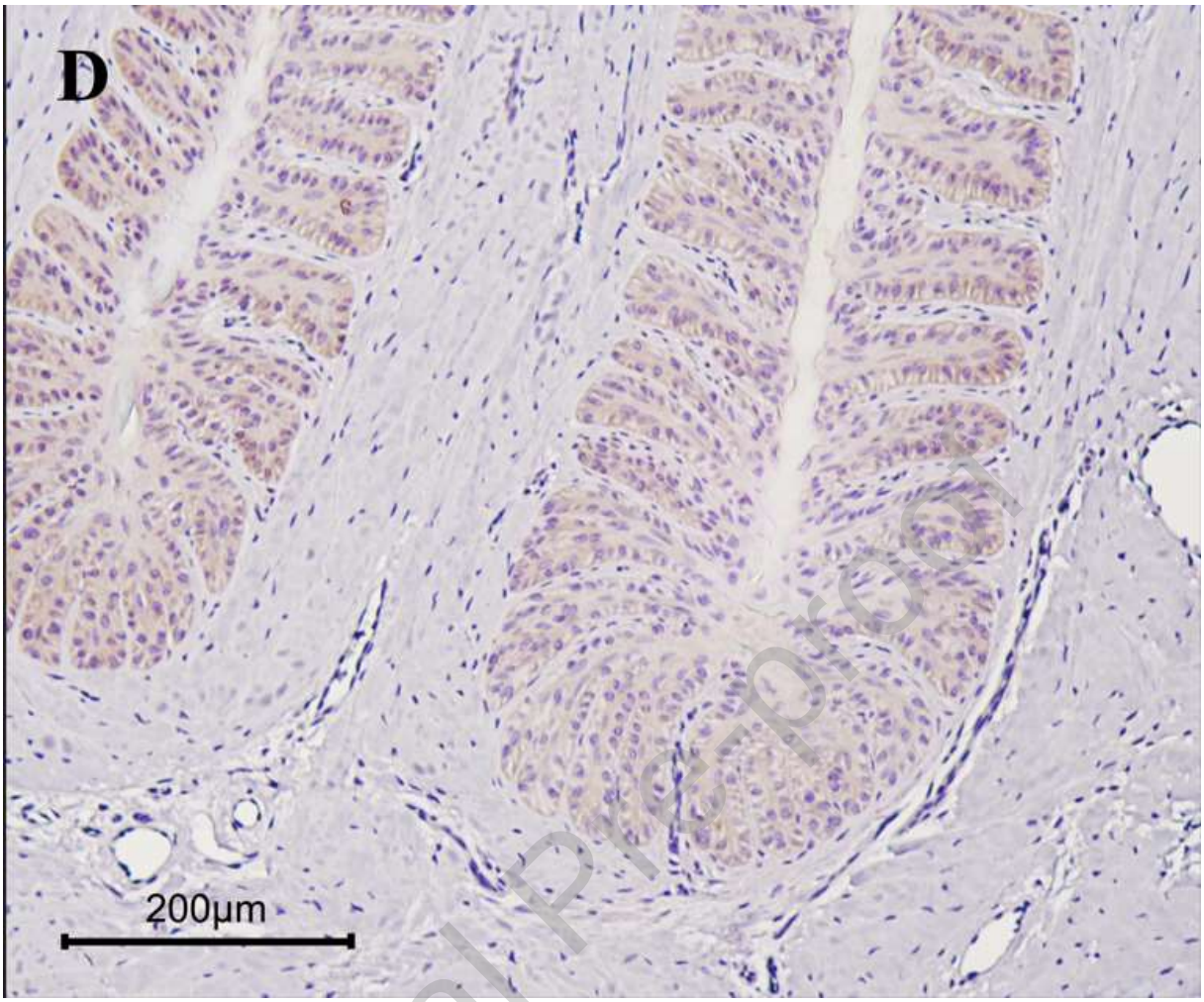
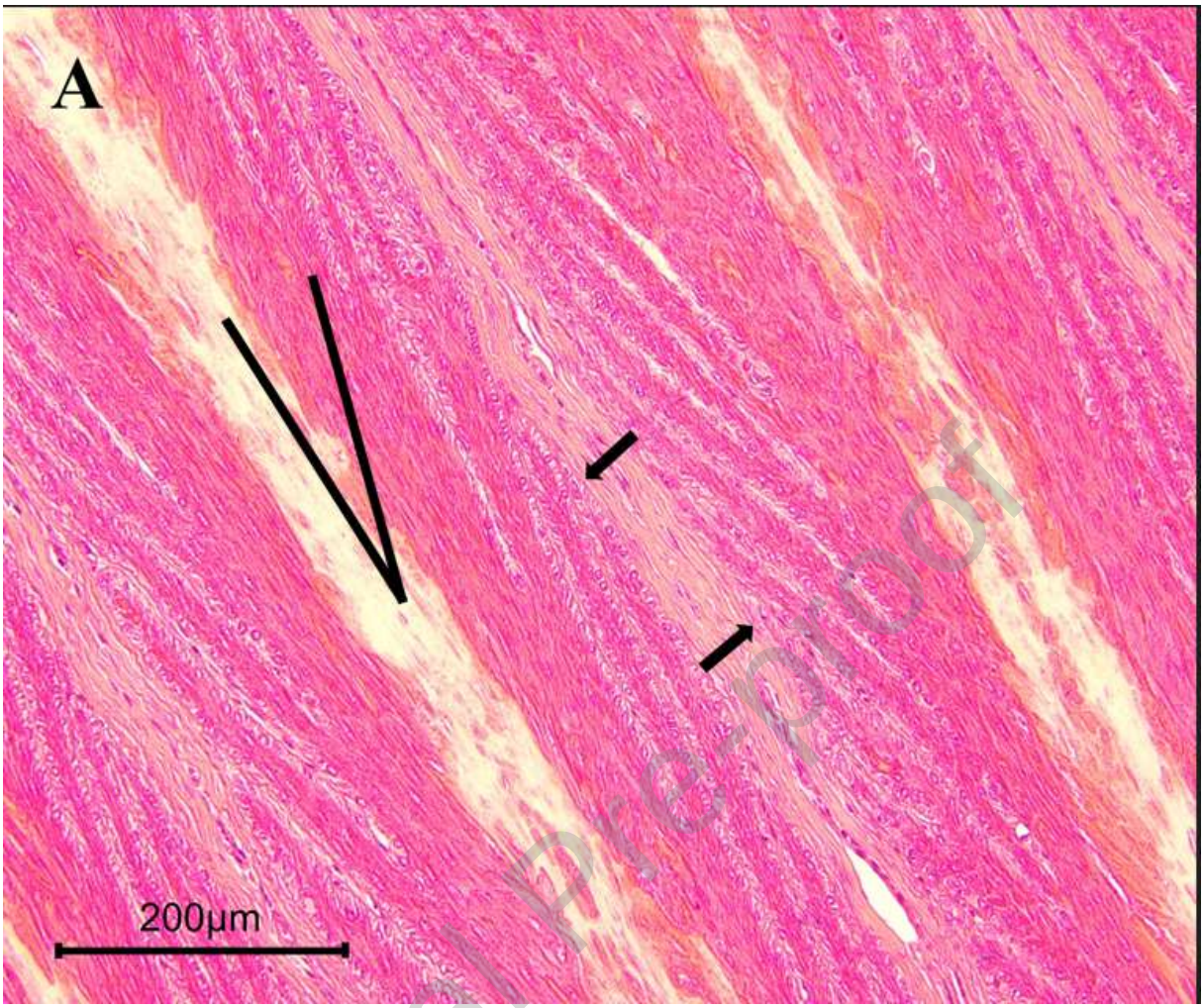
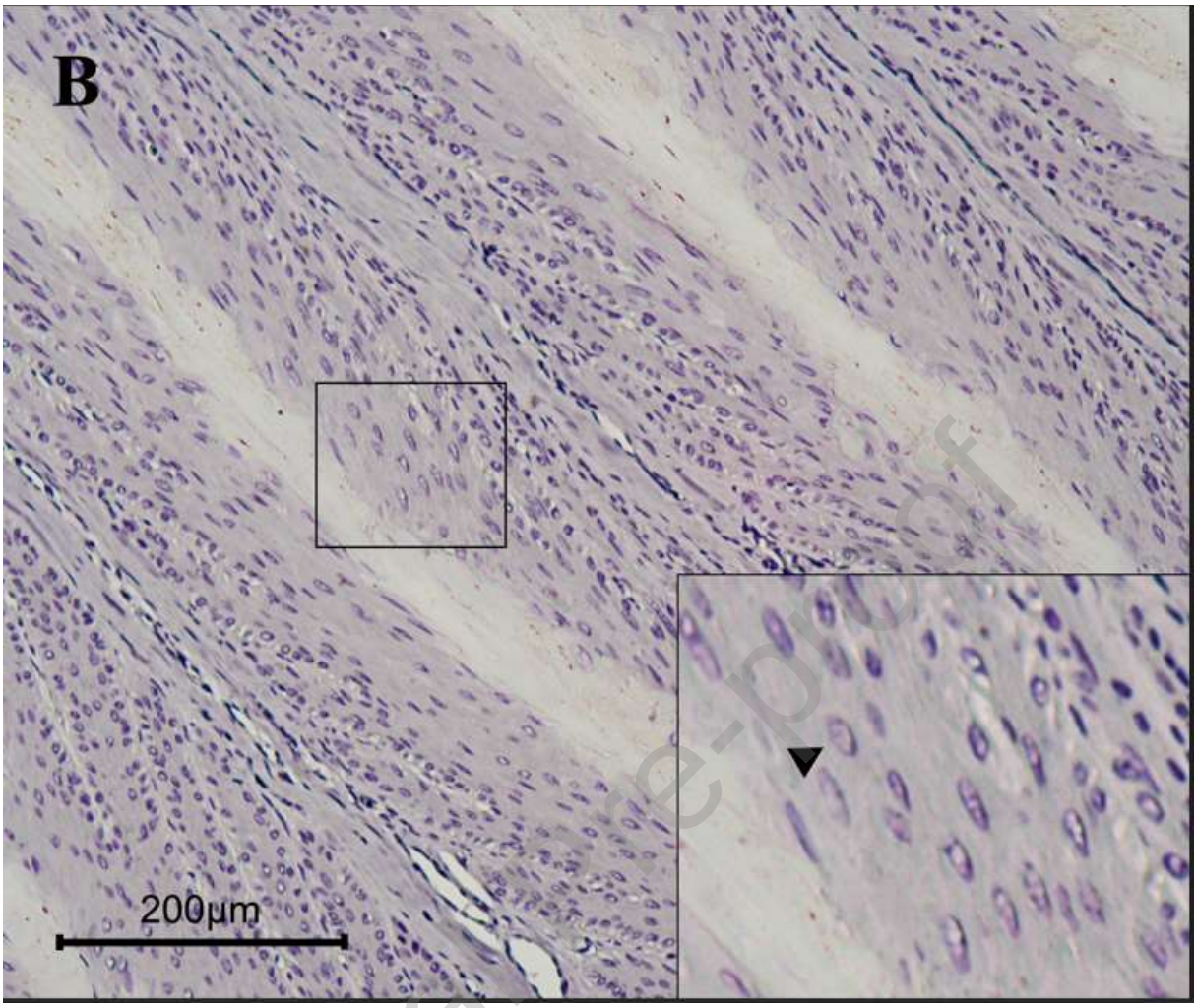
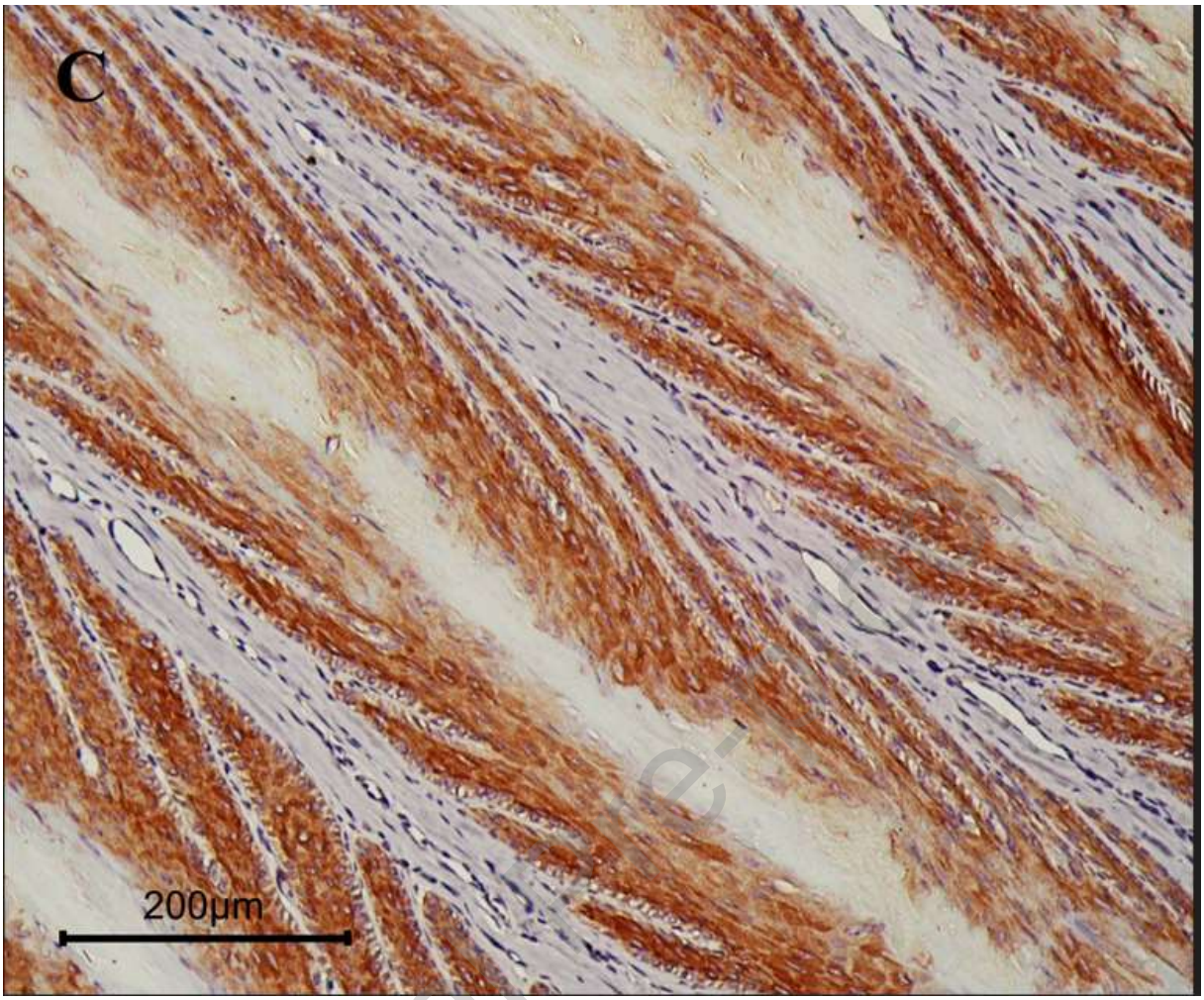


Figure 3 – Photomicrographs of a control horse stained with HE (A), negative control (B), MPO (C) and NET (D) immunohistochemical staining. The secondary epidermal and dermal lamellae are oriented perpendicularly on their primary axis (A, lines), have rounded tips (A, arrows) and the lamellar basal cells have elongated nuclei (B, arrowhead). There is only some minor brown staining visible on the MPO and NET stained slides (C, D). PDL: primary dermal lamellae, PEL: Primary epidermal lamellae







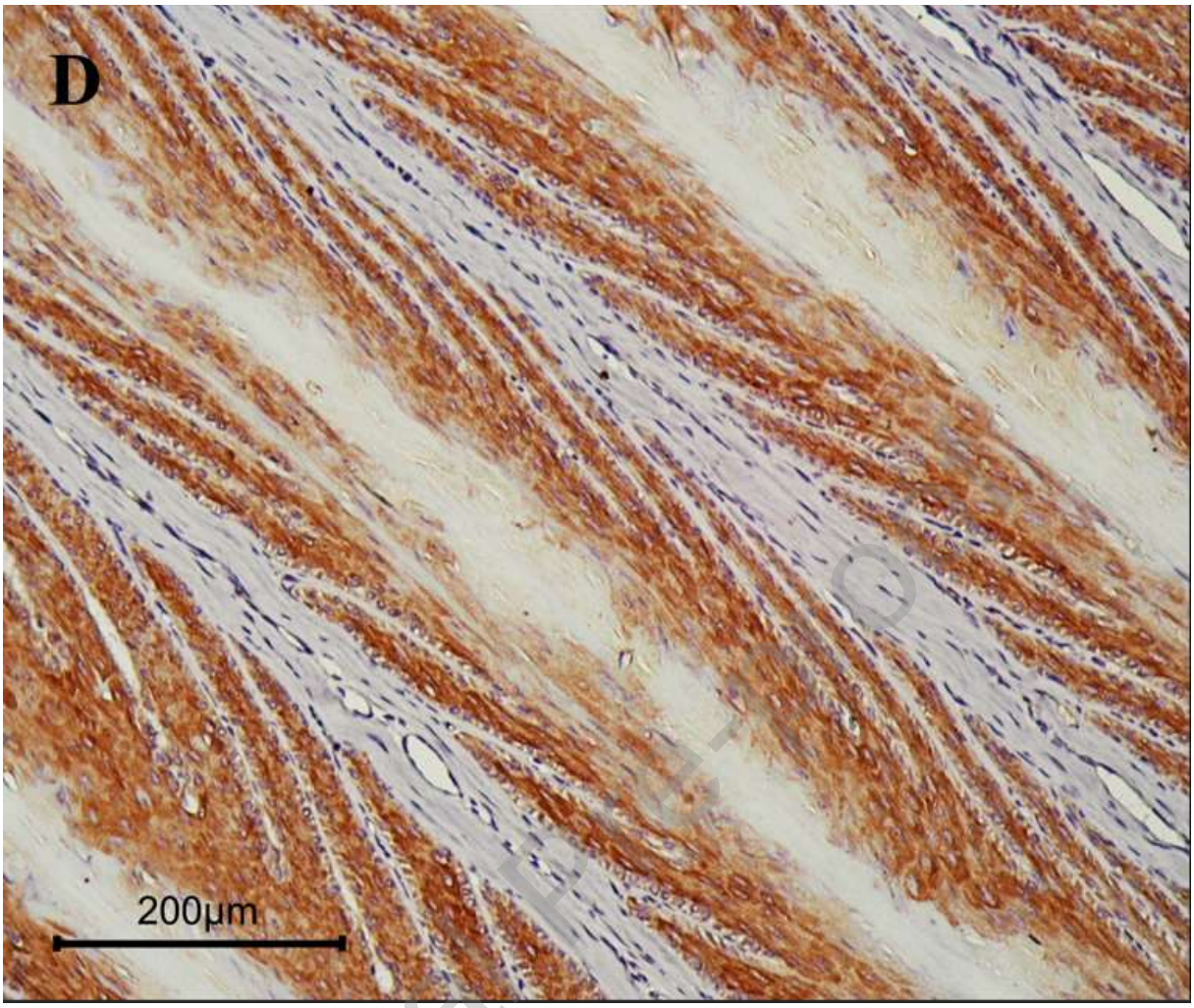
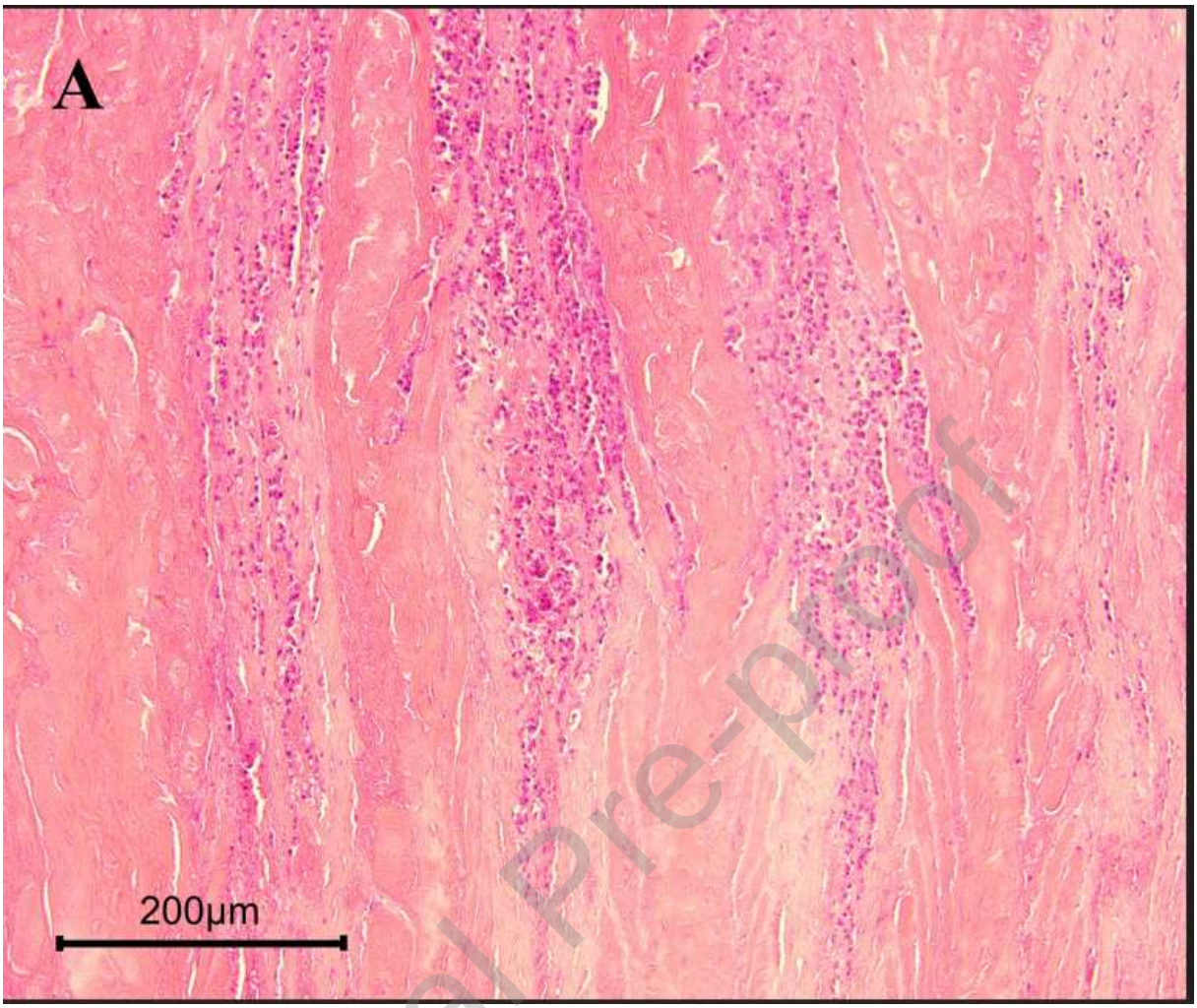
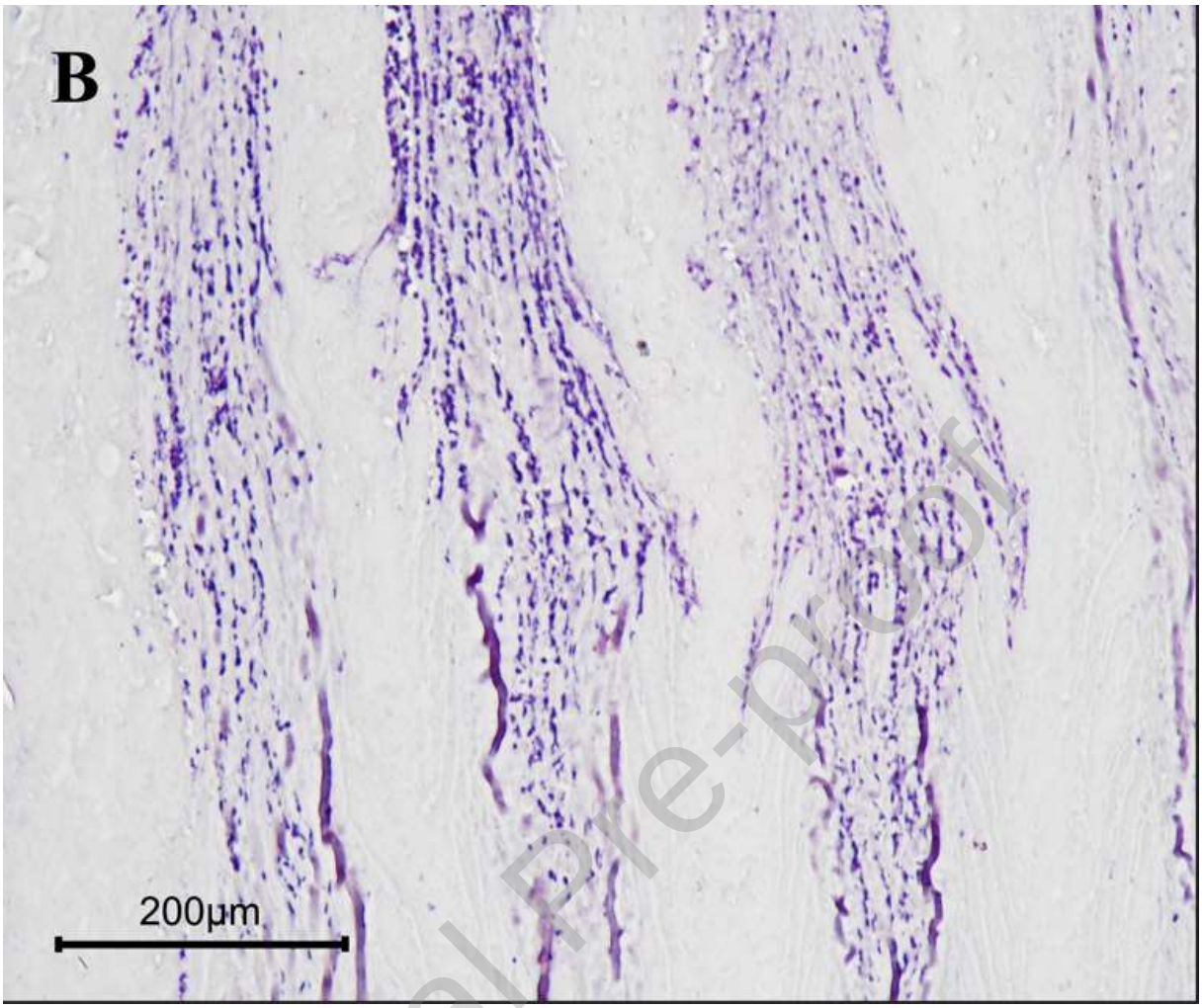
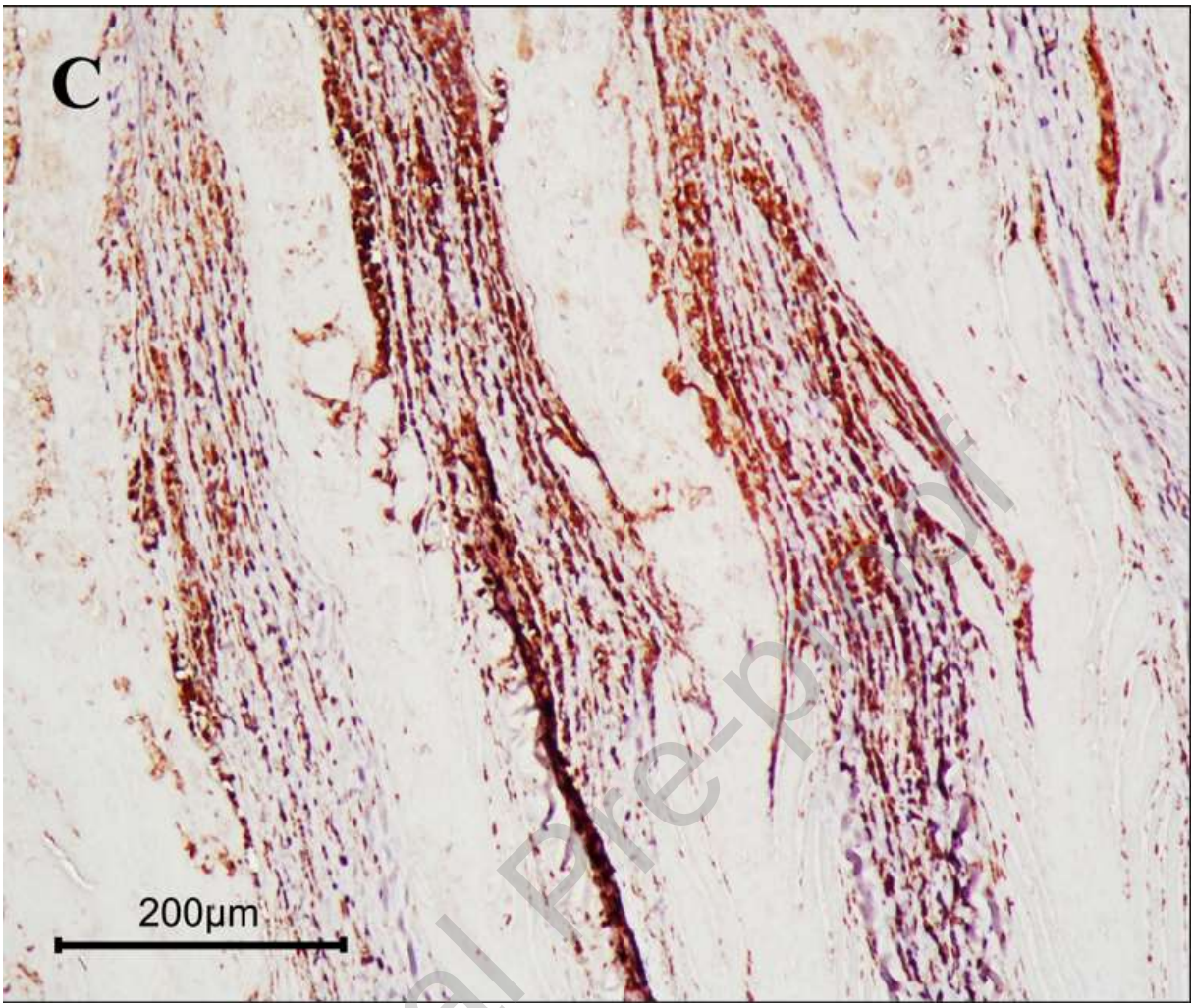


Figure 4 – Photomicrographs of a laminitis horse stained with HE (A), negative control (B), MPO (C) and NET (D) immunohistochemical staining. The secondary epidermal lamellae have tapered tips (A, arrows), appear elongated and narrow, and are acutely angled on primary epidermal lamellae (A, lines). The lamellar basal cells have round nuclei that are centrally located (B, arrowhead). Intense MPO and NET labelling is observed in the keratinocytes composing the secondary epidermal lamellae (C, D).







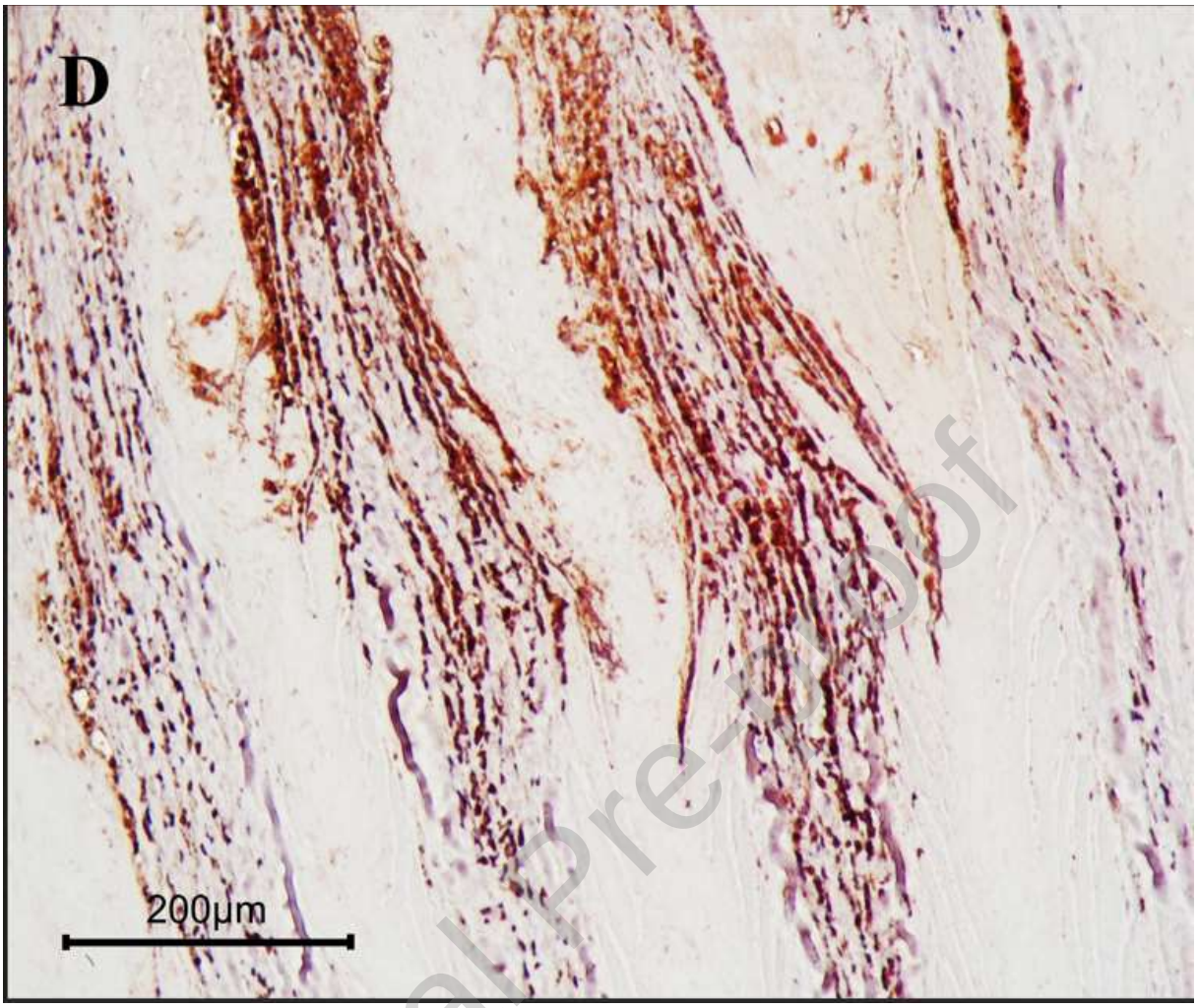
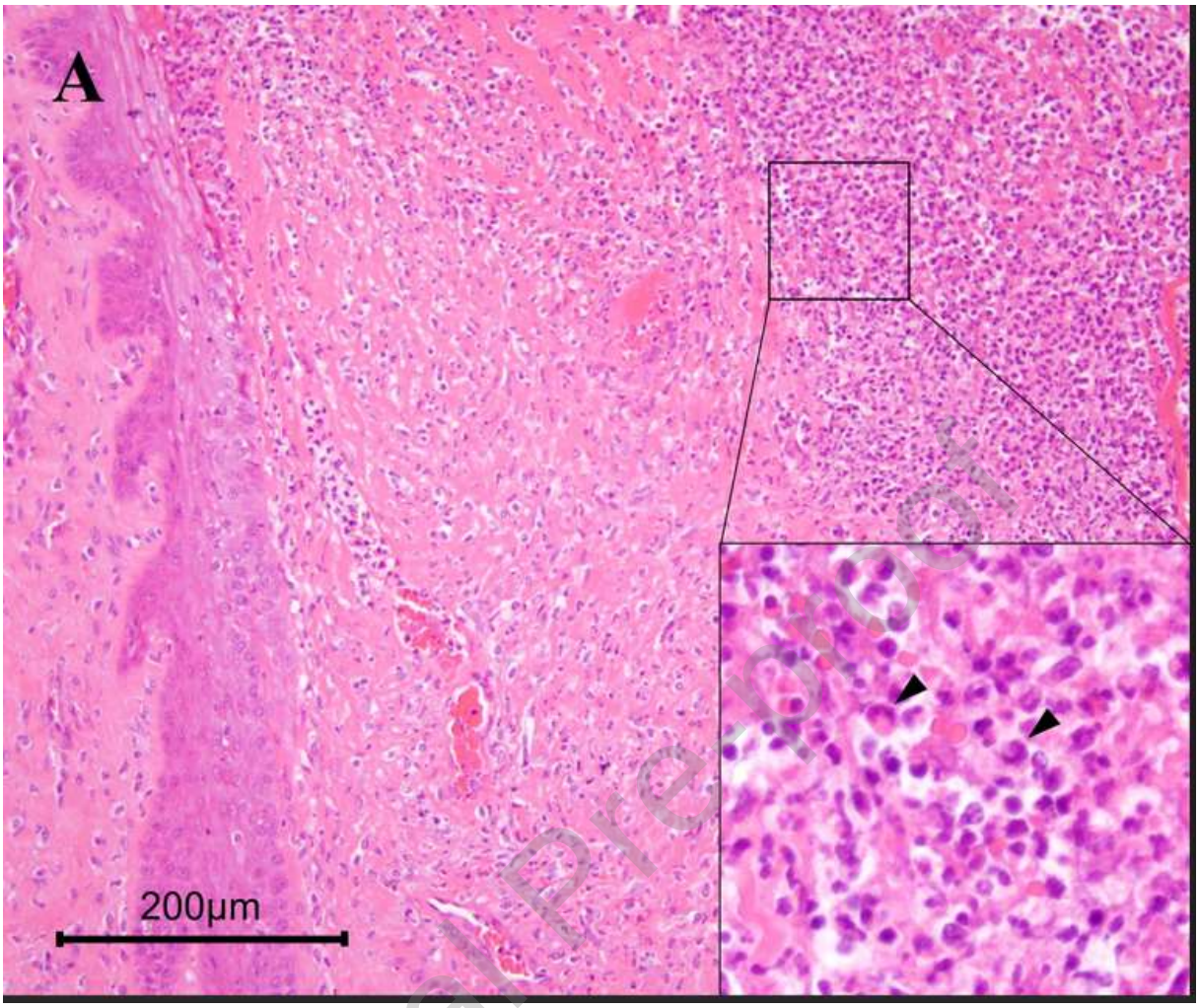


Figure 5 – Photomicrographs of a laminitis donkey stained with HE (A), negative control (B), MPO (C) and NET (D) immunohistochemical staining. Lamellar anatomy is almost unrecognisable due to severe tissue destruction. Intense MPO and NET labelling is observed in what looks like the remaining keratinocytes composing the secondary epidermal lamellae (C, D).



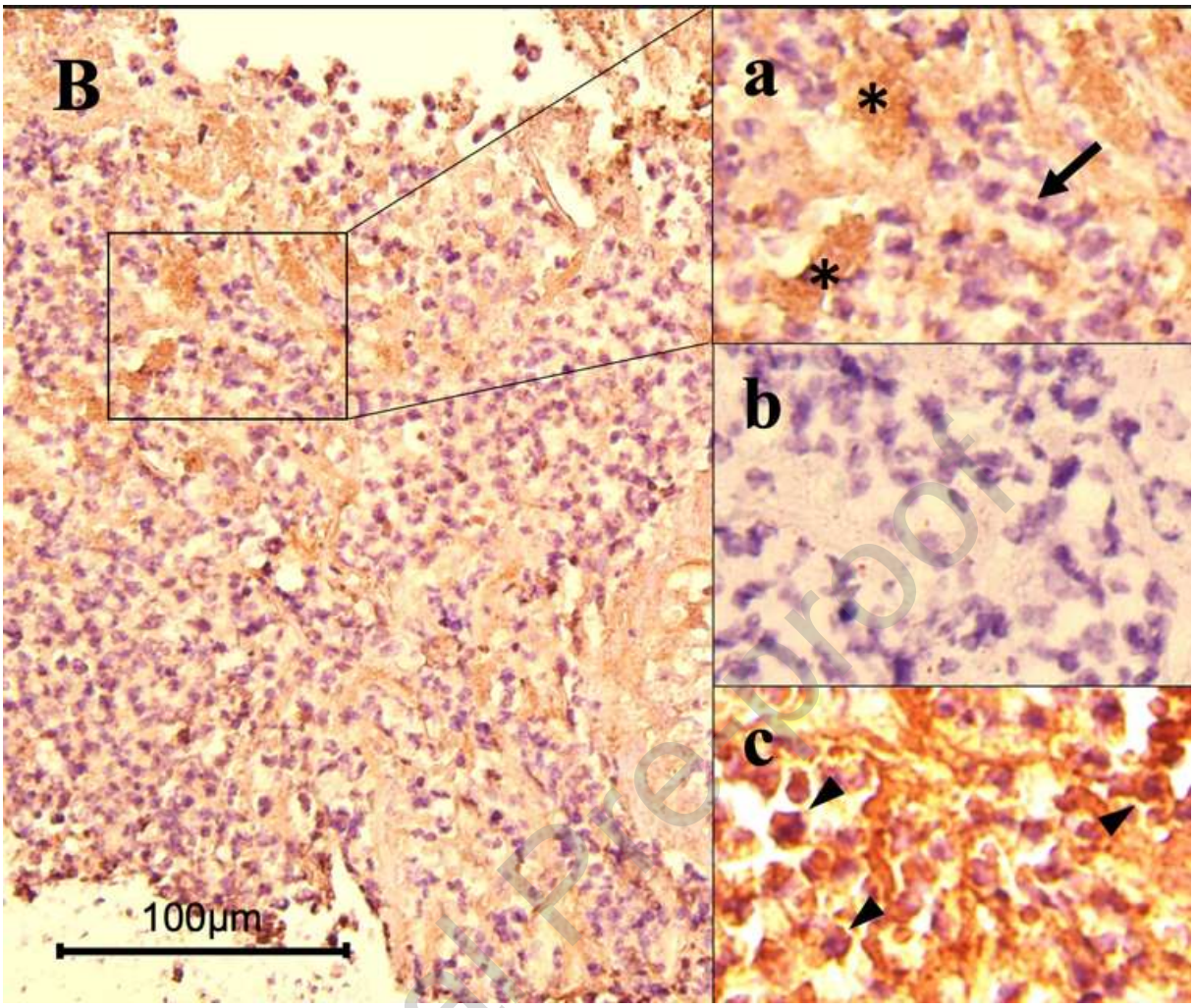


Figure 6 – Photomicrographs of a laminitis horse stained with HE (A) and NET (B) immunohistochemical staining. Figure 6A shows severe neutrophil infiltration (arrowheads) in the dermal tissue. Figure 6B shows NET labelling, visible as diffuse brown spots devoid of a nucleus and a cell wall, present around the neutrophils (B, insert a, asterisk). Inserts of the same area on the accompanying negative control slide (B, insert b) and on the MPO immunohistochemically stained slide (B, insert c) are displayed to show that neutrophils are not stained on the negative control slide (B, insert b), are intensely brown on the MPO immunohistochemical staining (B, insert c, arrowheads) and are not NET positive on the NET immunohistochemical staining (B, insert a, arrows).

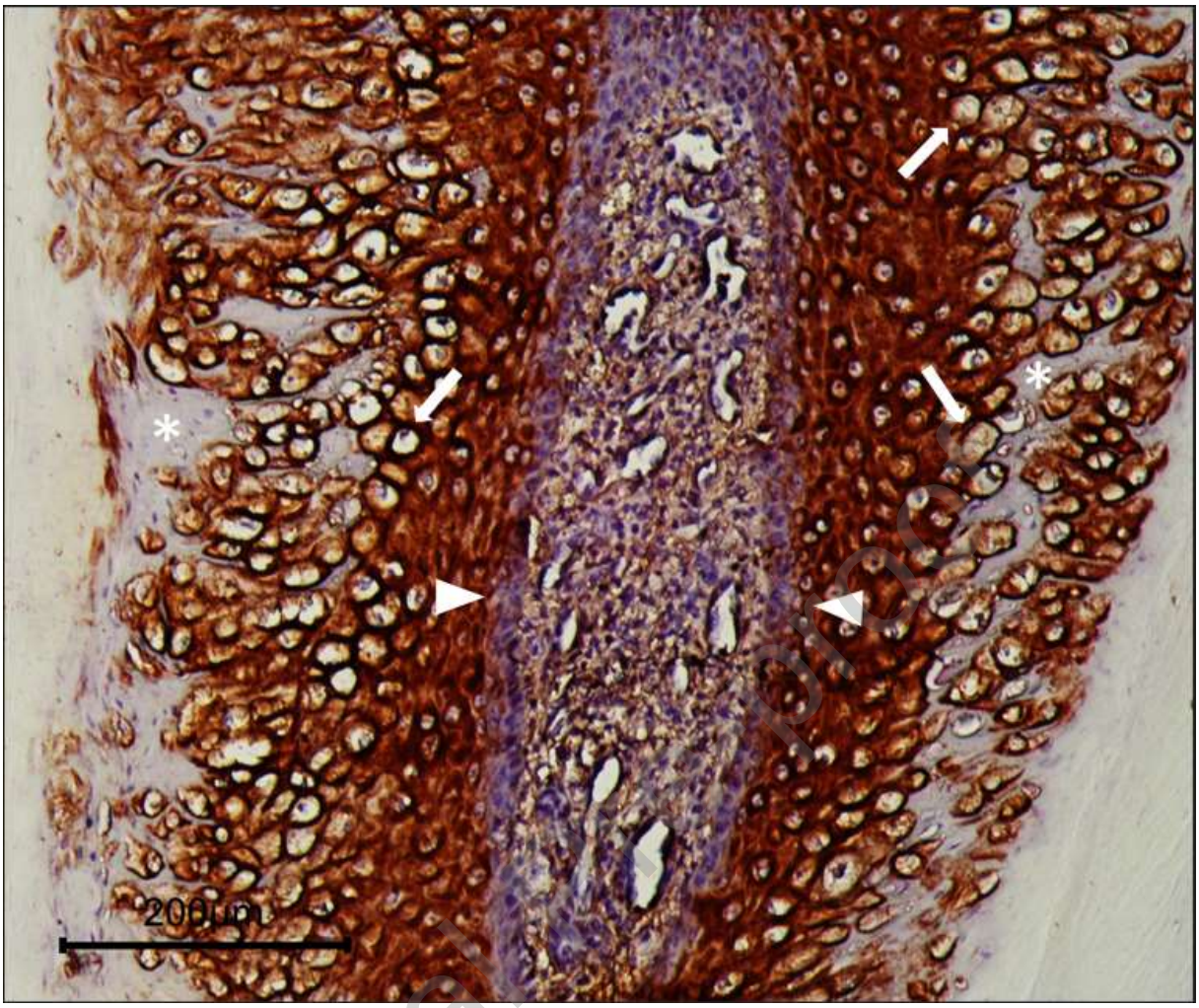


Figure 7 – Photomicrograph of a transverse section of the lamellae of a laminitis horse immunohistochemically stained for NET. The keratinocytes of the epidermal lamellae are strongly labelled for NET, whereas the lamellar basal cells are less intensely brown coloured (arrowheads). The dermal tissue is also slightly labelled for NET with presence of some neutrophils. Multiple phantom cells are noted (arrows) and some free fluid infiltration is present (asterisk).

Declaration of Competing Interest
none

Highlights

- MPO and NET are both present and active in lamellar tissue of laminitis cases
- The MPO activity is ~~mainly~~ partly due to NET-bound MPO
- MPO and NET are mainly located in lamellar keratinocytes and neutrophil infiltrates
- Both horses and donkeys with clinical laminitis are similarly affected
- Treatment options targeting MPO activity and NET formation might be explored