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L'implication des neutrophiles dans la pathogénie de la fourbure équine: une voie prometteuse pour une approche thérapeutique innovante

Targeting neutrophil involvement in equine laminitis pathogenesis: A promising approach for novel therapeutic development

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Cheers to the many adventures that lie ahead!

Abreviations

ACP	Acepromazine
AJ	Adherens junctions
ANOVA	Analysis of variance
ANS	Activated neutrophil supernatant
A/R	Anoxia-reoxygenation
ATP	Adenosine triphosphate
AVAs	Arteriovenous anastomoses
BCS	Body condition score
BM	Basement membrane
BSA	Bovine serum albumin
BWE	Black walnut extract
CB	Cytochalasin B
CHO	Carbohydrate overload
CL	Chemiluminescence
CNS	Cresty neck score
COX	Cyclooxygenase
DAMPs	Damage-associated molecular patterns
DDFT	Deep digital flexor tendon
DMEM	Dulbecco's modified eagle medium
DMPO	5,5′ -Dimethyl-pyrroline-Noxide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediamine tetraacetic acid
EHV-1	Equine herpes virus 1
ELISA	Enzyme-linked immunosorbent assay
EMS	Equine metabolic syndrome
EPR	Electron paramagnetic resonance
EV	Extracellular vesicules
FBS	Fetal bovine serum

fMLP	Formylmethionyl- leucyl-phenylalanine
GSH	Glutathione
H_2O_2	Hydrogen peroxide
H3Cit	Citrullinated histone 3
HaCaT	Human epidermal keratinocytes
HE	Hematoxylin-eosin
HOCl	Hypochlorous acid
IFs	Intermediate filaments
IGF-1	Insulin-like growth factor-1
IgG	Immunoglobulin G
IHC	Immunohistochemsitry
IL	Interleukine
IRI	Ischemic-reperfusion injury
KPEL	Keratinized primary epidermal lamellae length
LBECs	Lamellar basal epithelial cells
LMWH	Low molecular weight heparin
LPS	Lipopolysaccharide
mdMSC	Muscle-derived mesenchymal stem cells
MPO	Myeloperoxidase
MSC	Mesenchymal stem cells
MT	Masson's trichrome
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NADPH	Nicotinamide adenine dinucleotide phosphate
NANS	Non-activated neutrophil supernatant
NET	Neutrophil extracellular traps
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NSAID	Nonsteroidal anti-inflammatory drug
NSC	Nonstructural carbohydrates
OF	Oligofructose
P3	Third phalanx
PAD4	Peptidyl arginine deiminase 4

PAMP	Pathogen-associated molecular patterns
PAS	Periodic Acid-Schiff
PBS	Phosphate-buffered saline
PDL	Primary dermal lamellae
pEHC	Prolonged euglycemic hyperinsulinemic clamp
PEL	Primary epidermal lamellae
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear neutrophils
PPID	Pars pituitary intermedia dysfunction
ROI	Region of interest
ROS	Reactive oxygen species
SD	Standard deviation
SDL	Secondary dermal lamellae
SEL	Secondary epidermal lamellae
SEM	Standard error of the mean
SIEFED	Specific immune extraction followed by enzymatic detection
SIRS	Systemic inflammatory response syndrome
TNF-α	Tumor necrosis factor alpha
TPEL	Total primary epidermal lamellar length

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Résumé - Summary

Résumé

Dans le cadre de la recherche de nouvelles voies thérapeutiques concernant la fourbure chez le cheval, cette thèse de doctorat investigue l'implication de l'activation des neutrophiles dans la physiopathologie de la maladie. Caractérisée par une désolidarisation de l'interface dermo-épidermique, la fourbure est une pathologie grave et invalidante du pied du cheval qui est très répandue et pourtant mal comprise. Notre prisme d'étude de la maladie s'est tout d'abord focalisé sur la compréhension du rôle de l'activation des neutrophiles dans la pathogenèse de la fourbure, au travers de la présence et l'activité de la myéloperoxydase (MPO) et des « neutrophil extracellular traps » (NET). Ensuite, le potentiel thérapeutique des cellules souches a été exploré par le prisme spécifique de leurs propriétés immunomodulatrices. A noter que l'ensemble de ces études a été mis en œuvre par une approche plurielle, combinant modèles cellulaires *in vitro*, études animales *in vivo* et investigations cliniques.

Notre première étude *in vitro* se penche sur la juglone comme potentiel responsable de l'activation des neutrophiles dans le modèle dénommé « black walnut heartwood extract » (BWE). En effet, l'état de l'art met en évidence leur activation dans des modèles associés au syndrome de réponse inflammatoire systémique (SIRS). Parmi eux, le modèle BWE a mis en évidence la présence de MPO (Riggs *et al.* 2007) et d'élastase (de la Rebière de Pouyade *et al.* 2010) dans la peau, le sang et les tissus lamellaires des chevaux affectés par la fourbure. Notre première étude cible la juglone comme potentiel responsable de l'activation des neutrophiles dans ce modèle car ce composé naphtoquinone des noix possède des propriétés cytotoxiques et peut ainsi induire un stress d'oxydation impliquant cycle redox et génération de ROS. De manière intéressante, nous avons constaté que la juglone avait un effet inhibiteur sur la dégranulation des neutrophiles. De plus, elle réduit l'activité peroxydasique de la MPO, comme mis en évidence par des extractions immunologiques spécifiques par essais de détection enzymatiques (SIEFED). Ces résultats ont été confirmés par une étude de « docking » montrant le positionnement adéquant de la juglone sur le site actif de l'enzyme MPO. Ces résultats indiquent donc que la juglone n'est pas le déclencheur de la fourbure équine, du moins si l'on se concentre sur la modulation de l'activation des neutrophiles.

Ensuite, nous avons entrepris d'explorer l'activation des neutrophiles chez des chevaux dont la fourbure était induite par le modèle dénommé « prolonged euglycemic hyperinsulinemic clamp » (pEHC), imitant la fourbure endocrinopathique, une des causes prévalentes de la maladie. L'immunohistochimie sur des sections lamellaires a révélé que la MPO était largement présente dans le derme des chevaux affectés, bien que les neutrophiles soient remarquablement rares. Ces résultats suggèrent que l'activation des neutrophiles pourrait jouer un rôle plus important que précédemment soupçonné dans les cas de fourbure endocrinopathique.

L’étape subséquente du projet de recherche a consisté à évaluer l'activation des neutrophiles dans des cas cliniques de fourbure, afin de représenter aussi fidèlement que possible les scénarios réels. L’étude inclut deux espèces affectées par la maladie, des chevaux et des ânes, et posait l’hypothèse de la présence de MPO et de NET comme source de l’intense marquage de la MPO et de la rareté des neutrophiles observée précédemment. Nos résultats ont révélé une présence et une activité accrues de la MPO et des NET chez les chevaux et les ânes atteints de fourbure par rapport aux échantillons de contrôle. De plus, l'activité de la MPO a été démontrée comme en partie attribuable à la MPO liée aux NET. Enfin, l'analyse histologique a montré la sévérité des lésions lamellaires et la présence de neutrophiles dans ces cas de fourbure en phase terminale, contrastant ainsi avec les observations dans les modèles *in vivo* en stade plus précoce.

Finalement, nous avons tenté d’évaluer le potentiel thérapeutique des cellules souches en lien avec l’activité des neutrophiles. Sur base des propriétés immunomodulatrices et régénératrices des cellules souches mésenchymateuses dérivées du muscle équin (mdMSC), nous avons créé un modèle *in vitro* utilisant des kératinocytes et des surnageants de neutrophiles activés soumis à un stress d'anoxie-reoxygénation. Grâce à ce modèle, nous avons démontré que les mdMSC équines possèdent des propriétés anti-inflammatoires impliquant la modulation des NET et de l'activité de la MPO. Nous avons également observé un effet positif sur le rétablissemnent du métabolisme des kératinocytes. Cela souligne ainsi son potentiel dans le traitement de la fourbure via l’atténuation de l'inflammation et la potentielle restauration du métabolisme des kératinocytes.

En conclusion, cette thèse de doctorat soutient l'hypothèse de l'inflammation avec activation des neutrophiles en tant qu'élément général dans la physiopathologie de la fourbure, indépendamment des diverses causes déclenchantes. Le traitement avec mdMSC pourrait constituer une nouvelle voie de traitement pour améliorer le pronostic de cette maladie invalidante.

Summary

This PhD thesis investigates the implication of neutrophil activation in laminitis pathophysiology to propose a novel treatment appraoch. While prevalent, laminitis is a severe and debilitating pathology of the equine foot whose mechanisms are poorly understood and characterized by the desolidarisation of the dermo-epidermal interface. We focused on elucidating the role of neutrophil activation in the pathogenesis of laminitis, as evidenced by the presence and activity of myeloperoxidase (MPO) and neutrophil extracellular traps (NET). Additionally, the therapeutic potential of stem cells, particularly in light of their immunomodulatory properties, was explored. To comprehensively address these objectives, we have implemented a multifaceted approach, encompassing *in vitro* cellular models, *in vivo* animal studies, and clinical investigations.

In our first *in vitro* study we aimed to investigate if juglone could be the responsible element for inducing neutrophil activation in the black walnut heartwood extract (BWE) model. Existing literature indicates neutrophil activation in systemic inflammatory response syndrome (SIRS)-associated models of laminitis. For example, in the BWE model the presence of MPO (Riggs *et al.*, 2007) and elastase (de la Rebiètre *et al.,* 2010) has been demonstrated in the skin, blood and lamellar tissue of horses affected with laminitis. Juglone, a naphthoquinone compound in walnuts with cytotoxic properties can induce oxidative stress with redox cycling and reactive oxygen species (ROS) generation. Interestingly, we found that juglone had an inhibitory effect on the degranulation of neutrophils. Moreover, it also reduced the peroxidase activity of MPO, as evidenced by the specific immunological extraction followed by enzymatic detection (SIEFED) assays. These results were confirmed by a docking study showing the perfect positioning of juglone in the MPO enzyme active site. These results indicate that juglone is not the trigger for equine laminitis, at least if we focus on the modulation of neutrophil activation.

We then set out to explore neutrophil activation in horses with laminitis induced using the prolonged euglycemic hyperinsulinemic clamp (pEHC) model, mimicking endocrinopathic laminitis, another prevalent cause of laminitis. Immunohistochemistry on lamellar sections revealed that MPO was largely present in the dermis of affected horses, though neutrophils were notably sparse. One hypothesis for this observation could be the presence of NET. These findings suggest that neutrophil activation may play a more significant role than previously suspected in cases of endocrinopathic laminitis.

The subsequent step in our research project involved assessing neutrophil activation in clinical cases of laminitis to represent the live scenarios as closely as possible. This entailed the inclusion of both horses and donkeys as they are both affected by this disease. We aimed to investigate the presence and activity of MPO and NET in lamellar tissue. Our findings revealed an increased presence of MPO and NET and an increased activity of MPO and NET-bound MPO in horses and donkeys affected with laminitis

compared to controls. Furthermore, it was also shown that part of the MPO activity was attributable to NET-bound MPO. Additionally, the histologic analysis showed the severity of the lamellar lesions and the presence of neutrophils in these advanced cases of laminitis, likely related to the severity of the disease in these end-stage cases and contrasting with the earlier-stage observations in *in vivo* models.

Finally, we attempted to assess the therapeutic potential of stem cells in relation to neutrophil activity. Based on the immunomodulatory and regenerative properties of equine muscle-derived mesenchymal stem cells (mdMSC), we created an *in vitro* model using keratinocytes and activated neutrophil supernatant co-culture submitted to an anoxia-reoxygenation stress. Through this *in vitro* model we demonstrated that equine mdMSC were able to restore keratinocyte metabolism and possess anti-inflammatory properties implicating the modulation of NET and MPO activity, thereby highlighting its potential in the treatment of laminitis.

In conclusion, this PhD thesis provides evidence that inflammation with neutrophil activation is a key element in the pathophysiology of laminitis regardless of the various inciting causes. MdMSC therapy might prove a novel treatment approach for improving the outcome of this debilitating disease, through mitigating inflammation and restoring keratinocyte metabolism, although further research is necessary to validate these findings, optimize treatment protocols, and understand the long-term effects and safety of this therapeutic approach.

General preamble

In recent years, the understanding of equine laminitis has evolved significantly, driven by a growing recognition of its complex pathophysiology and far-reaching impact on equine health and welfare. Laminitis, characterized by structural disruption of the dermo-epidermal interface within the hoof, presents substantial challenges to equine practitioners, researchers, caregivers and horse owners.

This thesis aims to delve deeper into the multifaceted nature of laminitis, exploring its pathogenesis and possible therapeutic pathways. By combining available information with novel insights, this work contributes to the ongoing research surrounding laminitis, aiming to advance the understanding of this debilitating condition and improving the outcome for affected horses.

In essence, this thesis represents a dedicated effort to unravel some of the complexities in the pathophysiology of laminitis. This work will not only deepen our comprehension of laminitis but also lay groundwork for further research and innovative treatment strategies, ultimately paving the way toward more effective prevention and treatment for this complex and debilitating equine pathology.

Introduction

1. ANATOMY OF THE DIGIT

1.1 Macroscopic anatomy

The equine foot thrives in its natural environment but becomes vulnerable to disease and injury under the pressures of human domestication. A comprehensive understanding of equine foot anatomy is essential for addressing its disorders, encompassing pathophysiology, diagnosis, and treatment (Pollitt 1992, Parks 2017).

1.1.1 The foot

The equine foot comprises many specialized structures like the third phalanx, the distal extremity of the second phalanx, the navicular bone, part of the ungual cartilages of the distal phalanx, the distal interphalangeal joint, the navicular bursa, different ligaments and the deep digital flexor tendon. The hoof is the integument of the horse's foot, therefore three main layers can be considered: the epidermis, the dermis and the hypodermis (Parks 2017).

1.1.2 The epidermis

The hoof wall, thickest at the toe, gradually thins towards the heels and consists of three layers:

- The external layer (*stratum externum*)

The *stratum externum*, derived from the limbic germinal epidermis, is an exceptionally thin layer of tubular horn coating the surface of the hoof wall (Fig. 1). The narrow, soft, non-pigmented proximal portion, known as the periople expands caudally over the heel bulbs and has a soft and skin-like appearance. This layer provides toughness and impermeability while maintaining elasticity. The outermost layer covering the rest of the hoof wall, the stratum tectorium, lends the wall its smooth, glossy appearance due to its high lipid content, and reduces evaporative water loss. (Pollitt 1992, Parks 2017).

- The middle layer (*stratum medium*)

The *stratum medium*, derived from the coronary corium, is composed of prominent tubular and intertubular horn and forms the bulk of the hoof wall (Fig. 1, Pollitt 1992, Parks 2017). Its proximal part is excavated from the coronary groove, housing the convex coronary corium (Pollitt 1992).

Each horn tubule features a long cylindrical cavity, the medulla, bordered by keratinized cortical cells (Chateau *et al.* 2007). The proximal end of the medulla accommodates a papilla from the coronary

corium, which generates the keratinized cortical cells (Chateau *et al.* 2007). Horn tubules are visible on the hoof surface, appearing as fine longitudinal lines (Pollitt 1992). The intertubular horn sometimes contains melanin pigment, darkening this thick layer in some horses (Pollitt 1992, Parks 2017).

- The internal layer (*stratum internum or lamellatum*)

The *stratum internum* is derived from the parietal germinal epidermis (Parks 2017). This innermost layer is consistently non-pigmented, featuring about 600 keratinized primary epidermal lamellae (PEL) extending from the coronary groove to the ground surface. Each primary lamella bears 100-150 non-keratinized secondary epidermal lamellae (SEL), interlocking with lamellar dermal counterparts (Fig. 1, Pollitt 1992). This specific organization increases the surface significantly. The surface area of the lamellae of the inner hoof wall of standardbred horses is approximately 0.8 m^2 (Daradka 2000).

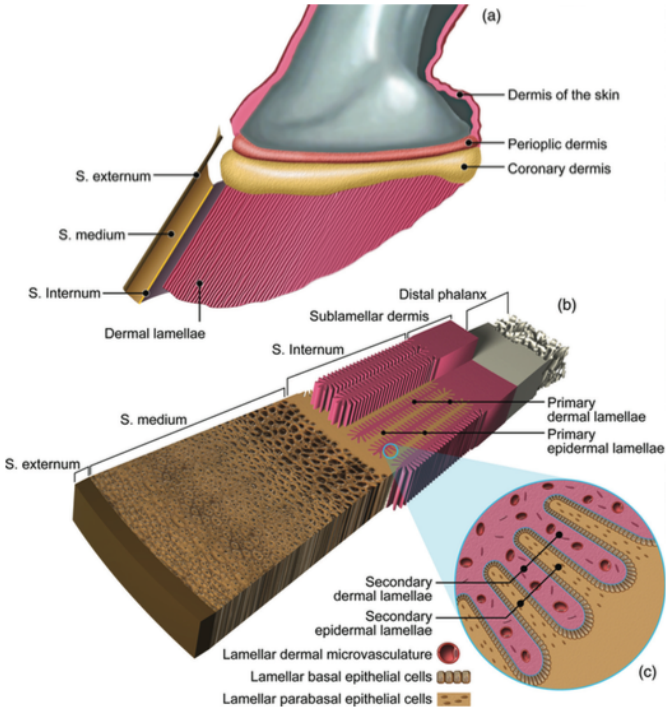

Fig. 1 Schematic representation of the dermo-epidermal interface. This interface consists of primary epidermal lamellae, secondary epidermal lamellae, primary dermal lamellae and secondary dermal lamellae (Parks 2017).

The hoof wall, a keratinized epidermal structure devoid of blood vessels and nerves, resembles other epidermis-derived structures like horn or hair. The hoof horn derives from the constant division and differentiation of keratinocytes (Pollitt 1992). This dynamic process leads to the development of three histological strata:

- The *stratum germinativum,* localised in the *stratum internum*, comprises germinal basal cells with large nuclei and scant cytoplasm that are permanently attached to the basement membrane (BM), thereby forming the dermo-epidermal junction. This stratum plays a crucial role in providing cells for the more superficial strata by mitosis and keratinization, facilitating growth and regeneration of the hoof wall (Pollitt 1992).
- The *stratum spinosum,* derived from the *stratum germinativum* and localised in the *stratum internum*, contains cells that acquire a more spiky cell appearance because of a large number of desmosomes (Pollitt 1992).
- The *stratum corneum,* composing the *stratum medium* and *stratum externum*, contains fully keratinized cells without a nucleus, called corneocytes and is at the end of the differentiation process. These mature corneocytes create a robust barrier preventing the passage of water and water-soluble substances inward and the loss of body fluids outwards. Additionally, the hoof corneocytes support the entire horse's weight through specialized arrangements in cylindrical tubules and leaf-like lamellae (Pollitt 1992).

1.1.3 The dermis - Corium

The highly vascular and sensitive corium, represents the dermal part between the epidermis and the subcutis, forming a robust matrix of connective tissue housing a network of arteries, veins, capillaries, and sensory and vasomotor nerves. The dermis consists of two main structures: (i) the coronary corium, situated in the coronary groove just below the perioplic corium and featuring multiple papillae and (ii) the lamellar corium, comprising approximately 600 primary dermal lamellae (PDL), each subdivided into 100 to 200 secondary dermal lamellae (SDL), the lamellar corium forms a critical component of the hoof structure. The attachment of these interdigitating epidermal and dermal lamellae is primarily dependent on the attachment of the epidermal lamellar basal cells to the BM connecting it to the dermis (Parks 2017). A dense, fibrous matrix of connective tissue connects the lamellar BM of the dermo-epidermal junction to the periosteal surface of the distal phalanx, establishing the crucial hoof-distal phalanx bond and providing a solid support for the entire appendicular skeleton and weight of the horse (Pollitt 1992). It is crucial to maintain this unity among the hoof wall epidermis, dermo-epidermal junction, BM, lamellar corium connective tissue, and periosteum of the distal phalanx, as disruption in this junctional integrity can lead to severe pathological consequences, such as laminitis (Pollitt 1992).

1.1.4 The hypodermis

The subcutaneous tissue is attached to the overlying dermis and epidermis and the underlying distal phalanx and ungual cartilages. This tissue is further specialized in the digital cushion at the level of the frog and in the coronary cushion at the level of the coronary band (Parks 2017). The coronary cushion

houses the coronary vascular plexus (Pollitt 1992). Only minimal subcutaneous tissue is present at the parietal aspect of the distal phalanx (Parks 2017).

1.1.5 The blood supply

The paired palmar/plantar digital arteries and veins provide a dense vascular plexus to the foot. Within the lamellar tissue, among the microcirculatory system, numerous arteriovenous anastomoses (AVAs) connect axial arteries and veins of the dermal lamellae, contributing to the control of dermal microcirculation in the equine digit. These AVAs are richly innervated by autonomic vasomotor nerves, feature thick walls of smooth muscle and a specialized, tall endothelium (Fig. 2, Pollitt and Molyneux 1990). These structures are crucial for thermoregulation and pressure modulation in the dermal microcirculation.

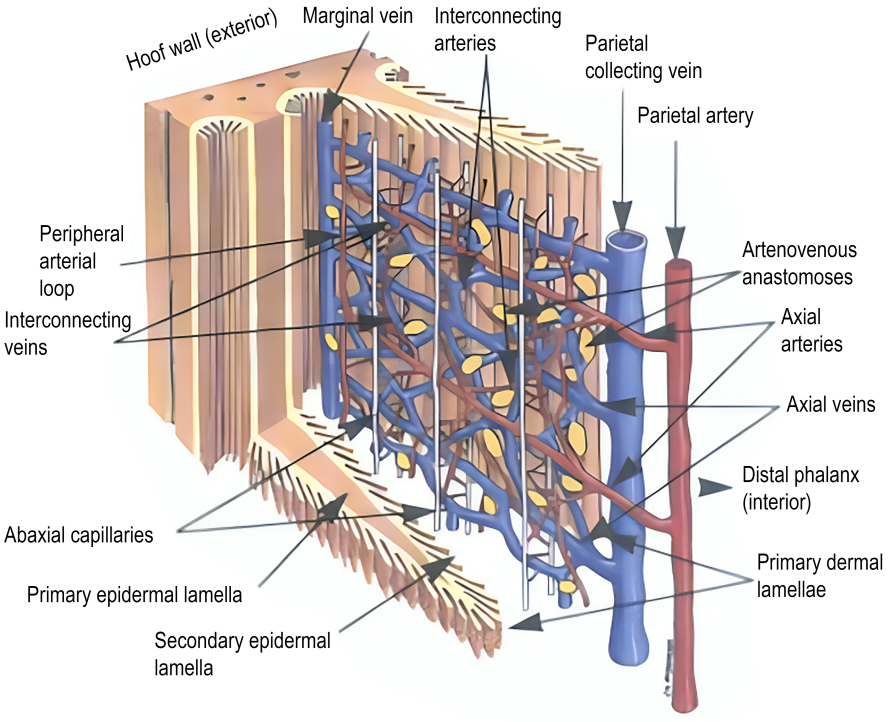

Fig. 2 Schematic representation of the lamellar microcirculation of the equine foot and of the arteriovenous anastomoses (Pollit 1992).

1.2 Microscopic anatomy

1.2.1 Microscopic lamellar characteristics

The lamellar anatomy of clinically sound horses has been extensively characterized using histology (Pollitt 1996, Pollitt 2004a). Each adaptation of the epidermis corresponds to a complementary

specialization in the underlying dermis, resulting in the pairing of each epidermal lamella with its corresponding dermal counterpart (Fig. 3, Pollitt 1992). Both the primary and secondary dermal and epidermal lamellae can be clearly distinguished histologically (Fig. 3). The PEL feature an acellular keratinized axis. Their SEL exhibit rounded tips in contrast to the tapered tips of the SDL. The tips of the primary and secondary lamellae orientate toward the distal phalanx, indicating the lines of tension to which the lamellar suspensory apparatus is subjected (Pollitt 1996, Pollitt 2004a). The SDL are narrower than the SEL and are filled with connective tissue until their very tips, between SEL bases. Fine, thin-walled capillaries, with a diameter equivalent to one or two erythrocytes, are present within the filamentous connective tissue of most of the SDL (Pollitt 1996). The BM, highlighted using special stains like Periodic Acid-Schiff (PAS), appears as a dark magenta line closely adherent to the lamellar basal cells of the SEL, penetrating deeply between each SEL base to lie close to the anuclear, keratinized axis of the PEL (Pollitt 1996, Pollitt 2004a, Pollitt 2017a). The proximity of the SDL tip to the keratinized axis is therefore readily appreciated, typically at a distance equivalent to one or two lamellar basal cells (Pollitt 1996). The nuclei of the lamellar basal cells of the SEL are oval and positioned at the apex away from the BM, with the long axis perpendicular to the SEL axis (Pollitt 1996, Pollitt 2004a). In normal tissue, polymorphonuclear leukocytes are not visible, either in capillaries or in the dermis (Pollitt 2004a).

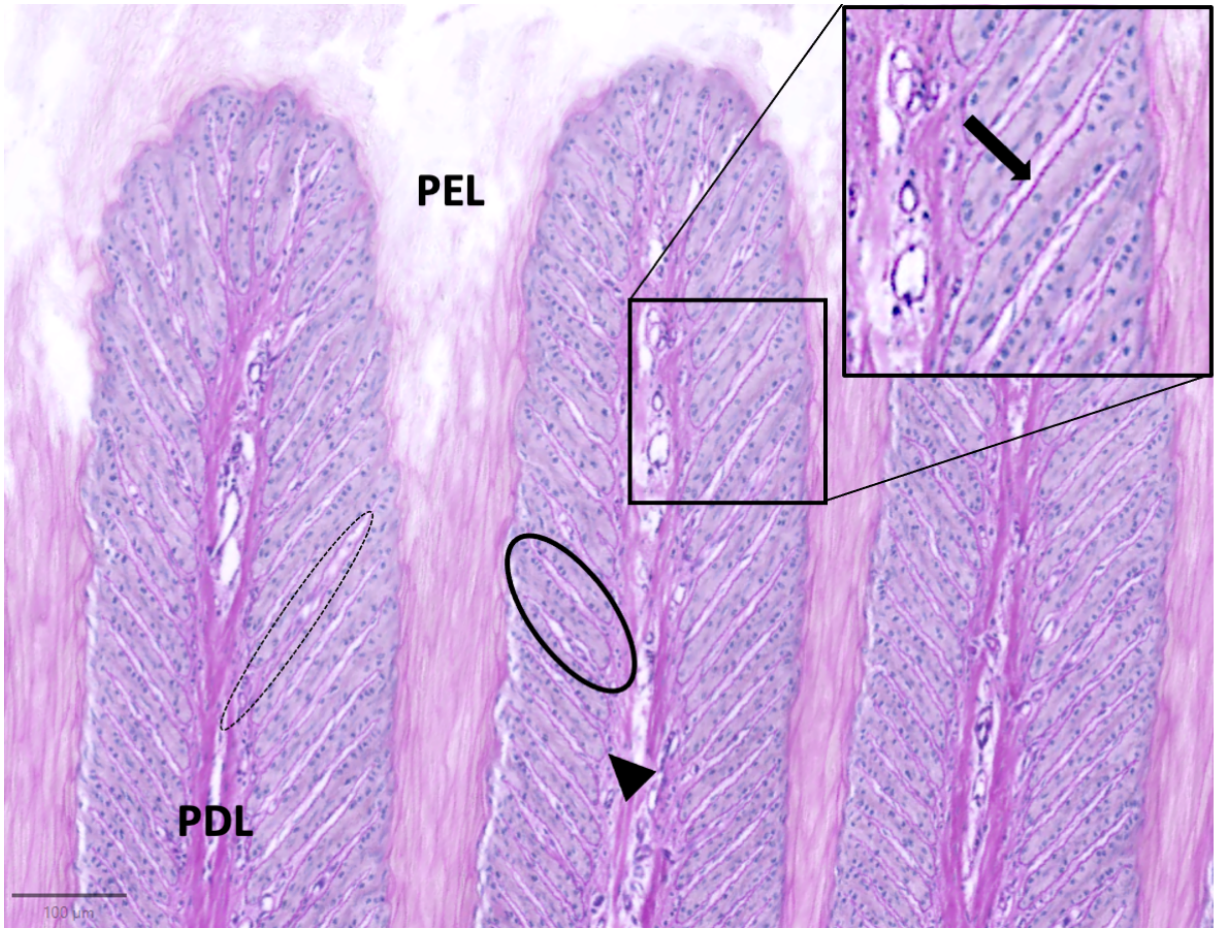

Fig. 3 Photomicrograph displaying normal anatomic features of lamellar tissue stained with periodic acid-Schiff. PEL: primary epidermal lamella, PDL: primary dermal lamella, full circle: secondary epidermal lamella, interrupted circle: secondary dermal lamella, arrowhead: rounded tip of the secondary epidermal lamella, arrow: basement membrane (personal illustration).

1.2.2 The basement membrane

At the junction of the epidermis and dermis lies the BM (Fig. 3 and Fig. 4), a thin, unbroken proteinaceous sheet of extracellular matrix (Pollitt 2004a). It separates the lamellar dermis and epidermis, with epidermal basal cells on the epidermal side and connective tissue on the dermal side (Pollitt 2004a). Besides providing a barrier and structural support for lamellar basal epidermal cells (LBECs), the BM is also involved in the regulation of cell attachment, growth, migration, differentiation and signalling (Abrahamson 1986, Durham 2017, Pollitt 2017a). Its major components are collagen type IV, laminin, nidogen/entactin, and perlecan (Parks 2017).

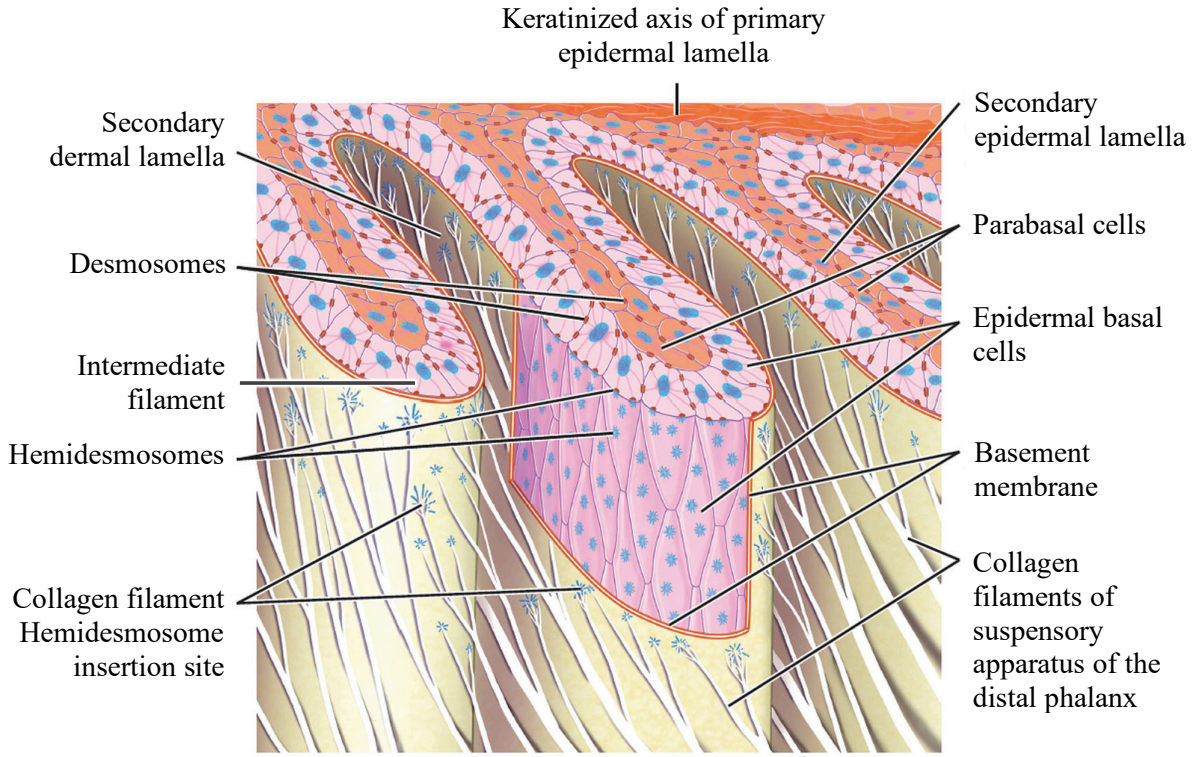

Fig. 4 Schematic representation of the secondary epidermal lamellae and suspensory apparatus of the distal phalanx (adapted from Pollit 2017a).

1.2.3 Lamellar basal cell attachments

A solid attachment between LBECs and the dermis is essential in weight-bearing hoof lamellae. LBECs are attached to their epidermal neighbours and to the underlying BM by different cell junctions (protein

complexes), called desmosomes, hemidesmosomes and adherens junctions. All of them consist of three elements: (i) one or more transmembrane proteins, (ii) cytosolic cytolinker proteins and (iii) the cytoskeleton (Pollitt 2017a).

Desmosomes (Fig. 4) are intercellular junctions that serve as pivotal structures not only in physically connecting cells, but also by interacting with signaling cascades that are involved in establishing cell polarity, cell shape, motility and cell proliferation and differentiation (Nekrasova and Green 2013, Pollitt 2017a). Under transmission electron microscopy, the transmembrane extracellular proteins manifest as an electron-dense line where they cross the intercellular space (Divers 2017, Pollitt 2017b).

Adherens junctions (AJ) are located at the cell-cell border, typically at the apical and basolateral membranes of each LBEC, and associate with the actin cytoskeleton. Multiple adherens junctions collaboratively form an actin belt called the zonula adherens (Harris and Tepass 2010, Pollitt 2017a). The primary role of AJ is providing cellular stability, but they are also involved in regulating the actin cytoskeleton, facilitating intercellular signaling and participating in transcription regulation (Hartsock and Nelson 2008). Under transmission electron microscopy, AJ manifest as mildly electron-dense transmembrane structures that associate with the actin cytoskeleton (Pollitt 2017b).

Hemidesmosomes (Fig. 4) serve as specialized structures that attach the plasma membrane of each LBEC to the underlying BM, creating a vital bridge between the cells' interior and the external connective tissue (Pollitt 2004a). In addition to their adhesive role, hemidesmosomes also participate in signal transduction (Borradori and Sonnenberg 1999). Unlike desmosomes and adherens junctions, hemidesmosomes are located exclusively along the basal plasma membrane of LBECs, connecting cytoskeletal intermediate filaments to the BM (Pollitt 2017b). When examined under the transmission electron microscope, the numerous hemidesmosomes appear as clusters of electron-dense plaques, linking the plasma membrane to the BM at the base of LBEC (French and Pollitt 2004, Pollitt 2004a).

1.2.4 The cytoskeleton

The cytoskeleton is a meshwork of crosslinked filaments (Fig. 4), composed of mainly keratin intermediate filaments (IFs, 85%) and actin microfilaments (11%) (Carter *et al.* 2010, Pollitt 2017a). It provides mechanical stability to the cell and to the entire epidermal lamellar system by being interconnected to all other LBECs and dermis (Carter *et al.* 2010, Pollitt 2017a). The IFs stabilize the nucleus by anchoring it to the basal plasma membrane via hemidesmosomes and to the remaining plasma membrane via desmosomes (Carter *et al.* 2010, Pollitt 2017a).

Besides providing mechanical stability, the cytoskeleton is also a regulator of cellular molecular signalling as it modulates cellular pathways by controlling the activity and localization of signalling

proteins and their targets (Kim and Coulombe 2010, Pollitt 2017a). Therefore, the cytoskeleton is not only a static structural entity that maintains cell architecture, but it also exhibits dynamic characteristics as it can be built up or broken down to enable various cellular processes such as cell migration and adhesion (Pollitt 2017a).

1.2.5 The endothelium

The vascular endothelium is a highly specialized, multifunctional organ, composed of a single cell layer, which covers the entire inner surface of the circulatory system. Each endothelial cell is a dynamic, intimate interface between the circulating blood and the underlying tissues. The endothelium can adapt its function as required to maintain homeostasis due to its strategic location and capacity to monitor chemical and physical stimuli of local and systemic origin. The endothelium is involved in maintenance of a selectively permeable blood–tissue barrier, modulation of vascular tone, regulation of hemostasis, regulation of inflammation, angiogenesis and wound healing. These functions are interconnected and can change in response to various stimuli such as inflammatory mediators, hypoxia and elements of coagulation (Duffy *et al*. 2004). For example, endothelial cells and leukocytes express complementary adhesion molecules. Under normal circumstances, leukocytes circulate freely without significant interaction with the microvascular endothelium. However, under pathological conditions, the inflammatory signalling can activate the endothelial cells and leukocytes, and this will cause their recruitement to sites of inflammation (Duffy *et al.* 2004). Furthermore, digital veins characterized by their high muscularity, low compliance, and high sensitivity to vasoconstrictive substances, predispose the equine digit to elevated venous pressures, increased hydrostatic pressure and oedema formation (Allen *et al.* 1988, Baxter *et al.* 1989, Katwa *et al.* 1999, Eades *et al.* 2002).

1.3 Differences between the horse and the donkey

Donkeys are usually considered as small horses and are therefore treated in the same way. However, some anatomic differences are important to acknowledge. Firstly, donkeys have an ‘upright boxy’ hoof contrasting with the ‘inclined rounded’ capsule of horses. The solar surface of the donkey hoof is oval-shaped and the dorsal hoof wall is 5-10° more upright. These characteristics differ from the circular solar surface, tapering heel and inclined heel angle encountered in horse hooves. Additionally, the frog is well-developed in the palmar/plantar aspect but extends less far rostrally under the third phalanx compared to horses (Fig. 5, Thiemann *et al.* 2021). Furthermore, donkey hooves feature a marked flare at the heels. Finally, the third phalanx sits more distally in the hoof capsule and therefore palpation of a depression at the coronary band to indicate sinking of the phalanx is less straightforward and the founder distance is increased compared to horses (Fig. 5, Collins *et al.* 2011). Interestingly, the internal horn structure in donkey hooves has a higher moisture content, easing the adaptation to the arid environment

that donkeys originate from (Hopegood *et al.* 2004). To the authors' knowledge, no studies are available describing the microscopic anatomy of the donkey hoof.

These anatomical and ultrastructural differences are likely to influence both the mechanical proprieties of the hoof and the development of foot pathology (Thiemann *et al.* 2021).

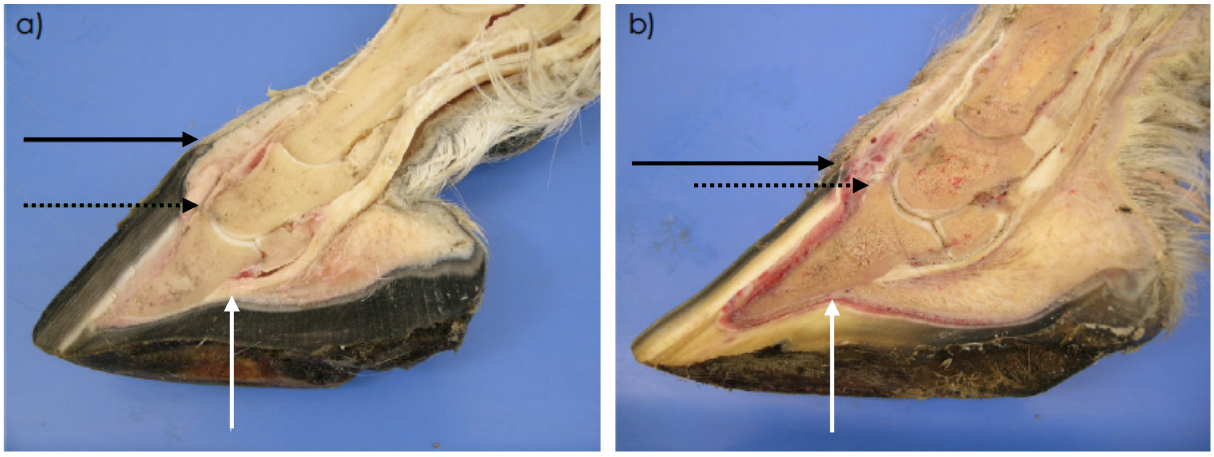

Fig. 5 Split sections of a donkey hoof (a) and a horse hoof (b). The distance between the coronary band (black arrow) and extensor process (black dotted arrow) in larger in the donkey hoof (a) than in the horse hoof (b) where both are almost aligned. The frog of the donkey hoof sits less far under the pedal bone than the frog of the horse hoof (white vertical arrows) (adapted from Thiemann *et al.* 2021).

1.4 Differences between horses, cattle and camelids

Bovine and equine hoof anatomy, while serving similar functions, differ significantly in structure due to their distinct evolutionary adaptations. The bovine digit is cloven, with two weight-bearing claws per foot, a medial and a lateral one, separated by a digital cleft and united by a cruciate ligament (Greenough 2007b). Cattle hooves encompass a third phalanx, that is attached to the hoof wall by similar dermal and epidermal lamellae as in equids, however, the absence of secondary lamellae is typical for the bovine hoof (Greenough 2007a). The dermal lamellae are most developed on the dorsal wall of the claw and there are no lamellae on the inside of the posterior half of the wall of the claw (Greenough 2007a). There is however an extensive network of fibers encasing the deep digital flexor tendon and the digital cushion (Greenough 20007a).

The camelid foot is unique, with two digits on each foot. The palmar/plantar surface is covered with a soft, cornifyed layer of epithelium, called the slipper. Lamas and alpacas have a separate slipper for each digit, while in the camel a single slipper covers the entire weightbearing surface. A small, nonweightbearing nail is located at the extremity of each digit. The toenail has laminae which attach the nail to the third phalanx (Fowler 2010a).

2. DEFINITION AND EPIDEMIOLOGY OF LAMINITIS

Laminitis is a common, severely debilitating, excruciatingly painful, potentially career-ending and life-threatening condition of the laminae of the digit of equids (Eades *et al.* 2002). It is characterized by a structural failure of the dermo-epidermal interface, causing destabilization and, ultimately, displacement of the distal phalanx, thereby leading to severe lameness and dramatic pain, sometimes requiring euthanasia (Menzies-Gow *et al.* 2010, Katz and Bailey 2012, Leise 2018).

Far from being an emerging pathology, laminitis was already described by Aristote more than 300 years BC (Rendle 2006). Accurately estimating the current prevalence of the disease remains challenging. Wylie *et al.* (2011) report a prevalence ranging from 1.5-34% in horses, 4.4-48.5% in donkeys (Thiemann *et al.* 2021, Menzies-Gow *et al.* 2022), 1.5% in cattle (Marti *et al.* 2021) and only rare cases are reported in camelids (Fowler 2010b). Eades *et al.* (2002) estimates that 15% of horses in the United States will be affected with laminitis during their lifetime, with 75% developing severe or chronic lameness potentially requiring euthanasia. Particularly painful and detrimental to the horse, it frequently leads to performance losses that can result in the premature end of a sports career or even death. Beyond its financial toll, the emotional cost of laminitis and the still limited knowledge of its pathophysiology, treatments, and prevention methods make this disease particularly frustrating for both veterinarians and horse owners.

3. ETIOLOGY OF LAMINITIS

Laminitis often has a multifactorial etiology and results from a combination of inflammation, decreased blood flow (ischemia) in the foot, endothelial/vascular dysfunctions, degradation of the extracellular matrix, and metabolic disturbances in keratinocytes (Peroni *et al.* 2005, Loftus *et al.* 2006, Serteyn *et al.* 2014).

Besides supporting limb laminitis, which is a rather uncommon cause of laminitis that stems from specific limb injuries causing severe lameness (e.g. fractures) and typically characterized by contralateral lameness in horses devoid of systemic abnormalities (Baxter 2017), more prevalent etiologies of laminitis include hormonal disturbances and diseases leading to systemic inflammatory response syndrome (SIRS).

Laminitis has been associated with various diseases related to SIRS such as metritis, broncho-/pleuropneumonia, and gastrointestinal disease (Eades 2017a). Gastrointestinal tract disease used to be a notable contributor, with pathologies such as surgical colic (Hunt *et al.* 1986), acute diarrhoea (Cohen and Woods 1999), and duodenitis/proximal jejunitis (Cohen 1994). Endotoxemia, characterized by the

presence of endotoxin (lipopolysaccharide, LPS) in the bloodstream, is a common trigger for systemic inflammation and is associated with diseases caused by gram-negative bacteria (Eades 2017a). Although endotoxemia is a significant risk factor for laminitis (Parsons *et al.* 2007), experimental induction of endotoxemia does not directly induce laminitis, suggesting the involvement of other toxins/mediators or other local or physiologic events (Eades 2017a). Local inflammation, pain, and hypovolemia may also play a role in the laminitis pathophysiology. Moreover, the release of damage-associated molecular pattern molecules (DAMPs) from injured host cells can trigger an inflammatory response similar to that induced by bacterial products (pathogen-associated molecular pattern molecules, PAMPs, like LPS), in diseases associated with tissue injury and cell death (e.g. compromised bowel wall, tubular necrosis in kidney disease) (Manson *et al.* 2011).

Gastrointestinal tract disease used to be the most common primary disease in 54% of horses that developed acute laminitis (Slater *et al.* 1995), but this has over time been replaced by the increased prevalence of laminitis associated with equine metabolic disorders, such as pituitary pars intermedia dysfunction (PPID), and equine metabolic syndrome (EMS). The primary feature of these conditions is insulin resistance with hyperinsulinemia (Asplin *et al.* 2007, de Laat *et al.* 2012). Multiple mechanisms are proposed to explain how hyperinsulinemia can lead to laminitis. Firstly, hyperinsulinemia could lead to the insulin-mediated stimulation of insulin-like growth factor-1 (IGF-1) receptors on basal epidermal cells, thereby promoting cell proliferation and structural weakening of this important attachment zone (de Laat *et al.* 2013b). Furthermore, studies also indicate that hyperinsulinemia induces increased vascular resistance and endothelin-1 expression in the equine digit, suggesting a role for abnormalities in lamellar perfusion in endocrinopathic laminitis (Gauff *et al.* 2013). Additionally, insulin resistance is associated with a chronic pro-inflammatory state as in the human metabolic syndrome, where increased adipose tissue mass amplifies the secretion of proinflammatory adipokines that decrease insulin sensitivity, induce oxidative stress, and impair microvascular function (Geor and Frank 2009). Similarly, EMS is characterized by regional adiposity, hyperinsulinemia, insulin resistance, hypertriglyceridemia, and recurrent laminitis (Johnson *et al.* 2004, Geor and Frank 2009, Frank *et al.* 2010).

Although studies agree that a multifactorial etiology for equine laminitis pathology is very likely, with a great variety of different causes, inflammation seems to be a common element reuniting the different etiologies in the pathophysiology and therefore warrants further investigation.

Equids and bovines both have similar size and weight, are both herbivores and ungulates, and eventhogh their digestive system largerly differs, they both suffer from laminitis. Simalarly to the horse, laminitis in cattle is a systemic disease with local manifestation in the claws (Greenough 2007a). Laminitis comes in acute, subacute and chronic forms (Greenough 2007a). Acute laminitis is rare in cattle and associated with grain overload causing rumen acidosis and rumenitis (Greenough 2007a). Interestingly, grain overload and rumen acidosis are less likely to cause laminitis in camelids compared to cattle (Fowler

2010b). Furthermore, in cattle, laminitis is encountered in the peri-partum period with a probable multifactorial origin combining environmental, dietary and hormonal changes (Greenough 2007a).

4. PATHOPHYSIOLOGY OF LAMINITIS

4.1 Models for laminitis induction

An understanding of the pathophysiology of a disease process is essential for clinicians to establish effective therapies, whether employed prophylactically in animals at risk or therapeutically in animals already suffering from the disease. One step in unravelling the pathophysiologic mechanisms involves creating an experimental model to effectively study the disease. Different models have been developed, each considering different etiologies.

4.1.1 Starch overload

The starch overload model, developed by Harold Garner *et al.* in the early 1970s, serves as an experimental model for acute sepsis-related laminitis accompanied by acute alimentary disease resulting from the oral intake of large quantities of digestible carbohydrates. A slurry mixture of 80% corn starch and 20% wood flour is administered through a nasogastric tube (Garner *et al.* 1975). Starch overload results in enterocolitis caused by an overload in soluble carbohydrates, which overwhelms the digestive capability of the small intestine and subsequently causes dysbacteriosis in the caecum and colon (Garner *et al.* 1978). Laminitis induction proved generally successful in 90% of the horses. Horses also presented mild to moderate diarrhoea and colic signs (Garner *et al.* 1975).

4.1.2 Oligofructose overload

The oligofructose (OF) model used a commercially available fructan extracted from chicory roots (*Cichorium intybus*) administered through a nasogastric tube, to replicate laminitis potentially resulting from the ingestion of fructan-rich pasture (van Eps and Pollitt 2006, Longland and Byrd 2006). Besides complex structural carbohydrates, ingested pasture also contains nonstructural carbohydrates (NSC) in the form of fructans that are usually fermented in the hindgut (caecum and colon) (Ince *et al.* 2014). However, during specific times of the year and under certain environmental conditions, horses may ingest excessive pasture fructans.

The administration of OF consistently induced laminitis, without excessive colonic gas production or colic. Therefore, the OF laminitis induction model has proved reliable and reproducible, effectively mimicking field cases of laminitis when fructan concentrations are notably high (Pollitt and Milinovich 2017).

4.1.3 Black walnut heartwood extract model

The development of the black walnut heartwood extract (BWE) model of laminitis, similar to the carbohydrate overload model, took its origin from clinical reports highlighting laminitis cases in animals bedded on wood shavings from black walnut (*Juglans nigra*) trees (Peroni 2017). The BWE is made by soaking shavings made from the heartwood of a branch of a black walnut tree in water for 12 hours. The resulting dark-red, slightly viscous solution was separated from the shavings and administered to the study subjects via a nasogastric tube (Minnick *et al.* 1987). The BWE model can be performed in a consistent manner, resulting in laminitis developing in 80% of horses, with the primary variability lying in the degree of toxicity of the tree from which shavings were obtained (Peroni 2017).

Currently, the responsible molecule for the laminitis induction in the BWE model remains unknown. Juglone (5-hydroxy-1,4-naphthoquinone), a phenolic compound in walnuts, possesses cytotoxic properties like other naphthoquinones, such as the induction of oxidative stress with redox cycling, cell membrane damage, apoptosis, and necrotic cell death (Ollinger and Brunmark 1991, Aithal *et al.* 2009) and could therefore be a trigger capable of inducing laminitis.

4.1.4 Prolonged euglycemic hyperinsulinemic clamp

Endocrinopathic laminitis, associated with insulin resistance and hyperinsulinemia in diseases like EMS and PPID, is currently the most common cause of laminitis in horses presented for lameness (Donaldson *et al.* 2004, Karikoski *et al.* 2011). In 2007, Asplin *et al.* introduced the prolonged euglycemic-hyperinsulinemic clamp (pEHC) technique to mimic endocrinopathic laminitis. This model involves administering a constant rate infusion of insulin, while simultaneously maintaining euglycemia through a glucose infusion. All ponies exposed to this technique consistently developed laminitis with a relatively slow onset, experiencing Obel grade 2 lameness at 55h. Notably, there was no evidence of gastrointestinal involvement or systemic illness in these cases (Asplin *et al.* 2007). Subsequent repetitions of the technique in horses showed a faster development of laminitis than in ponies, with Obel grade 2 lameness observed at 46h (de laat *et al.* 2010).

The precise mechanism by which hyperinsulinemia induces laminitis, with rapid and profound histological changes observed in various studies, remains elusive. It is unclear whether these effects are due to direct insulin toxicity or are secondary to other mechanisms such as rapidly induced insulin resistance or alterations in intracellular signaling pathways (McGowan and Patterson-Kane 2017). Nevertheless, the presence of microscopic lesions has provided several theories that warrant further investigation.

4.2 Anatomical abnormalities at a macroscopic and microscopic level

Various experimental models have been utilized to explore both macroscopic and microscopic changes in laminitis, providing valuable insights into its pathophysiology. These observations have significantly contributed to our understanding of the condition and are described in the following sections.

4.2.1 Macroscopic abnormalities and clinical signs

Laminitic horses and donkeys usually present with a stilted gait, lameness involving multiple hooves, stiffness, weight shifting, a typical 'saw horse' stance and reluctance to move (Dyson 2011). Their clinical examination usually reveals increased digital arterial pulses and elevated hoof temperature (Dyson 2011). Macroscopic abnormalities of their hooves include the presence of growth lines with divergent rings (Fig. 6), a thickened white line and in some acute cases a redder color can be noted in this area (Pollitt 2004b, Karikoski *et al.* 2015). Furthermore, the foot can have a convex sole and a painful palpation with the hoof tester in the toe area (Fig. 6, Dyson 2011). In horses, depression at the coronary band with finger pressure is indicative of founder, however, as the third phalanx (P3) sits more distally in the hoof capsule in donkeys, this technique is difficult to interpret. If there is pain associated with depression at the dorsal coronary band, this should be considered abnormal and warrants further investigation (Thiemann *et al.* 2021).

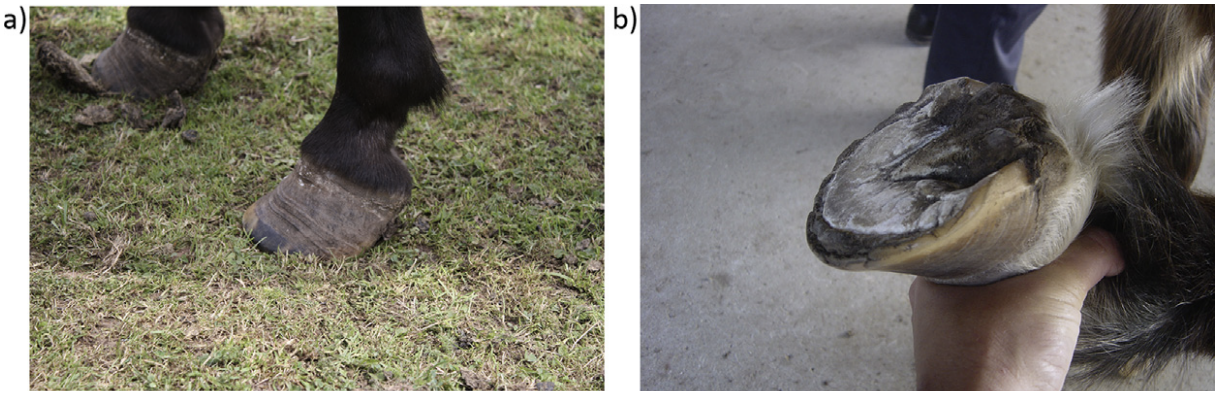

Fig. 6 Macroscopic aspect of hooves of laminitic horses. (a) Divergent rings on the outer hoof wall of a pony without current lameness. (b) Solar hoof surface of a pony showing a convex or 'dropped' sole and a widened white line (Patterson-Kane *et al.* 2018).

4.2.2 Microscopic abnormalities

- Structural lamellar changes

Microscopically, multiple abnormalities have been observed regardless of the etiology. Documented early histological changes in endocrinopathic and SIRS-associated laminitis models have included loss of the perpendicular orientation of SEL nuclei relative to their BMs, nuclear rounding, a more centrally located nucleus within the cytoplasm (vs. apical), with a more random orientation and prominent nucleoli (Pollitt 1996, Asplin *et al.* 2010, de Laat *et al.* 2011, de Laat *et al.* 2013a). SEL were observed to elongate, narrow, develop tapered (vs. club-shaped) tips and to become more acutely angled to the PEL axis, with irregularity of the PEL/SEL interface (Fig. 7). The PEL and SEL were more closely apposed and frequently became difficult to distinguish (Pollitt 1996, Asplin *et al.* 2010, de Laat *et al.* 2011, de Laat *et al.* 2013a). SEL elongation was thought to occur partly due to epidermal cells sliding past each other (Pollitt, 2004) or because of increased proliferative activity provoked by insulin (de Laat *et al.* 2013b). However, lamellar epithelial cell stretching is now regarded as a key early structural event and was the earliest noticeable histological change at the 6h time point, suggesting cytoskeletal deformation and eroded mechano-protection (de Laat *et al.* 2013a).

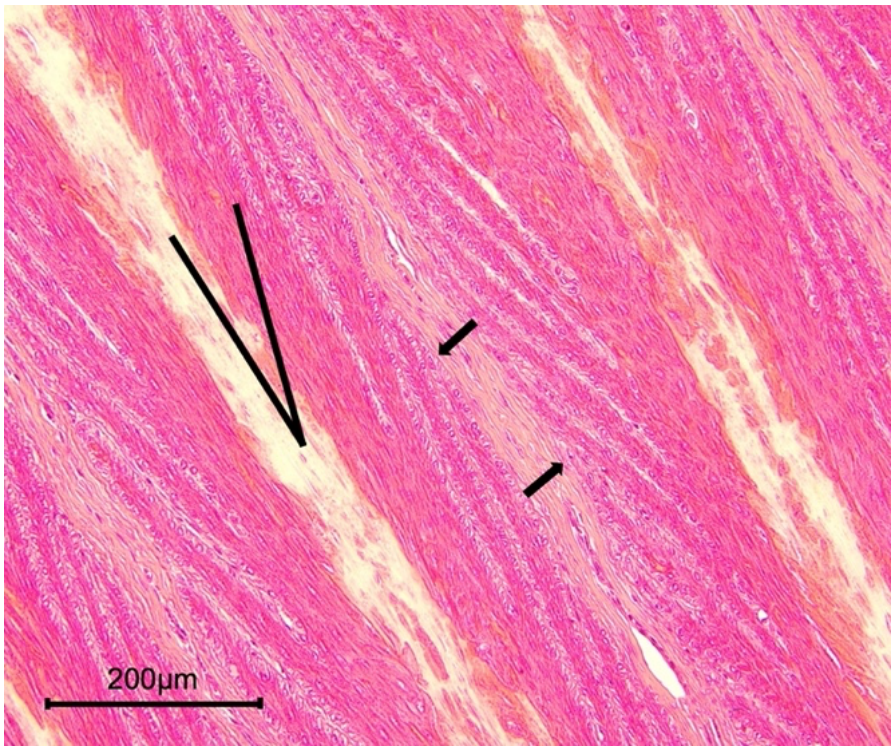

Fig. 7 Photomicrograph of lamellar tissue of a laminitic horse stained with hematoxylin and eosin showing increased SEL length, an acutre PEL-SEL angle (lines) and tapered SEL tips (arrow) (personal illustration).

- Cell death and proliferation

The spatial and temporal relationship between SEL stretching, cell death, and proliferation is complex (Karikoski *et al.* 2014). Cellular stretching was accompanied and followed by an accelerated cell death-proliferation cycle (de Laat *et al.* 2013a, Karikoski *et al.* 2014). While apoptotic cells are rare in normal lamellar tissue, their increased presence in hyperinsulinemic models suggests a response to mechanical stress caused by lamellar epithelial cell stretching (Asplin *et al.* 2010, Karikoski *et al.* 2014). Retrospective examination of specimens from multiple hyperinsulinemia models suggests that this process commences axially (adjacent to the distal phalanx), spreading abaxially (toward the hoof wall) in a 'wave' that is followed by proliferation (Asplin *et al.* 2010, de Laat *et al.* 2013a). Additionally, vacuolar cell swelling (reversible pathology) has also been noted in both the carbohydrate overload (CHO) and hyperinsulinemia models. Thus, there appears to be a spectrum of reversible and irreversible cellular injury and apoptotic cell death that varies markedly between individuals (Patterson-Kane *et al.* 2018).

- Basement membrane lesions

In laminitis, marked by the failure of the dermo-epidermal junction, the disintegration and separation of the lamellar BM leads to the loss of attachment to basal cells and detachment from epidermal lamellae. Notably, laminin-1 and collagen IV vanish during laminitis thereby progressively losing the attachment of the BM to the basal cells (Pollitt 2004a, Divers 2017).

Structural BM lesions develop in different degrees with subtle lesions in endocrinopathic laminitis in comparison with a more rapid failure in SIRS-associated laminitis. In some studies of CHO laminitis, BM blebbing was evident at the tips of the SEL at 18h and progressively worsened (with areas of 'blurring,' and discontinuity) before progressing into a 'degloving' process, with the BM forming large vesicles at the SEL tips and then pulling away from the SEL, taking the dermal tissue and its blood supply with it (Pollitt 1996). In most hyperinsulinemia models, BM damage was minimal, with mild and multifocal BM blebbing and separation predominantly localised to the most axial SEL only; neither all SEL nor all specimens were affected (Asplin *et al.* 2010, de Laat *et al.* 2011, de Laat *et al.* 2013a, Patterson-Kane *et al.* 2018). BM separation was absent ultra-structurally in hyperinsulinemic ponies, there were reduced numbers of hemidesmosomes per unit length of BM, but this was to a lesser degree than that measured in a CHO model (Nourian *et al.* 2009, Patterson-Kane *et al.* 2018).

- Endothelial dysfunction

Alterations in endothelial cell function during the onset of acute laminitis may affect endothelium-dependent vasodilatory actions, altering the responsiveness of digital vasculature to vasoactive agents

favouring a vasoconstriction (Peroni *et al.* 2017). Furthermore, platelet activation is also observed in early stages of laminitis particularly in the CHO model (Weiss *et al.* 1995). Interestingly, in CHO models, the presence of microthrombi doesn't seem to be related to systemic activation of coagulation, as likely occurs in gastrointestinal inflammatory and ischemic pathologies associated with SIRS and multiple organ dysfunction syndrome (Bailey 2017). Intravascular thrombosis, causing a lack of energy and blood flow to the lamellar tissues, has been evocated as a process causing laminitis to progress and caused severe lamellar damage with tissue necrosis as a consequence (Bailey 2017). However, this finding has been inconsistently described in literature and does not seem to be confirmed in endocrinopathic laminitis (Weiss *et al.* 1994, Weiss *et al.* 1995, Asplin *et al.* 2010, de Laat *et al.* 2011). Interestingly, vascular dysfunction was noted in horses with endocrinopathic laminitis with decreased response in vascular relaxation and increased vascular constriction in both lamellar vessels and facial arteries. The systemic nature of the abnormalities suggested that this dysfunction was possibly associated with the underlying endocrinopathy rather than a local process in the lamellar tissue (Morgan *et al.* 2016).

- From experimental models to naturally occurring laminitis and beyond

Many of the above findings stem from experimental laminitis conditions and only very limited information is available about naturally occurring laminitis pathology. One study describes the histopathology of naturally occurring endocrinopathic laminitis in 14 horses and ponies and 25 control horses (Karikoski *et al.* 2015). Microscopic lesions were primarily localised abaxially (close to the hoof wall) and included apoptosis, lamellar fusion, hyperplasia and partial replacement with aberrant keratin containing nucleated debris and proteinaceous lakes. While the lesions align with the progression of the lamellar stretching and deformation noted acutely and possibly primarily in hyperinsulinemic models, they still do not indicate if endocrinopathic and SIRS-associated laminitis are fundamentally different diseases (Patterson-Kane *et al.* 2018). It is suspected that these lesions represent sequelae that emerge regardless of the inciting pathology, likely related to the physical tearing of tissue once the lamellar structure is weakened (Baxter 1986). However, there remains a limited understanding of the progression of these lesions to end-stage laminitis cases commonly encountered in equine hospitals. Additionally, research predominantly focuses on horses, despite naturally occurring laminitis also affecting donkeys.

4.3 Inflammation

4.3.1 The role of neutrophils in general

Neutrophils are derived from hematopoietic cells of the bone marrow (Demaret *et al.* 2014). In the case of inflammation, neutrophils are attracted to the site of inflammation by chemotactic substances. Neutrophils are capable of phagocytosis of micro-organisms in a phagosome that becomes a

phagolysosome after fusion with lysosomal granules. The activation of neutrophils leads to the digestion of micro-organisms by two pathways. The first is independent of oxygen and results from the liberation of cationic proteins, lactoferrin and hydrolytic and proteolytic enzymes. The second involves the activation of oxygen consumption through a respiratory burst. The latter involves three enzymes: nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, myeloperoxidase (MPO) and nitric oxide (NO) synthase, acting by producing reactive oxygen species (ROS) capable of destroying bacterial polysaccharide capsules resistant to proteolytic enzymes (Babior 1999, Babior 2000, Serteyn *et al*. 2003, Mantovani *et al*. 2011).

Neutrophils contain several types of granules. The primary or azurophilic granules contain MPO, elastase, defensins and proteins capable of increasing the permeability of bacteria. Secondary granules contain antimicrobial proteins such as lactoferrin, whereas the tertiary granules mainly contain gelatinases (Borregaard and Cowland 1997, de la Rebière de Pouyade *et al*. 2010).

MPO is a hemic enzyme present in high concentrations in neutrophils (Borregaard and Cowland 1997, Klebanoff 2005) and a small amount in monocytes (Mathy-Hartert *et al.* 1996). The mature enzyme is a glycosylated symmetric homodimer, formed of two hemi-enzymes, each with two subunits (a light chain and a heavy chain) (Nauseef *et al.* 2000). MPO is responsible for the direct or indirect synthesis of many oxidizing species that participate in the defense of the host. Among them, hypochlorous acid (HOCl), a powerful oxidizing agent necessary for the destruction of micro-organisms in the phagolysosome which is produced from hydrogen peroxide (H_2O_2, derived from the superoxide anion itself produced by NADPH-oxidase) and chloride anion. Thanks to MPO's double activity of peroxidation and chlorination, the derived products of the enzyme (HOCl, nitrogen dioxide, …) are capable of nitrating, chlorinating and oxidizing protein residues (Fig. 8, Taurog and Dorris 1992, Dunford 2000, Podrez *et al.* 2000, Serteyn *et al.* 2003).

When the inflammatory reaction becomes uncontrolled, consequently to the degranulation or the death of neutrophils, MPO is released into the extracellular environment where it causes damage, in particular by attaching to endothelial cells and by the interaction of HOCl with many substances like amino acids and enzymes. Due to its toxic effects, MPO was shown to be implicated in acute inflammatory pathologies, but also in the development of cancers, atherosclerosis and Alzheimer's disease in human medicine (Nagra *et al.* 1997, Reynolds *et al.* 1999, Song and Santanam 2001, Serteyn *et al.* 2003).

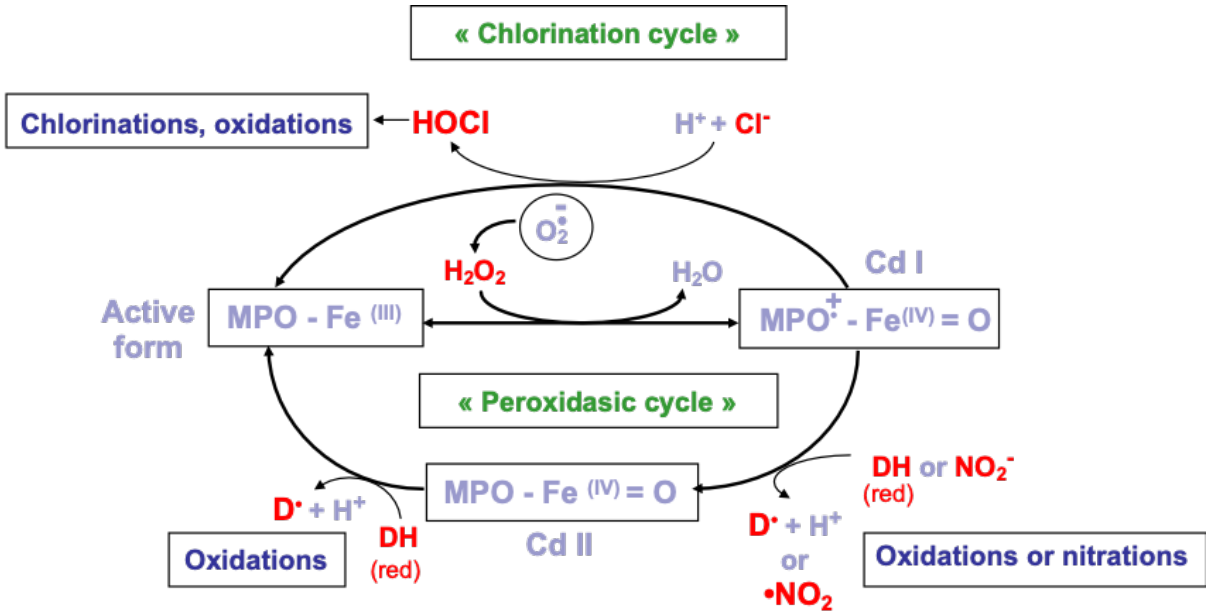

Fig. 8 Detailed steps of MPO [MPO-Fe (III): native enzyme] enzymatic activity and formation of intermediate compounds (Cd). Cd I: compound carrying a free radical on an amino acid of the protein chain, DH: electron donor substrate (red: reduced form), D•: radical derived from DH (by loss of H^+). NO_2^-: nitrite, $^\bullet NO_2$: nitrogen dioxide (modified image based on Deby-Dupont *et al.* 1999).

Besides MPO being liberated from neutrophil granules of activated neutrophils, it can also be found in tissues as a part of neutrophil extracellular traps (NETs). NETs are composed of extracellular strands of decondensed DNA in complex with histones, mainly H3 and H4, and neutrophil granule proteins, such as elastase, MPO, cathepsin G and gelatinase, which were expelled from neutrophils to ensnare and kill microbes (Takei *et al.* 1996, Brinkmann *et al.* 2004, Sørensen and Borregaard 2016, Delgado-Rizo *et al.* 2017). "NETosis", considered a form of cell death, is the term commonly used to describe the sequence of cellular events leading up to the release of NET (Yipp and Kubes 2013, Li and Tablin 2018). Various stimuli have been reported to induce NETosis. NETs are released after infection with bacteria, large pathogens and fungi (Hoppenbrouwers *et al.* 2017). The size of the microorganisms, as well as their virulence factors, and released inflammatory molecules regulate NET formation. Small microorganisms are usually removed by the neutrophil through phagocytosis and fusion with their granules. Larger microorganisms that are not easily digested, can block phagocytosis and trigger cells to commit to NET formation (Papayannopoulos 2018). Other distinct stimuli leading to NETosis include NO, autoantibodies, proinflammatory cytokines such as interleukine (IL)-1, IL-6, IL-8, tumor necrosis factor- α (TNF- α), and interaction with activated platelets or endothelial cells (Kaplan and Radic 2012).

In NETosis, 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" is dependent on ROS for histone citrullination by PAD4 before releasing DNA as extracellular traps. As the nuclear and plasmatic membranes are lost in the process, the neutrophil itself does not survive. In contrast, "vital NETosis" features neutrophil survival as all NET components are released in the extracellular environment by the fusion of vesicles containing NET components with the plasmatic membrane of the neutrophil (Yipp and Kubes 2013, Delgado-Rizo *et al.* 2017). In the ROS-dependent pathway of NETosis two enzymes, MPO and elastase, have critical roles. ROS generated by NADPH oxidase stimulate MPO to trigger the activation and translocation of elastase from azurophilic granules to the nucleus (Papayannopoulos 2018). More precisely, MPO converts hydrogen peroxide to hypochlorous acid, activating elastase, which in turn degrades the cytoskeleton and dismantles the nuclear membrane, allowing for NET expulsion (Papayannopoulos *et al.* 2010, Klopf *et al.* 2021). Furthermore, elastase proteolytically processes histones to disrupt chromatin packaging (Papayannopoulos 2018). Subsequently, MPO binds chromatin and synergizes with elastase in decondensing chromatin (Papayannopoulos *et al.* 2010). A study using human neutrophils showed 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). Interestinly, elastase, an essential player in the process of NETosis, 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).

4.3.2 Relation of inflammation to laminitis

- Inflammation in SIRS-associated laminitis

Different models have been used to investigate the various mechanisms causing laminitis. The systemic and the local inflammatory responses and the release of cytokines and enzymes such as MPO or elastase have been described in laminitis models such as the CHO model and the BWE model (Johnson *et al.* 1998, van Eps and Pollitt 2006, Riggs *et al*. 2007, de la Rebière de Pouyade *et al.* 2010), both mimicking sepsis-related diseases like enterocolitis or septic metritis which are common causes of laminitis (Eades 2017b). Overall, significant inflammatory events occur systemically and in the lamellar tissue after BWE administration, with rapid increases of lung and liver proinflammatory cytokines (IL-6, IL-8 and TNF-α) occurring during the developmental period (Stewart *et al.* 2009).

Systemic neutrophil activation is encountered in equine laminitis, as demonstrated by the up-regulation of circulatory cytokine expression and neutropenia, the dynamic changes in blood neutrophil phenotype, the formation of neutrophil-platelet aggregates and the infiltration of inflammatory cells in lamellar tissue and skin (Black *et al.* 2006, Hurley *et al*. 2006, Loftus *et al.* 2007). A study by Riggs *et al.* (2007) confirmed neutrophil activation, using MPO as a marker, in the blood, the skin and lamellar tissue in the first hours after BWE administration. MPO concentrations were analysed using an enzyme-linked

immunosorbent assay (ELISA) test and a specific immune extraction followed by enzymatic detection (SIEFED) method was used to analyse its activity (Franck *et al.* 2006). Furthermore, inflammation localised in the hoof lamellae has been confirmed using elastase as a marker in a BWE model. Elastase is a serine protease released by degranulating neutrophils and its concentration was increased in plasma, skin and laminar tissue after BWE administration (de la Rebière de Pouyade *et al.* 2010). Therefore, it was concluded that neutrophil activation could be an early step in the pathogenesis of laminitis.

- Inflammation in endocrinopathic laminitis

Although inflammation, including leukocyte migration and activation, has been extensively investigated in models mimicking sepsis and SIRS (Leise 2018), horses also develop laminitis related to endocrine disturbances like hyperinsulinemia encountered in PPID and EMS. Less is known about the inflammatory component in these cases (McGowan and Patterson-Kane 2017).

The human metabolic syndrome is associated with multiple factors like insulin resistance, hypertension, dyslipidemia and abdominal obesity (Mottillo *et al.* 2010). Similarly, EMS is characterized by regional adiposity, hyperinsulinemia, insulin resistance, hypertriglyceridemia, and recurrent laminitis (Geor and Frank 2009, Frank *et al.* 2010). In human medicine, hyperinsulinemia has been associated with a chronic pro-inflammatory state (Geor and Frank 2009). The increased presence of adipose tissue amplifies the secretion of proinflammatory adipokines that decrease insulin sensitivity, induce oxidative stress and impair microvascular function (Geor and Frank 2009). However, inflammation was thought to be limited with only moderate neutrophil infiltration in hoof lamellae of horses with laminitis induced using a hyperinsulinemia model (McGowan and Patterson-Kane 2017). Some degree of neutrophil infiltration in laminar tissue was confirmed in hyperinsulinemic-induced laminitis by immunohistochemical staining for calprotectin, a protein complex in the cytoplasm of neutrophils and on the membrane of monocytes (de Laat *et al.* 2011). Furthermore, increased serum concentrations of TNF-α have been documented in ponies with a history of pasture-associated laminitis (Treiber *et al.* 2009), and increased expression of IL-1β and TNF-α has been associated with the development of insulin resistance in obsese horses (Vick *et al.* 2007). In contrast, expression of IL-1, IL-6 and TNF-α in adipose tissue from insulin-resistant mares was similar to that from insulin-sensitive mares (Burns *et al.* 2010). Additionally, ponies that were overfed carbohydrates (provoking hyperinsulinemia) did not show general up-regulation of lamellar pro-inflammatory cytokine gene expression or leucocyte infiltration (Burns *et al.* 2015). However, this same study reported increased gene expression of cyclooxygenase (COX)-2 and COX-2 signalling has been linked with insulin-like growth factor-1 (IGF-1) stimulation (Tian *et al.* 2012). Taking the above-mentioned information into account, the implication of inflammation in endocrinopathic laminitis warrants further investigation.

5. CURRENT TREATMENT OPTIONS FOR LAMINITIS

The treatment of laminitis is complex and challenging and is performed at multiple levels. The objectives of treatment are controlling the inflammation, alleviating the pain, preventing further alterations in the orientation of P3 within the hoof capsule and finally restoring a more normal relationship between P3 and the hoof capsule. Despite the array of available therapies, the treatment of refractory acute and chronic laminitis remains very challenging.

5.1 Medical treatment and cryotherapy

5.1.1 Controlling the inflammation

Inflammation is a significant factor in the pathophysiology of laminitis. Consequently, controlling inflammation is essential for managing the disease effectively. Various therapeutic options are available:

- Non-steroidal anti-inflammatory medication

Nonsteroidal anti-inflammatory drugs (NSAIDs), like phenylbutazone and flunixin meglumine, are COX inhibitors which have anti-inflammatory, analgesic, and antipyretic properties. There are mainly two forms of the COX enzyme: COX-1 and COX-2. While the COX-1 pathway helps to maintain mucosal perfusion and acid resistance in the gastrointestinal tract, kidney perfusion and platelet function, COX-2 is primarily induced and found at sites of inflammation. Therefore, NSAIDs with greater selectivity for COX-2 (such as firocoxib and meloxicam) are potentially associated with fewer side effects and, therefore, their use is preferred in chronic laminitis cases.

Beyond their anti-inflammatory effects, NSAIDs also have potent analgesic properties. It is suggested that NSAIDs alleviate pain in laminitis cases not only by controlling lamellar inflammation but also by inhibiting the central sensory neurons (Driessen *et al.* 2010). Experimental evidence suggests that inhibition of both COX-1 and COX-2 yields superior analgesia than inhibition of either alone (Martinez *et al.* 2010). This helps to explain the anecdotally superior analgesic effect of nonselective NSAIDs like phenylbutazone for laminitis pain in horses, a phenomenon that is widely recognised in clinical practice (Hopster and van Eps 2019).

- Acepromazine

Acepromazine (ACP), a phenothiazine derivative, is a frequently used tranquillizer in horses. It is commonly used in the treatment of laminitis (Stashak 1987). Besides its debated vasodilatory effects on the foot (Leise *et al.* 2007, Medina-Torres *et al.* 2016), ACP also possesses antioxidant and anti-inflammatory properties. Indeed, *in vivo* administration of acepromazine to horses has been shown to decrease the ROS production in subsequently isolated neutrophils (Péters *et al.* 2009). Furthermore, an *ex vivo* study demonstrated the modulating effects of ACP on the production of ROS produced by stimulated neutrophils (Sandersen *et al.* 2011).

- Low molecular weight heparin

In addition to its anti-thromboembolic properties (Hirsh *et al.* 2001), low molecular weight heparin (LMWH) also exhibits anti-inflammatory properties as demonstrated in an *in vitro* study where LMWH inhibited MPO-associated damage of endothelial cells (de la Rebière *et al.* 2008). This study proposed that heparins could be able to block the active site of the MPO enzyme or modify the structure of the enzyme enough to render it inactive. Interestingly there were differences in MPO uptake between arterial and venous endothelial cells that were attributed to their different distribution pattern of glycosaminoglycans. It has indeed been shown that the endothelium-MPO interactions were dependent on the glycosaminoglycans of the glycocalyx (Daphna *et al.* 1998, Baldus *et al.* 2001). Other studies have reported that heparins had a protective effect against the activation of neutrophils by decreasing the superoxide anion production and MPO release (Leculier *et al.* 1993). In horses, evidence shows that the prevalence and severity of laminitis as a postoperative complication of colic surgery were both reduced in patients who received LMWH (de la Rebière de Pouyade *et al.* 2009).

5.1.2 Pain management

Laminitis, depending on its severity, is an immensely painful disease. Therefore, the comfort of the patient must be closely monitored and pain management is a vital part of the disease management. However, it is important to recognize that pain also serves a protective function. Hence, while ensuring patient comfort through adequate analgesia is essential, it must be accompanied by appropriate confinement and restriction of ambulatory activity, considering the compromised integrity of the hoof lamellae.

Inflammation is likely the primary origin of disease-related pain (Orsini *et al.* 2009, Driessen and Zarucco 2017). Nevertheless, conventional NSAID therapy often fails to sufficiently relieve pain, prompting the preference for multimodal analgesia. This approach involves the use of different types of analgesic drugs with different mechanisms and sites of action in the nervous system to achieve superior

analgesia with fewer side effects. Common therapeutic options include morphine, ketamine, lidocaine constant rate infusions and gabapentin.

- Opioids

Opioids are potent and effective pain relievers, typically considered as an adjunctive therapeutic to NSAIDs, especially in severely painful cases. There are two main opioid receptors relevant for the analgesic effects: μ and κ. Morphine, a full μ-receptor agonist, demonstrates analgesic efficacy in horses based on cutaneous and visceral pain models (Kalpravidh *et al.* 1984). Its onset is slow, typically a few minutes, and the duration of action is dose-dependent but usually 3–4 h. Butorphanol is a κ-agonist and μ-antagonist, and it is commonly used in equine medicine. It has a short duration of action of 60–90 min and may have fewer negative effects on the intestinal tract, the cardiopulmonary system and behavior in horses (Kalpravidh *et al.* 1984, Roger *et al.* 1994).

- Systemic lidocaine

In addition to its well-studied local anaesthetic effects in the peripheral and central nervous system, lidocaine also exhibits analgesic properties through inhibition of spinal and supraspinal nociception when administered systemically and, therefore, is used as an adjunctive analgesic in multimodal pain therapy (Ness 2000). Experimental studies have found intravenous lidocaine to suppress the development of peripheral hyperalgesia (increased sensitivity to pain) and allodynia (increased sensitivity to nonpainful stimuli) (Ness 2000). Moreover, it has shown efficacy in managing chronic pain characterized by hyperalgesia and/or allodynia (Gormsen *et al.* 2009). In horses, lidocaine infusions have been shown to produce moderate analgesia in somatic pain models (Robertson *et al.* 2005). While the requirement for intravenous infusion renders lidocaine unsuitable for long-term administration in chronic laminitis cases, it serves as a valuable adjunctive analgesia in horses with acute pain from an active laminitis episode (Hopster and van Eps 2019).

Lidocaine is also known for having anti-inflammatory properties although the exact mode of action remains to be clarified. In humans, lidocaine reduces neutrophil adhesion, inhibits the release of superoxide anions, inhibits prostaglandin biosynthesis and release and blocks the release of inflammatory mediators in *in vitro* and *in vivo* studies (Castro *et al.* 2023). Another study showed that local anesthetics exhibit modest free radical scavenging activity in aqueous environments, with lidocaine demonstrating the highest activity. However, in lipophilic environments, such as cellular membranes, myelin sheets, and adipose tissue, their antioxidant activity appeared to be negligible (Kavcic *et al.* 2023). A study using murine neutrophils discovered that lidocaine inhibits neutrophil adhesion and migration through influencing the pivotal function of neutrophil sodium channels (Poffers

et al. 2018). In contrast, an *in vitro* experiment using equine neutrophils showed that lidocaine did not inhibit neutrophil adhesion and migration at therapeutic concentrations (Cook *et al.* 2009).

- Ketamine

Ketamine, a dissociative anaesthetic, induces analgesia at subanaesthetic doses mainly through N-methyl-D-aspartate (NMDA) receptor antagonism (Craven 2007). Ketamine has been shown to regulate inflammatory responses and reduce hyperalgesia, and its administration results in almost complete remission of symptoms of refractory neuropathic pain in human patients (Craven 2007). In an orthopaedic pain model in otherwise healthy experimental horses, responses to ketamine varied from no analgesia to marked improvement of comfort within 6–12h after starting the drug infusion (Lankveld *et al.* 2006).

- Gabapentin

Gabapentin is used in human medicine as analgesia for neuropathic and chronic pain (Harding *et al.* 2005, Arendt-Nielsen *et al.* 2007). Gabapentin may be suitable for long-term oral dosing in horses with chronic laminitis. As gabapentin has low bioavailability in horses (16%), it must be used at high doses orally (at least 5–20 mg/kg bwt 2–3 times per day) (Terry *et al.* 2010).

5.1.3 Cryotherapy

Cryotherapy has been widely recognized for its preventive (van Eps and Pollitt 2009, van Eps *et al.* 2012, Kullmann *et al.* 2014) and therapeutic use (van Eps *et al.* 2014) in equine laminitis management. Its efficacy may stem from reducing lamellar inflammation, lamellar oxygen and nutrient requirements and inhibiting destructive enzymes and oxidative reactions (Divers 2017). Therapeutic hypothermia may also provide analgesia through a combination of its profound anti-inflammatory effects and its direct effects on nerve conduction (van Eps 2010). Current evidence supports recommendations of maintaining hoof temperatures below 10°C for the duration of therapy. Adequate methods include immersing the entire hoof and a portion of the distal limb in ice and water or utilizing a prototype dry sleeve application over the hoof and distal limb, in which a cooling medium is continuously recirculated (van Eps and Orsini 2016). Additionally, a sleeve-style ice boot, not including the hoof, also appears capable of cooling lamellar tissues to recommended levels (Burke *et al.* 2018).

5.1.4 Management of the inciting causes

As discussed earlier, laminitis can arise from various causes, with PPID, EMS, and endotoxemia being the most common ones. When managing a laminitis patient, it is important to investigate the underlying cause that triggered the episode. Once identified, addressing this inciting factor is essential for

controlling the disease progression and avoiding future relapse. Although the treatments for these specific inciting causes are beyond the scope of this thesis, it is crucial to acknowledge their importance in comprehensive case management.

5.2 Farriery and support and stabilization of the distal phalanx

In addition to pharmacological treatment options, several other important factors should be considered when addressing pain in laminitis cases. Providing a soft and deformable ground surface that supports the sole and frog (e.g. sand) can significantly alleviate pain in many acute and chronic cases. Propper trimming of overgrown or neglected feet is essential, focusing on concentrating the load under the foot. Trimming is done using radiographic guidance whenever possible (O'Grady and Parks 2008). Utilizing a rasp to bevel the foot dorsally shifts the breakover palmarly, reducing the forces on the lamellae created by the deep digital flexor tendon (DDFT) (O'Grady and Parks 2008). Furthermore, foot support can be provided using many methods or devices varying in complexity and expense, ranging from relatively simple homemade bandages or polyurethane supports to commercially available boots with deformable inserts (e.g. Soft Rides). Whatever the chosen method, encompassing all structures in the palmar/plantar section rather than solely the frog is important (O'Grady and Parks 2008).

In more chronic cases trimming aims to restore normal hoof function and shape including realignment of the distal phalanx and the hoof capsule in a dorsopalmar plane and realigning the ground surface of the hoof capsule with the solar margin of the distal phalanx (O'Grady and Parks 2008). Different shoe types, such as egg-bar, heart-bar, reverse, and wooden shoes, have shown varying degrees of success, highlighting the need for individualized approaches (O'Grady and Parks 2008).

5.3 Surgical management: deep digital flexor tendon tenotomy

The DDFT tenotomy aims to reduce the forces causing distopalmar (or distoplantar) rotation of the dorsodistal aspect of the P3, to reduce the pain associated with lamellar separation and to enable aggressive corrective trimming. By transecting the DDFT, the forces favouring lamellar separation are reduced. This procedure is typically reserved for severe refractory acute laminitis cases or chronic cases where conventional therapies have failed to address the rotational displacement of the distal phalanx. Radiographic evaluation and venograms aid in decision-making (Floyd 2007, Rucker 2007). The procedure can be performed either at the mid-metacarpus or at the level of the proximal interphalangeal joint, with the former being preferred due to simplicity, reduced complications, and avoidance of general anaesthesia (Waguespack 2017). However, performing the procedure at the proximal interphalangeal joint may be necessary for recurrent cases (Waguespack 2017).

6. EQUINE STEM CELLS AND IMMUNOMODULATION

Decades of research contributed to contemporary standards of care that include systemic and local therapies as well as mechanical hoof support. Despite this, consistent mechanisms to restore healthy tissue formation following a laminitic insult are lacking.

Although a plethora of treatment options exists for managing the laminitis patient, laminitis is still not always treated successfully. Therefore, continuous research endeavours seek innovative treatment modalities to enhance the efficacy of laminitis management. Among these advancements, regenerative medicine stands out as a promising approach. In addition to their well-known regenerative properties, mesenchymal stem cells (MSCs) exhibit anti-inflammatory properties, which have propelled their expanding therapeutic use (Saeedi *et al.* 2019, Regmi *et al.* 2019). MSCs are known to migrate to sites of tissue damage, where they engage in tissue remodelling, wound healing and immune response modulation (Jiang and Xu 2020). Rather than engraftment and differentiation into functional cells, the paracrine activity of MSCs appears to be the predominant mechanism by which they participate in tissue repair (Maumus *et al.* 2013, Keshtkar *et al.* 2018). Moreover, MSCs influence the immune system through anti-inflammatory properties by modulating the activity and function of macrophages and neutrophils, then reducing neutrophil-induced tissue damage (Jiang *et al.* 2016, Mittal *et al.* 2018). Recent studies indicate that MSCs achieve their anti-inflammatory and cytoprotective effects by modulating the redox environment and oxidative stress, offering potential antioxidant mechanisms for MSC therapies (Stavely and Nurgali 2020). In a mouse model of acute endotoxin-induced lung inflammation, MSCs reduced inflammation and inhibited the formation of NETs (Pedrazza *et al.* 2017). Human MSCs from bone marrow inhibited neutrophil apoptosis and ROS production in a cellular model (Raffaghello *et al.* 2008). Feline bone marrow-derived and apidpose-derived MSCs and their supernatant inhibited ROS production in cultured neutrophils (Mumaw *et al.* 2015). Equine MSCs showed a modulatory effect on neutrophil respiratory burst with less ROS production in co-culture with equine MSCs and a strong decrease in ROS production after incubaction with MSC supernatant (Espinosa *et al.* 2020). In addition, equine MSCs derived from skeletal muscle have been shown to inhibit the activity of free and NET-bound MPO *in vitro* (Franck *et al.* 2021). However, the specific exploration of the effect of MSCs on keratinocytes and hoof lamellae in horses remains scant.

Promising results were obtained in a study reporting the contribution of adipose tissue-derived MSCs in combination with platelet-rich plasma injected intravenously three times with one-month interval, to treat nine chronic laminitis cases (Angelone *et al.* 2017). Repeated venograms showed progressive amelioration of the vascularization in the foot and in addition the structure and function of the hoof improved. Another study reports 6 horses with bilateral laminitis treated with adipose-derived MSCs in

one limb and saline control in the opposite limb. MSC-treated limbs had improved hoof growth and vascular perfusion when compared with controls (Oliveira *et al.* 2021).

Objectives

The introduction has outlined the challenges in understanding and treating equine laminitis. Despite many years of intensive ongoing research on the pathophysiology of laminitis, several questions remain regarding the implication of inflammation, and more specifically of activated neutrophils and their degranulation products, in the disease process.

- Is inflammation, including neutrophils and their degranulation products, present in all the various types of laminitis?
- At which stage of the disease are neutrophils and their byproducts encountered?
- Are current laminitis models an accurate representation of naturally occurring laminitis?
- How do microscopic lesions observed in the initial stages of laminitis evolve in more severe naturally occurring laminitis not included in these induced models?
- Can laminitic donkeys be considered small laminitic horses histologically?
- Is MSC therapy a worthwhile perspective in the treatment of this debilitating condition?

We hypothesized that inflammation with neutrophil activation, evidenced by MPO and NET, would be a common pathway in the pathophysiology of laminitis regardless of the inciting cause and that the immunomodulatory properties of muscle-derived MSCs would provide them with the capacity to mitigate this neutrophil-mediated inflammatory response.

Therefore, the first objective of this PhD thesis was to demonstrate that inflammation, evidenced by the presence of activated neutrophils and their byproducts such as MPO and NET, was not only present in SIRS-related laminitis but also in endocrinopathic laminitis and clinical cases regardless of the etiology.

In addition, because laminitis models only focus on the early stage of the disease and include only ponies and horses, this thesis will explore the evolution of histological injuries in advanced clinical cases both in horses and donkeys.

Finally, if inflammation is confirmed as a common pathway in laminitis, the final part of this PhD thesis will aim to explore the therapeutic potential of muscle-derived MSCs as a novel treatment in modulating neutrophil-mediated inflammation in laminitis.

To comprehensively address these objectives, we have implemented a multifaceted approach, encompassing *in vitro* cellular models, *in vivo* animal studies, and clinical investigations.

Experimental section

Experimental section

Summary of the research results

This chapter provides a concise summary of the findings derived from our different studies conducted with the aim of addressing our research questions.

1. Neutrophil activation in models of equine laminitis

This section explores the significance of neutrophil activation across various equine laminitis models. Our first study seeks to elucidate, through an *in vitro* approach, the molecule responsible for inducing neutrophil activation in the BWE model, mimicking SIRS-related laminitis. In the second study we investigate the presence of neutrophil activation *in vivo* within the pEHC model for endocrinopathic laminitis.

Study 1 – Effects of juglone on neutrophil degranulation and myeloperoxidase activity related to equine laminitis

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Introduction – Literature has demonstrated the presence of neutrophil activation, evidenced by MPO and elastase, in lamellar tissue of horses with laminitis induced using the BWE model (Riggs *et al.* 2007, de la Rebière de Pouyade *et al. 2010)*. However, the active principle responsible for inducing laminitis in this model remains elusive. Juglone, a compound found in plants of the walnut tree family (*Juglans*), and present in the largest amounts in the *Juglans nigra*, used in the BWE model, was considered as an important toxic component involved in laminitis induction (Soderquist 1973, Uhlinger 1989, Ahmad and Suzuki 2019). The cytotoxic properties of naphthoquinones, like juglone, involve the induction of oxidative stress with redox cycling, cell membrane damage, apoptosis, and necrotic cell death (Ollinger and Brunmark 1991, Aithal *et al.* 2009). *In vitro* studies have shown juglone inhibiting the respiration rate of keratinocytes in conjunction with the production of intermediate radicals (Inbaraj and Chignell 2004). Therefore, juglone could be a trigger capable of inducing laminitis.
In the BWE model, Chiavaccini *et al.* (2011) demonstrated that oral administration of BWE induced an early leukocyte infiltration in the colonic mucosa of horses (McConnico *et al.* 2005). The changes in the colonic mucosa may allow the absorption of several molecules of intestinal origin, exacerbating

systemic inflammation and possibly leading to distant tissue injury such as laminitis. However, per os juglone administration inconsistently induced signs of laminitis and juglone was identified in the bark and in the nuts but not in the heartwood of the *Juglans nigra* tree (True and Lowe 1980, Minnick *et al.* 1987, Peroni 2017). Therefore, it was concluded that BWE was 'laminitogenic', but a doubt remains regarding the involvement of juglone. Interestingly, other studies showed that the topical application of juglone to the equine digit caused local skin irritation, and intravenous administration caused acute pulmonary edema (True and Lowe 1980). In the following *in vitro* study, we aimed to clarify if juglone could be responsible for inducing neutrophil activation, evidenced by the presence of MPO found in the BWE model.

Materials and methods – First, equine neutrophils were isolated and incubated with different concentrations of juglone, their viability was then assessed using trypan blue. Then, ROS production by phorbol myristate acetate (PMA)-activated neutrophils incubated with or without juglone was assessed using chemiluminescence and electron paramagnetic resonance spectroscopy. In addition, MPO activity was assessed using the classical enzymatic assay and the SIEFED assay. Finally docking of MPO on juglone was performed.

Results – Chemiluminescence and electron paramagnetic resonance techniques demonstrated that juglone was able to inhibit ROS and superoxide anion free radical formation in PMA-activated polymorphonuclear neutrophils (PMNs). Juglone, at 25 and 50 µM, significantly inhibited (>90%) MPO release on a cytochalasin B and formyl-methionylleucyl-phenylalanine (fMLP)-induced degranulation model. Moreover, it also reduced the peroxidase activity of MPO by interacting with the intermediate "π cation radical," as evidenced by the classical enzymatic assay and SIEFED assays. These results are confirmed by a docking study showing the perfect positioning of juglone in the MPO enzyme active site and its interaction with one of the amino acids (Arg- 239) of the MPO apoprotein.

Conclusion – These results indicate that, through the prism of the modulation of neutrophil activation, juglone is unlikely to be the trigger for equine laminitis. On the contrary, juglone seems to have a rather anti-inflammatory effect through its antioxidant properties than a pro-inflammatory effect on equine neutrophils and MPO. Additionally, to our knowledge, our results indicate, for the first time, that juglone can inhibit ROS production by PMA-stimulated neutrophils. Thus, the cause of laminitis induction in the BWE model remains unknown and further studies are needed to elucidate the exact mechanisms. Potential pathways include the presence of other toxic principles in the BWE, the development of enteritis with resorption of LPS of bacteria or other mechanisms responsible for the development of systemic inflammation, endothelial abnormalities and cytotoxicity.

Study 2 – Presence of myeloperoxidase in lamellar tissue of horses induced by an euglycemic hyperinsulinemic clamp

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Introduction – Neutrophil activation is recognized as a major factor in SIRS-associated laminitis. Less is known about the role of neutrophil activation in laminitis associated with metabolic disorders. Based on the association between inflammation and human metabolic disease and preliminary evidence of the possible presence of inflammation related to equine metabolic disease, we investigated the presence of neutrophil activation, evidenced by MPO, in the hoof lamellae of laminitic horses induced using the pEHC model, mimicking endocrinopathic laminitis.

Materials and methods – Three horses were subjected to 48h of pEHC-treatment and two healthy horses were used as controls. After euthanasia, lamellar tissue samples were collected and processed. Histological sections of lamellar tissue from all horses were immunohistochemically stained for MPO and counterstained with hematoxylin. The sections were examined for histopathological evidence of laminitis and myeloperoxidase.

Results – Histopathological changes that characterize insulin-induced laminitis, such as elongated and narrow SEL with tapered tips and acutely angled on the PEL, were observed in all pEHC-treated horses. Furthermore, they all showed an increased presence of MPO, especially in the dermal lamellae (Fig. 1). Only very mild staining occurred in control horses (Fig. 2). Interestingly, neutrophil infiltration remained very limited.

Conclusion – Inflammation with neutrophil MPO release may contribute to the pathophysiology of endocrinopathic laminitis. Further experiments are needed to confirm the origin of the MPO present in lamellar tissue and the possible toxic role that the enzyme could play.

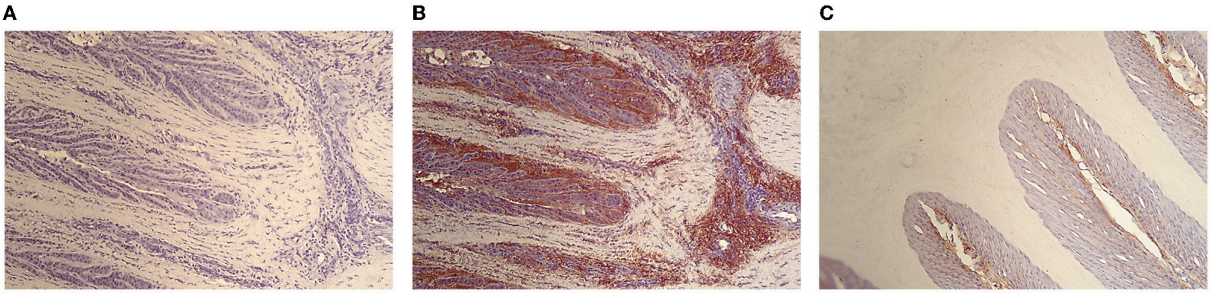

Figure 1 - Photomicrograph of the hoof lamellae of a pEHC-treated horse. Hematoxyllin staining without primary MPO antibody (A) and hematoxyllin staining with complete MPO immunohistochemical protocol (B, C) (x100).

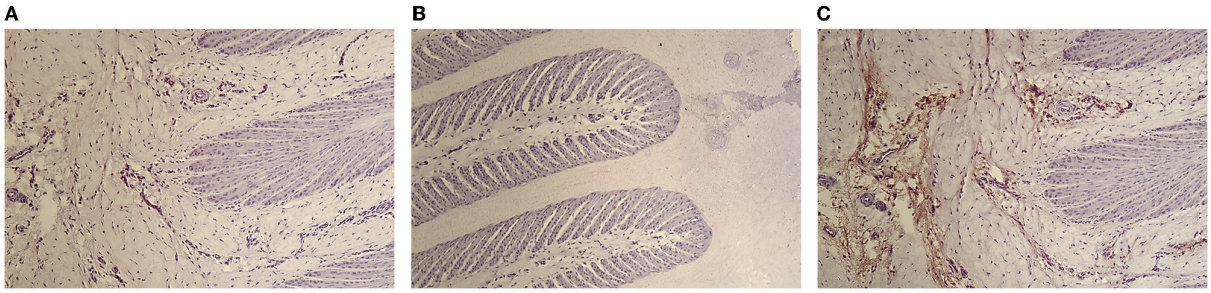

Figure 2 - Photomicrograph of the hoof lamellae of a control horse. Hematoxyllin staining without primary MPO antibody (A, B) and hematoxyllin staining with complete MPO immunohistochemical protocol (C) (x100).

2. Clinical studies

This section focusses on neutrophil activation and lamellar lesions encountered in patients with naturally occurring laminitis. It aims to validate the relevance of laminitis models in clinical contexts and investigate the evolution of lamellar lesions. In addition, in an equine hospital setting, not only horses are admitted for laminitis, donkeys also largely suffer from this pathology and are therefore included in the subsequent studies.

Study 3 – Neutrophil extracellular traps and active myeloperoxidase concentrate in lamellar tissue of equids with naturally occurring laminitis

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Introduction – The integration of the evidence for neutrophil activation in literature in SIRS-related laminitis combined with the evidence of our *in vivo* pEHC study has shown the implication of MPO-mediated inflammation in the pathophysiology of both SIRS-associated and endocrinopathic laminitis. However, information regarding neutrophil activation in naturally occurring laminitis remains scant. Furthermore, the presence of MPO was not accompanied by visible neutrophil infiltration in the *in vivo* pEHC model. One hypothesis to explain this discrepancy is that the observed MPO was associated with NET formation. This clinical study aimed to assess the presence of MPO and NET regardless of the etiology of the laminitis pathology both in horses and donkeys with naturally occurring laminitis.

Materials and methods – Samples of lamellar tissue of five horses and five donkeys presented with laminitis were collected along with eight control horses without laminitis. Lamellar tissue extracts were submitted to ELISA and SIEFED assays to confirm the presence and activity of both MPO and NET-bound MPO. Lamellar sections were also immunohistopathologically stained for MPO and NET.

Results – 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 partly originated from NET-bound MPO.

Immunohistochemical staining showed that MPO and NET labelling in control horses was minimal (Fig. 1), while labelling in laminitis cases was moderate to marked (Fig. 2), primarily in the epidermis and in inflammatory infiltrates containing neutrophils (Fig. 3).

Conclusion – This article constitutes the first 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.

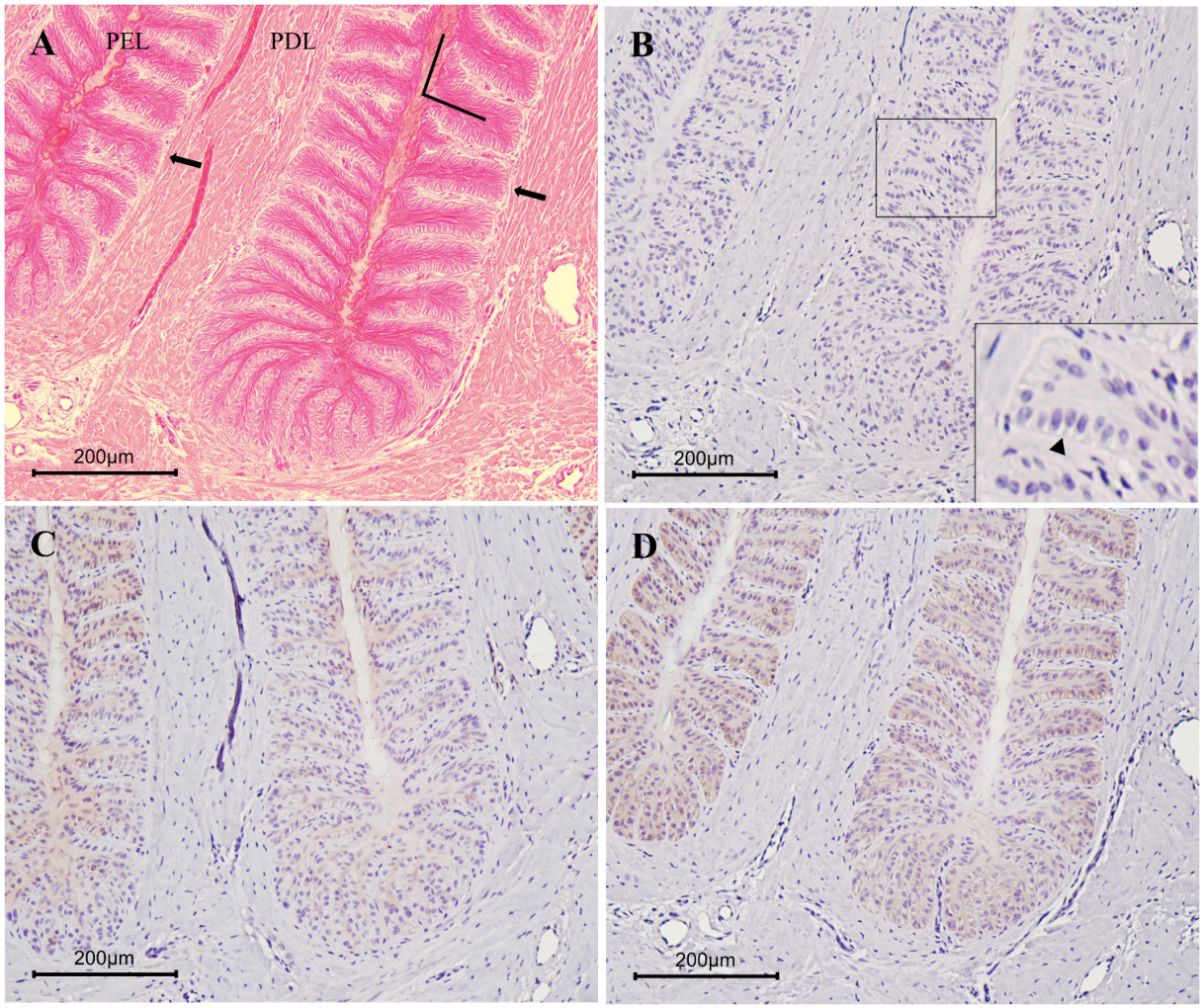

Figure 1 – Photomicrographs of a control horse stained with hematoxylin-eosin (HE) (A), negative control (B), MPO (C) and NET (D) immunohistochemical staining. The SEL and SDL are oriented perpendicularly on their primary axis (A, lines), have rounded tips (A, arrows) and the lamellar basal cells have 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.

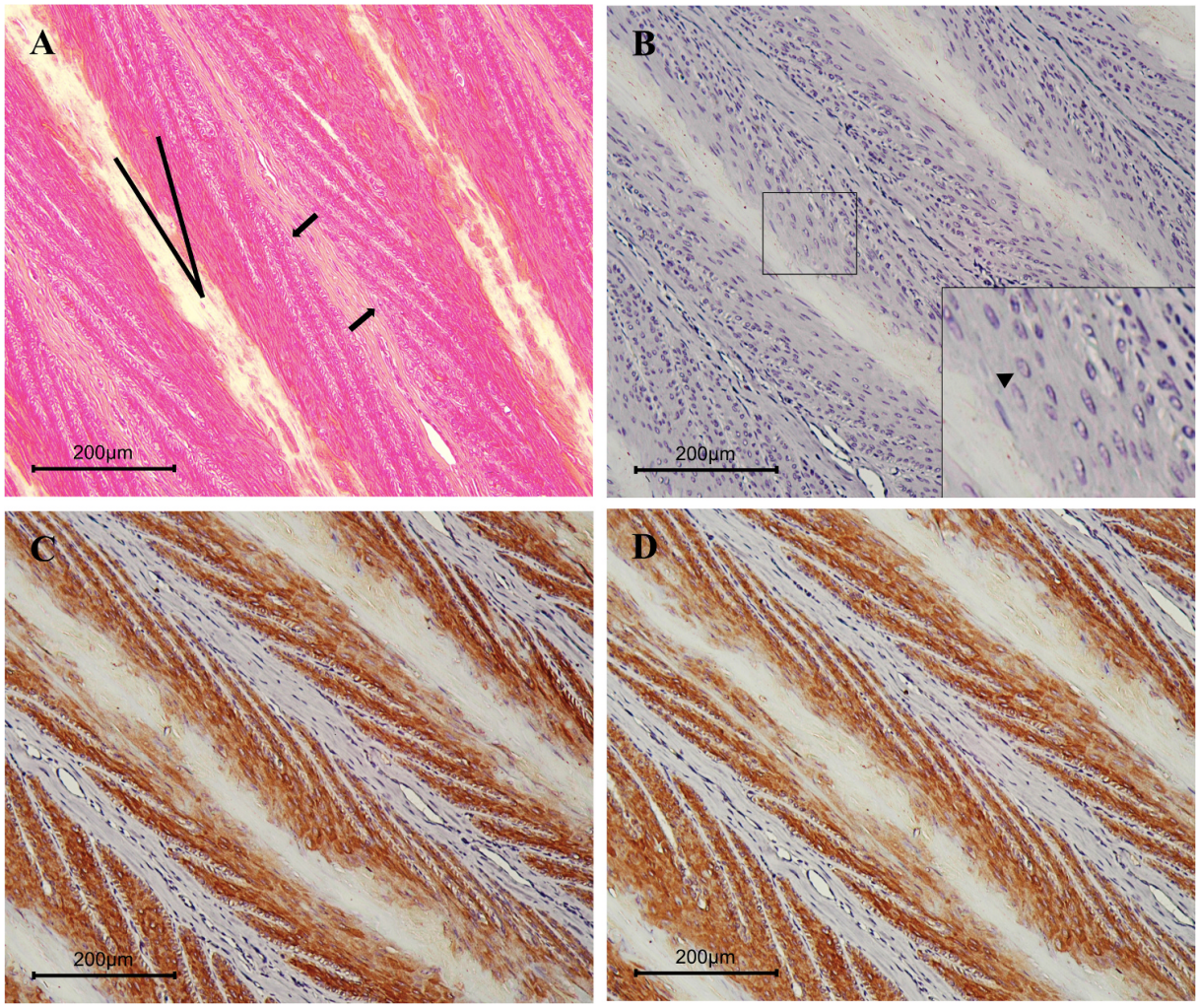

Figure 2 – Photomicrographs of a laminitic horse stained with HE (A), negative control (B), MPO (C) and NET (D) immunohistochemical staining. The SEL have tapered tips (A, arrows), appear elongated and narrow, and are acutely angled on primary epidermal lamellae (A, lines). The lamellar basal cells have round nuclei that are centrally located (B, arrowhead). Intense MPO and NET labelling is observed in the keratinocytes composing the SEL (C, D).

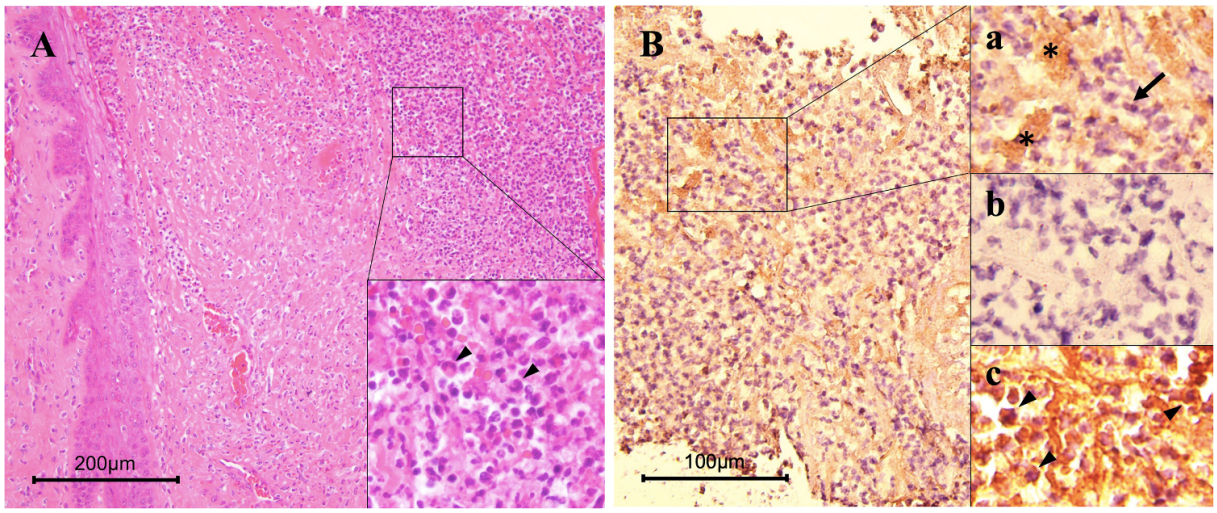

Figure 3 – Photomicrographs of a laminitic horse stained with HE (A) and NET (B) immunohistochemical staining. Fig. 3A shows severe neutrophil infiltration (arrowheads) in the dermal tissue. Fig. 3B shows NET labelling, visible as diffuse brown spots devoid of a nucleus and a cell wall, present around 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).

Study 4 – Macroscopic and microscopic histopathological characteristics of hoof lamellae of horses and donkeys with severe naturally occurring laminitis

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Under preparation for publication in Veterinary Pathology, 2024.

Introduction – The mechanisms involved in the pathophysiology of laminitis have been investigated using histopathology in the developmental phase of the disease in equine models. However, information regarding the evolution of these lesions in naturally occurring laminitis is lacking both in horses and in donkeys. This study aimed to describe macroscopic and microscopic lesions in equids with naturally occurring laminitis.

Methods – Lamellar samples were collected in 9 horses and 5 donkeys euthanized due to severe laminitis and in 8 control horses and one control donkey. Samples were processed for histology and stained with haematoxylin-eosin (HE), periodic acid-Schiff (PAS) and Masson's trichrome (MT) before light microscopy observation and analysis with digital pathology software (QuPath).

Results – Macroscopic analysis of laminitic samples revealed an increased thickness of the lamellar area, hemorrhage, abnormal lamellar architecture, lamellar separation, exudate, focal abscess formation and a soft consistency on palpation (Fig. 1). Histomorphometric analysis showed significant thickening of the area containing the lamellar tissue ($p<0.001$), an increased number of epidermal bridges ($p<0.001$), increased total primary epidermal lamellar lengths ($p=0.02$) and narrower primary dermal lamellae ($p=0.004$). The proportion of keratin increased while the proportion of dermal tissue decreased with worsening pathology (Fig. 2). The descriptive analysis showed severely altered lamellar architecture, varying degrees of hyperkeratosis progressively developing into severe dyskeratosis, cellular degeneration and necrosis, basement membrane detachment, oedema and inflammatory cell infiltration. None of these abnormalities were seen in controls.

Conclusions – This study constitutes the first to provide a detailed description of macroscopic and microscopic lesions in horses and donkeys with naturally occurring laminitis, thereby providing additional insights in the progression of the disease.

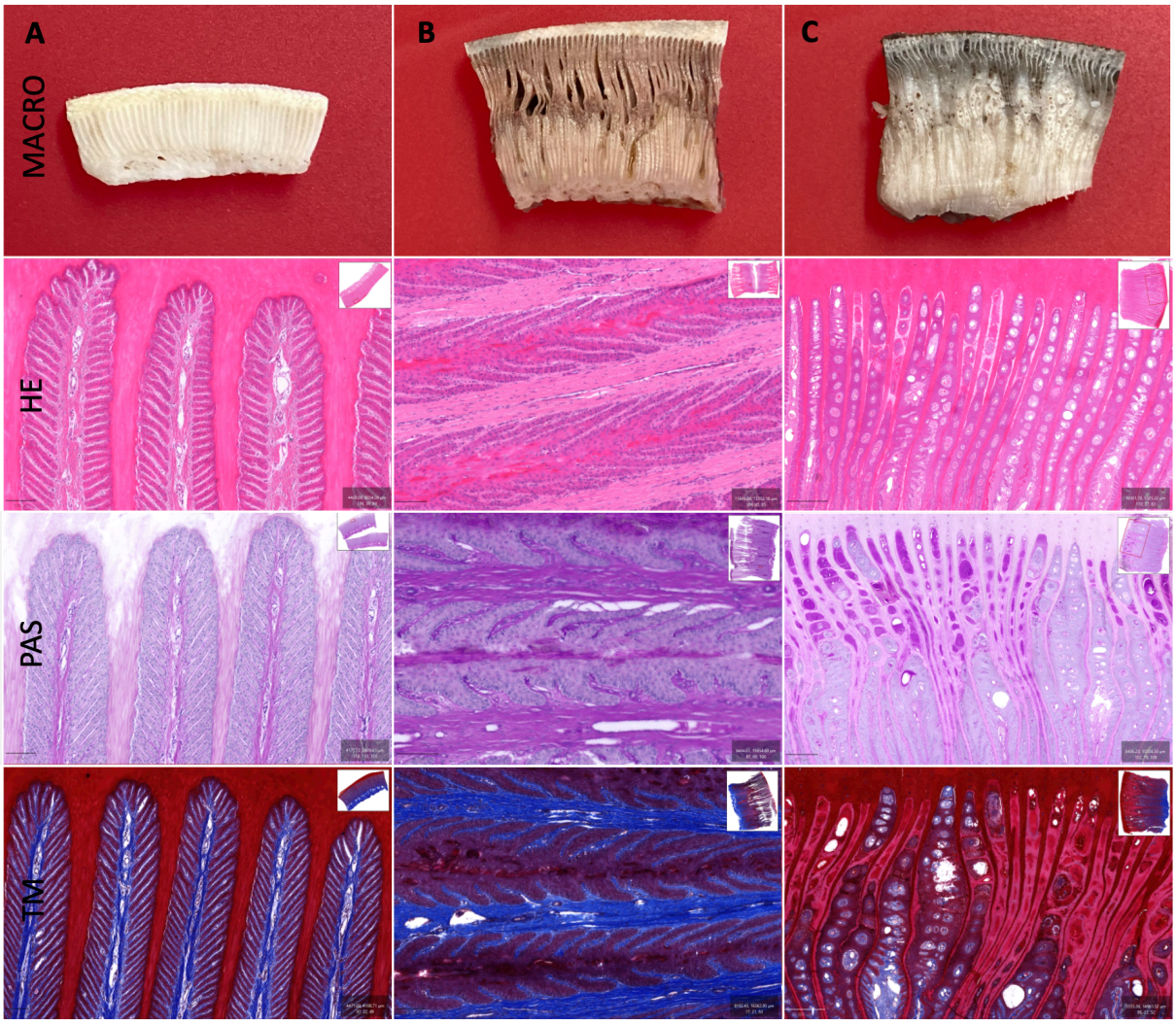

Figure 1 – Macroscopic pictures and photomicrographs of lamellar tissue with HE, PAS and MT staining of a control horse (A), laminitic donkey (B) and laminitic horse (C) showing increasing severity of lamellar pathology (A<B<C). Sample A shows normal macroscopic and microscopic anatomy. Sample B depicts significant macroscopic lamellar separation that is observed as well microscopically. Sample C indicates abnormal lamellar architecture with severe hyperkeratosis visible on the macroscopic and microscopic images.

Dermal tissue	39.15 %
Epithelium	35.50 %
Keratin	19.80 %

Dermal tissue	23.71 %
Epithelium	45.32 %
Keratin	14.72 %

Dermal tissue	18.80 %
Epithelium	17.86 %
Keratin	60.91 %

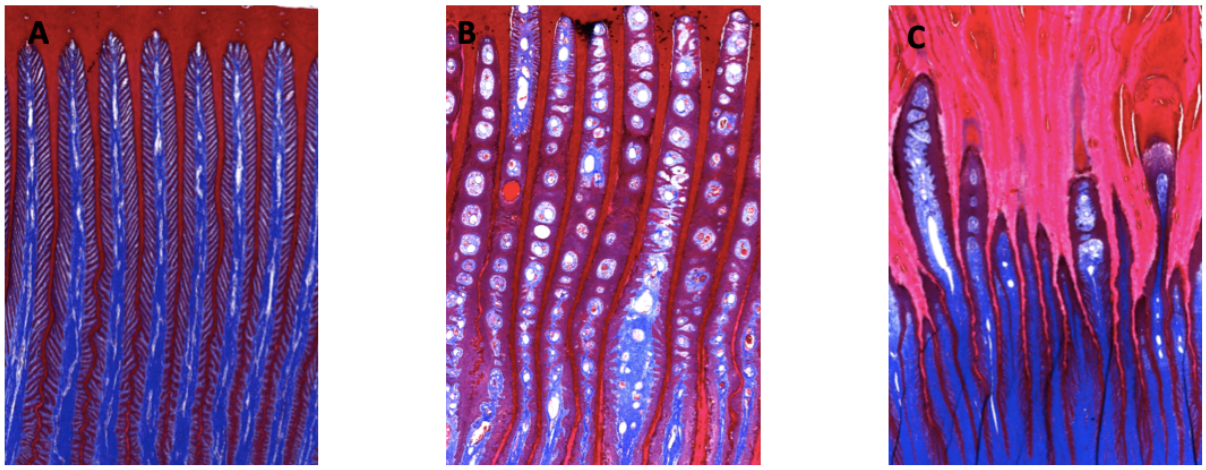

Figure 2 – Photomicrographs with MT stain showing the evolution of the proportions of dermal tissue and keratin content in control (A), moderate (B), and severe (C) laminitis cases. The proportion of keratine markedly increases while the proportion of dermal tissue decreases with the severity of the patology. A shows normal architecture and content, B presents moderate abnormalities including multiple epidermal bridges, while C depicts severe dyskeratosis.

4. Treatment perspectives

Drawing upon the insights garnered from our *in vivo* and clinical studies, in this section we aimed to explore whether mdMSCs could provide an innovative treatment approach for the laminitis patient.

Study 5 – Revealing the therapeutic potential of muscle-derived mesenchymal stem/stromal cells: an *in vitro* model for equine laminitis based on activated neutrophils, anoxia-reoxygenation and myeloperoxidase

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Introduction – NETs are extracellular strands of decondensed DNA in complex with histones and neutrophil granule proteins such as elastase and MPO (Brinkmann *et al.* 2004). NETs can induce mitochondrial dysfunction in cardiomyocytes associated with an increase of ROS release (He *et al.* 2023). Given the observed neutrophil activation in early laminitis stages and the identification of NETs in severe clinical cases, this study aimed to investigate the effect of mdMSCs on neutrophil activation and keratinocyte metabolism through an *in vitro* model for laminitis. MSCs are renowned for their regenerative and immunomodulatory properties, making them a promising candidate for a novel therapeutic approach to the laminitis patient. MSCs are indeed able to reduce inflammation and inhibit NET formation (Pedrazza *et al.* 2017), inhibit the detrimental effect of NETs on mitochondria (Magana-Guerrero *et al.* 2017) and decrease of ROS production by activated neutrophils with significant inhibition of the NET-bound MPO activity (Franck *et al.* 2021).

Materials and methods – The *in vitro* model was composed of keratinocytes (HaCat) exposed to anoxia-reoxygenation in conjunction with activated neutrophil supernatant (ANS). First, MPO and NET-bound MPO activity were measured in the supernatant using the SIEFED assay. Then, the metabolism of keratinocytes was evaluated using the MTS assay in the presence or absence of mdMSCs. Finally, after 2h incubation of HaCat with MPO and cell washing, the presence of MPO in HaCat cells was detected by immunohistochemistry (IHC) and by the measurement of the *in situ* peroxidase activity in the presence or absence of mdMSCs.

Results – In HaCat treated with ANS and submitted to anoxia then reoxygenation, a significant decrease of cell metabolism was noted, indicative of cellular stress (Fig. 1). However, adding mdMSCs during reoxygenation demonstrated a protective effect, restoring the HaCat metabolic activity (Fig. 2). Moreover, we showed that HaCat captured active MPO from ANS or purified equine MPO and that their co-culture with mdMSCs allowed to decrease the activity of the MPO captured by the cells.

Conclusions – These findings underscore the potential of mdMSCs in mitigating inflammation and restoring keratinocyte metabolism, offering insights for future cell therapy research in laminitis treatment.

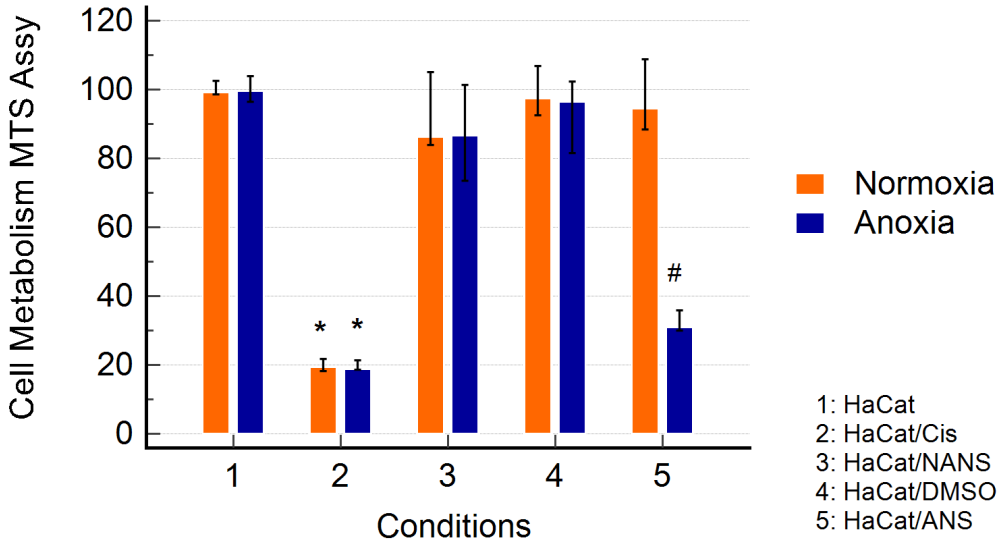

Figure 1 – Effect of non-activated neutrophil supernatant (NANS) and CB/fMLP activated one (ANS) on the metabolism of HaCat using MTS assay. HaCat were cultured for 48 h in normoxia or anoxia (5% CO2) and then incubated for 24 h with fresh medium under normoxia followed by measurement of metabolic activity after 4 h (MTS assay). Ctrl DMSO: control with DMSO used for the solubilization of CB and fMLP. Results are presented as medians with 95% confidence intervals of 12 experiments and are expressed as relative values (%) compared to control groups, which were standardized to 100%. (* $p<0.01$ vs HaCat alone; # $p<0.01$ vs normoxia).

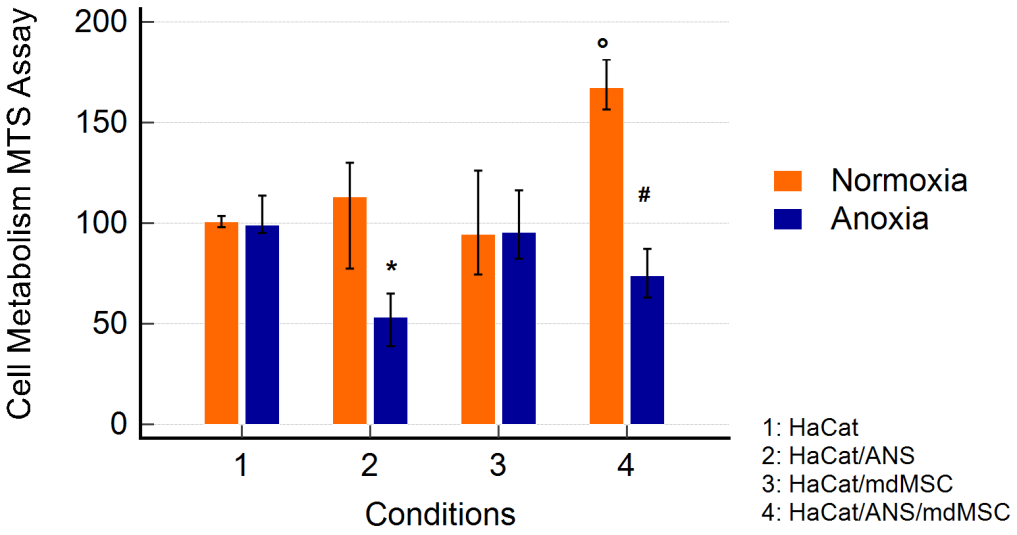

Figure 2 – Effect of mdMSCs on HaCat cultured in normoxia or anoxia for 48 h in the presence of activated neutrophil supernatant (ANS). MdMSCs were added at the reoxygenation period of 24h just after 48 h of normoxia or anoxia. After medium removal the MTS solution was added for the measurement of metabolic activity. Results consider the subtraction of the metabolic response due to mdMSCs. Results are presented as medians with 95% confidence intervals of 15 experiments and expressed in relative % vs condition 1 set as 100 % response. (* $p<0.01$ vs normoxia. ° $p<0.01$ vs other conditions; # $p<0,01$ between condition 2 and 4 in anoxia).

Experimental section

Systematic presentation of the research results

Experimental section

Study 1

Effects of juglone on neutrophil degranulation and myeloperoxidase activity related to equine laminitis

Frontiers in Veterinary Science, July 2021, vol. 8, 677675

Ange Mouithys-Mickalad, Nazaré Storms, Thierry Franck, Justine Ceusters, Geoffroy de la Rebière de Pouyade, Ginette Deby-Dupont and Didier Serteyn

Abstract

Experimental laminitis, characterized by a failure of the dermal–epidermal interface of the foot, can be induced in horses by the oral administration of a black walnut extract (BWE). In the early phase of this severe and painful disease, an activation of neutrophil occurs, with the release of myeloperoxidase (MPO), a pro-oxidant enzyme of neutrophils, in plasma, skin, and laminar tissue. Juglone, a naphthoquinone derivative endowed with redox properties, is found in walnuts and has been incriminated in this neutrophil activation. We report for the first time the inhibitory activity of juglone on the degranulation of neutrophils induced by cytochalasin B and formyl-methionylleucyl-phenylalanine as monitored by the MPO release (>90% inhibition for 25 and 50µM). Moreover, it also acts on the peroxidase activity of MPO by interacting with the intermediate "π cation radical," as evidenced by the classical and specific immunological extraction followed by enzymatic detection (SIEFED) assays. These results are confirmed by a docking study showing the perfect positioning of juglone in the MPO enzyme active site and its interaction with one of the amino acids (Arg- 239) of MPO apoprotein. By chemiluminescence and electron paramagnetic resonance techniques, we demonstrated that juglone inhibited reactive oxygen species (ROS) and superoxide anion free radical produced from phorbol myristate acetate (PMA)-activated polymorphonuclear neutrophils (PMNs). These results indicate that juglone is not the trigger for equine laminitis, at least if we focus on the modulation of neutrophil activation.

Keywords: neutrophil degranulation, myeloperoxidase, juglone, reactive oxygen species, electron paramagnetic resonance spectroscopy

Introduction

Laminitis is a common and very severe disease of the foot of horses, characterized by a failure of the dermal–epidermal interface that induces dramatic pain and, in severe cases, requires euthanasia (Menzies-Gow *et al.* 2010, Katz and Bailey 2012). Laminitis results from a combination of factors most often generated during an excessive and systemic inflammatory reaction, such as decreased blood flow (ischemia) and inflammation in the foot involving endothelial/vascular dysfunctions, degradation of the extracellular matrix, and metabolic abnormalities in keratinocytes (Peroni *et al.* 2005, Loftus *et al.* 2006, Serteyn 2014). The activation of neutrophils (polymorphonuclear neutrophils, PMNs) plays an important role in the initial stages of laminitis (Lunn and Hurley 2009, Leise 2018). Two models are usually used in research to induce experimental laminitis: an oral administration of black walnut extract (BWE) simulating sepsis or a hyperinsulinemia model to mimic endocrinopathic laminitis (Minnick *et al.* 1987, Thomsen *et al.* 2000, Hurley *et al.* 2006, Belknap 2010, Peroni 2017). Our group previously demonstrated neutrophil activation in a BWE model of laminitis: myeloperoxidase (MPO), a pro-oxidant enzyme of neutrophils, and neutrophil elastase were present in plasma, skin, and laminar tissue in correlation with the emigration of white blood cells from the vasculature, supporting a role for neutrophil activation and systemic inflammation in the early phase of the disease (Riggs *et al.* 2007, de la Rebière de Pouyade *et al.* 2010). More recently, similar pictures were obtained with the hyperinsulinemia model (unpublished work).

Several teams have considered that juglone, present in some BWE, could be the trigger capable of inducing laminitis, but other groups did not find this compound in active BWE (True and Lowe 1980, Minnick *et al.* 1987, Peroni 2017). In order to resolve this controversy, this work was designed to study the effects of juglone on the activation of neutrophils involved in the pathophysiology of equine laminitis regardless of the mechanism responsible for the onset of the disease.

Juglone (5-hydroxy-1,4-naphthoquinone), a phenolic compound found in walnuts, has been extensively studied not only for its redox properties but also for its antimicrobial properties and its implication in anticancer activity through the signaling pathway and reactive oxygen species (ROS) production (Clark *et al.* 1990:199, Wulf *et al.* 2002, Jin 2010, Tan *et al.* 2012, Zakavi *et al.* 2013, Ahmad and Suzuki 2019, Wellenstein *et al.* 2019). On the basis of *in vitro* studies, juglone has been described to inhibit the respiration rate on a cellular model (keratinocytes) in conjunction with the formation of semiquinone intermediate radicals (Inbaraj and Chignell 2004). It is well-known that the generation of ROS and the modulation of redox signaling are properties of quinones (Klotz *et al.* 2014, Ahmad and Suzuki 2019). The cytotoxic properties of naphthoquinones, like juglone, involve the induction of oxidative stress with redox cycling, cell membrane damage, apoptosis, and necrotic cell death (Ollinger and Brunmark 1991, Aithal *et al.* 2009). However, other authors like Dehorty and his group reported that redox cycling did not play a role in the mechanism of the toxicity of naphthoquinone derivatives, including juglone

(Doherty *et al.* 1987), raising the question of its action depending on the type of cells and pathological situation. An increasing body of interest has been focused on the mechanism of action of neutrophil degranulation, but little is known regarding the effect of juglone on the activity of MPO, a neutrophil oxidant enzyme also considered as a marker of inflammation (Hampton *et al.* 1998, Winterbourn 2002).

This work investigated the direct effect of juglone on the equine neutrophils activated by the cytochalasin B (CB)/formylmethionyl- leucyl-phenylalanine (fMLP) system or phorbol 12- myristate 13-acetate (PMA) in order to verify whether or not juglone can trigger ROS production and neutrophil degranulation and act on the release and activity of MPO. We also compared the effects of juglone on equine MPO activity as well as on human MPO activity before performing docking assays on human MPO.

Material and methods

Reagents

All the reagents were of analytical grade. Dimethyl sulfoxide (DMSO), ethanol, CaCl2, KCl, NaCl, hydrogen peroxide (H2O2) 30%, and Tween-20 were all supplied by Merck (VWRI, Leuven, Belgium). Sodium nitrite (NaNO2), bovine serum albumin (BSA), PMA, gallic acid, CB, and fMLP were all purchased from Sigma-Aldrich (Bornem, Belgium). Percoll was from GE Healthcare (VWR, Leuven, Belgium). 8-Amino-5-chloro- 7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H)dione (L-012) was from Wako Chemicals (Neuss, Germany) and Trypan blue was from ICN Biomedicals, Inc. (Aurora, OH, USA). MTS Cell Titer 96R was purchased from Promega REF G5430 (Madison, WI, USA), and human MPO (200 U/mg protein) was obtained from Calbiochem Millipore (Billerica, MA, USA). Equine MPO was purified according to the procedure previously reported (Franck *et al.* 2005). Juglone was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Amplex Red was from Molecular Probes Inc. (Leiden, Netherlands). 5,5′ -Dimethyl-pyrroline-Noxide (DMPO) was obtained from Enzo Life Sciences (Brussels, Belgium). All aqueous solutions were prepared with water previously purified in a Milli-Q water system (Millipore, Bedford, MA, USA).

Preparation of Juglone Solutions

The stock solution of juglone was prepared at a concentration of 10mM by dissolving (1.74 mg/ml) in DMSO. Appropriate dilutions were then performed to obtain 1, 0.1, 0.01mM, etc., by using DMSO as a solvent and not as a buffer in order to avoid any precipitate at the highest juglone concentrations. To obtain the final concentrations in the reaction vials, each dilution was diluted 100 times with buffer solution. DMSO was thus at 1% in all the samples at the final dilution.

Equine Neutrophil Isolation and Juglone Toxicity Assay on Neutrophils

Equine neutrophils were isolated from whole blood using Ethylenediamine tetraacetic acid EDTA disodium salt (1.6 mg/ml) as anticoagulant. The blood was drawn from the jugular vein of healthy horses bred and fed under identical conditions and without medical treatment. All the experiments were realized with approval from the ethics committee of the Faculty of Veterinary Medicine of the University of Liege (agreement no. 1474). Briefly, the neutrophils were isolated at room temperature (18–22◦C) by centrifugation (400 x g, 45min at 20◦C) on a discontinuous Percoll density gradient according to the method previously described (Benbarek et al. 1996). The cells were gently collected and washed with two volumes of physiological saline solution. After removal of the supernatant, the cell pellets were resuspended in 2ml phosphate-buffered saline (PBS) and counted for further use.

A cell viability test was performed by using the Trypan blue exclusion by viable cells, as described by Strober (2015). The samples were prepared as follows: PMNs were incubated for 30min with juglone at different concentrations. Afterwards, the cellular suspension was centrifuged and the supernatant eliminated. The cell pellets were resuspended in 450 µl PBS, to which was added 50 µl of Trypan blue. Ten microliters of the cell suspension was transferred into the Bürker chamber for colored dead cell counting under light microscopy.

Neutrophil ROS Production

Luminescence Investigation

The ROS produced by PMA-activated neutrophils were measured by L012-enhanced chemiluminescence (CL) under adaptation of the method previously described by Benbarek *et al.* (1996) and Derochette *et al.* (2013). Neutrophil suspensions were distributed in the wells (106 neutrophils/well, 143 µl PBS) of a 96-well microtiter plate (White Combiplate 8; Fisher Scientific, Merelbeke, Belgium) and incubated for 10min at 37◦C with 2 µl of juglone to reach final concentrations of 0.001, 0.01, 0.1, and 0.5µM. After incubation, 25 µl CaCl2 (10mM) and 20 µl L-012 (10−4 M) were added into the wells (final volume, 200 µl). Then, the suspensions were activated with 10 µl PMA (16µM) just before CLmeasurement (Derochette *et al.* 2013). The CL response of the neutrophils was monitored for 30min at 37◦C with a Fluoroskan Ascent spectrophotometer (Fisher Scientific, Merelbeke, Belgium) and expressed as the integral value of the total CL emission. The control was performed with neutrophils activated with PMA [positive control (Ctrl)] in the presence of PBS instead of juglone. Another control was performed with PMA-activated neutrophils in the presence of the vehicle solution of juglone (1% DMSO, final concentration) and was taken as 100% CL response. The negative control was done with unstimulated neutrophils (non-activated, NA) with the CL probe alone.

EPR Spin Trapping Investigation

In parallel to the CL assay, the effect of juglone on ROS production by PMA-activated neutrophils was evidenced by using electron paramagnetic resonance (EPR) spectroscopy in combination with the spin trapping technique (DMPO was used as the spin trap agent). The EPR assay was performed according to the protocol previously described (Mouithys-Mickalad *et al.* 1997). Neutrophil suspensions were distributed in Eppendorf tubes (4Å~106 neutrophils/ml, 143 µl PBS) in the presence of 10 µl of DMPO (50mM) and 5 µl of CaCl2 (10mM). The reaction was triggered upon the addition of 10 µl PMA (5 x 10−7 M). The solutions of juglone were tested at final concentrations of 0.5, 1, 2.5, and 10µM and compared to the complete system without juglone or with 1% DMSO used as a vehicle control. The sample was then transferred in the capillary, which was put into the EPR quartz tube and placed into the

cavity for measurement using an EMX-micro EPR (Bruker, Rheinstetten, Germany). The following settings were used for the analysis: microwave frequency, 9.78 GHz; microwave power, 18.9 mW; modulation amplitude, 1.0G; modulation frequency, 100 kHz; receiver gain, 2 x 104; conversion time, 40ms; time constant, 81.92ms; magnetic field centered at 3,480G; and number of scans, 4.

Neutrophil Degranulation Activity

The active MPO fraction released by PMNs was measured by the specific immunological extraction followed by enzymatic detection (SIEFED) method (Franck *et al.* 2006). Briefly, neutrophils (106 cell/ml) in 20mM PBS (pH 7.4) were incubated for 10min at 37◦C with 5 µl CB (1 mg/ml) in the presence or absence of increasing concentrations of juglone (0.1, 0.5, 1, 2.5, 5, 10, and 50µM) and 1% DMSO used as a vehicle control. Then, the cell suspensions were stimulated with 1 x 10−6 M fMLP (Saeki *et al.* 2001). A negative control assay was performed with neutrophils without any addition of juglone and stimulating agents. All the samples were incubated at 37◦C for 30min and then centrifuged at 450 x g for 10min at 37◦C. The supernatant was collected and transferred into a 5-ml tube and stored at −20◦C until further measurement of the active MPO.

Myeloperoxidase Activity

Purification of Equine MPO

Pure equine MPO was obtained as previously described by Franck *et al.* (2005). Briefly, packed neutrophils were homogenized in acetate buffer (pH 4.7) added with 1% detergent. After the dialysis of the supernatant, MPO was purified by two successive chromatographic steps: ion exchange on Sepharose gel (acetate buffer, pH 4.7, NaCl gradient) and gel filtration (same acetate buffer). After the final dialysis, MPO was >98% pure (as established by electrophoresis with enzymatic detection on electrophoretic bands). The enzyme-specific activity determined by the ortho-dianisidine test at pH 5.5 was 70.4 U/mg protein.

Measurement of MPO Activity

Measurement of the peroxidase activity of MPO was performed with a classical enzymatic assay and the SIEFED assay as described by Nyssen *et al.* (2018). The MPO solution was prepared with purified equine or human MPO in the dilution buffer (20mM PBS, pH 7.4, with 5 g/L BSA and 0.1% Tween-20). The solutions of juglone, at final concentrations ranging from 0.1 to 50µM, were incubated for 10min with equine or human MPO at a final concentration of 5 mU/ml before further use. The revelation of MPO activity was performed by monitoring the enzyme-catalyzed oxidation of Amplex Red in the presence of H2O2 (10µM) and nitrite (4.5mM) in phosphate buffer, pH 7.4. Similar assays were

performed in the same conditions with gallic acid instead of juglone. Gallic acid was used at final concentrations ranging from 0.1 to 0.5µM, dissolved in Milli-Qdistilled H2O; this molecule was chosen as a positive control for its known inhibitory properties on MPO activity (Franck *et al.* 2013).

Classical Assay of MPO Activity

After incubation, the mixtures containing 100 µl of juglone/vehicle and MPO were loaded into the wells of microtiter plates (transparent) and the peroxidase activity measured by adding 10 µl sodium nitrite solution (4.5mM, final concentration) and 100 µl of the reaction solution containing 10µM H2O2 and 40µM Amplex Red in phosphate buffer (50mM) at pH 7.5. The oxidation of Amplex Red into the fluorescent adduct resorufin ($\lambda_{excitation}$ = 544 nm, $\lambda_{emission}$ =590 nm) was monitored for 30min at 37◦C with a fluorescent plate reader (Fluoroskan Ascent, Fisher Scientific). A control assay set as relative percentage value of MPO activity was performed with purified MPO in the presence of PBS instead of the increasing concentrations of juglone and 1% DMSO used as the solvent of juglone. To eliminate the possibility of artifact reactions, which might arise from the MPO activity or its natural substrate (H2O2), direct reaction of juglone with H2O2 was performed in phosphate buffer (PBS) without the addition of equine or human MPO.

SIEFED Assay of MPO Activity

Samples with MPO and various concentrations of juglone were prepared and incubated as for the classical assay. One hundred microliters of each mixture (MPO alone or MPO + juglone/DMSO) was then loaded into the wells of a SIEFED microtiter plate coated with rabbit polyclonal antibodies (3µg/ml) against equine MPO or against human MPO and incubated for 2 h at 37◦C in darkness. After washing up the wells, the activity of the enzyme captured by the antibodies was measured by adding 10 µl sodium nitrite solution (4.5mM, final concentration) and 100 µl of a reaction solution containing 10µM H2O2 and 40µM Amplex Red in phosphate buffer (50mM) at pH 7.5. The oxidation of Amplex Red into the fluorescent adduct resorufin ($\lambda_{excitation}$ = 544 nm, $\lambda_{emission}$ =590 nm) was monitored for 30min at 37◦C with a fluorescent plate reader (Fluoroskan Ascent, Fisher Scientific). As for the MPO direct assay, a control assay set as relative value of MPO activity was performed with purified MPO in the presence of PBS instead of the samples of juglone dissolved in DMSO. In this SIEFED assay, MPO was bond by the antibodies into the wells and juglone was discarded by the washing step before starting the measurement of the enzymatic activity of the enzyme.

Docking of MPO–Juglone

Docking simulations were realized to determine the capacity of juglone to enter into the active site of the human enzyme MPO, based on the inhibitory effect on MPO activity previously analyzed by SIEFED, and to investigate its binding mode with amino acids near the heme cavity. This assay was performed on the human MPO as its crystallographic (X-ray) structure is wellknown in contrast to that of equine MPO (Carpena *et al.* 2009). The potential inhibitory effect of juglone was docked in the heme pocket using the GOLD program as described by (Nyssen *et al.* 2018). Five runs have been performed with the aim to determine the most frequent solutions and ensure their reproducibility.

Statistical Analysis

Data are given in relative values (in percent) in reference to control groups (DMSO or distilled H2O) defined as 100%. All data are expressed as the mean ± standard error of the mean (SEM) of at least two independent experiments made with different cell batches: in each independent experiment, the assays were performed in duplicate. For the MPO activity, the assays were performed in quadruplicate. Statistical analysis was performed with the corresponding solvent vehicle control group as the reference. One-way analysis of avariance (ANOVA) with Dunnett's multiple comparison test was performed. A $p < 0.05$ was considered as significant.

Results

Neutrophil Toxicity

The Trypan blue test showed that the viability of the cells was not significantly affected after 40min, which corresponds to the total incubation time used when neutrophils were treated with various concentrations of juglone during the stimulating assays (CL and degranulation). Only a weak cellular toxicity is observed upon exposure to the highest concentrations of juglone: 10–20% loss of viable cells at the tested concentrations of juglone (from 0.001 to 50 μM).

Neutrophil ROS Production

Luminescence Investigation

The CL response resulting from ROS production by PMAstimulated PMNs was inhibited by juglone in a dose-dependent manner, and this inhibition reached 78% for the highest concentration of 0.5μM. In the absence of PMA (NA neutrophils, negative Ctrl NA), only a weak light emission was observed compared to activated neutrophils (A, positive Ctrl A) with PMA. The control test with 1% DMSO, used as the solvent of juglone, did not induce a significant change compared to the positive control (Figure 1). In contrast, upon the exposure of juglone with PMNs without PMA, in the presence of the CL probe (L-012), no ROS production was observed (data not shown).

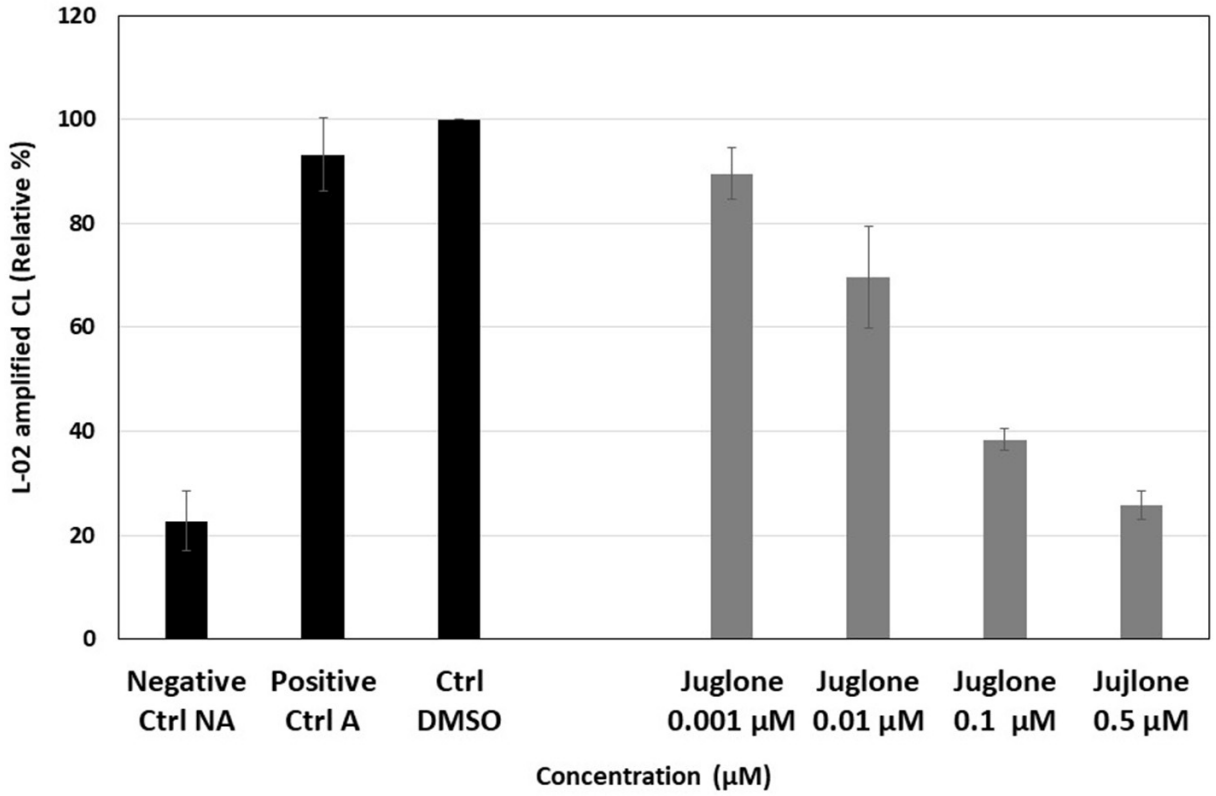

FIGURE 1 | Effect of increasing concentrations (0.001–0.5µM) of juglone on the reactive oxygen species (ROS) produced by phorbol myristate acetate (PMA)-activated neutrophils (1 million/well in PBS in the presence of 10mM calcium and the chemiluminescent probe, 10−4 M L-012). The results are the mean ± SD of triplicate experiments (n = 6). Negative Ctrl (NA), non-activated cells (no PMA); positive Ctrl (A), PMA-activated cells (+PMA); Ctrl DMSO, control of activated cells with 1% DMSO taken as 100% of ROS production.

EPR Spin Trapping Investigation

In the absence of juglone, a strong EPR signal was observed, which was a little bit increased in the presence of 1% DMSO (Figure 2). In contrast, when PMA-activated neutrophils were pre-incubated with increasing concentrations of juglone (from 0.5 to 10µM), a decrease of the EPR signal was observed in a dose-dependent manner, with total disappearance at the highest concentration of 10µM (Figure 2). The EPR signal resulting from the activation of PMNs is a mixture of two EPR spectra: a very weak signal attributed to the DMPO–OH adduct (1 in Figure 2, scan A) and the high signal of the DMPO–OOH adduct (2 in Figure 2, scan A). As expected, no EPR signal was observed with the non-activated neutrophils (not shown).

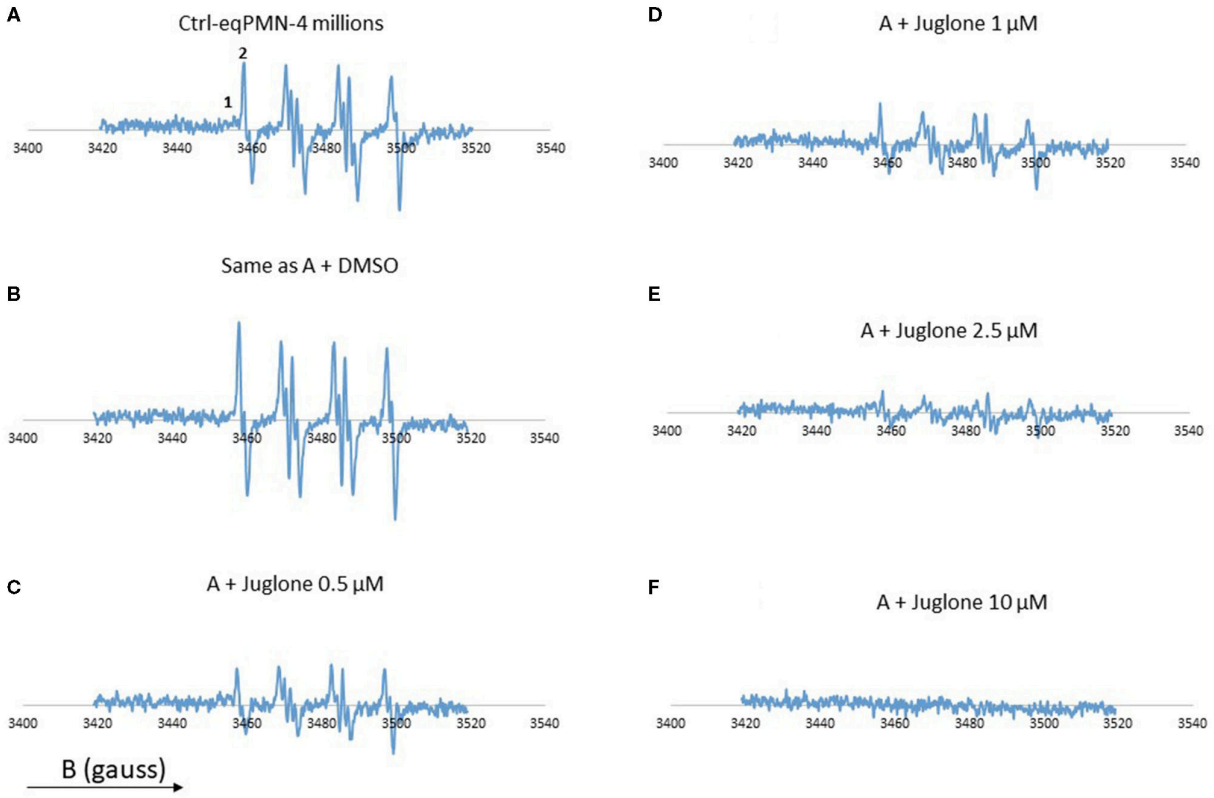

FIGURE 2 | Electron paramagnetic resonance (EPR) spectra from neutrophils (4 x 106 cells/ml) stimulated with phorbol myristate acetate (PMA, 5 x 10−7 M) in the presence of the spin trap 5,5′ -dimethyl-pyrroline-N-oxide (DMPO, 100mM). Scan (A) complete system which generates oxygen free radicals. Scan (B) same as (A), but with 1% DMSO. Scan (C) same as (A), but with 0.5µM juglone. Scan (D) same as (A) + juglone 1µM. Scan (E) same as (A) + juglone 2.5µM. Scan (F) same as (A) + juglone 10µM. Hyperfine splitting for scan (A) aH = 14.3G and aN = 14.87G. The results represent the mean of two experiments with two different cell batches. (1) DMPO–OH adduct. (2) DMPO–OOH adduct. The total number of scans is 4.

Neutrophil Degranulation Activity

The activation of neutrophils (106 cells/ml) with the mixture of CB and fMLP induced a strong release of active MPO measured in the cell supernatant. Figure 3 presents, for each experimental condition, the individual values obtained for six batches of neutrophils isolated from different horses. Upon exposure to increasing concentrations of juglone (from 0.1 to 50µM), a dose-dependent decrease of the active equine MPO release was observed in comparison to the control tests (cells alone and cells + 1% DMSO).

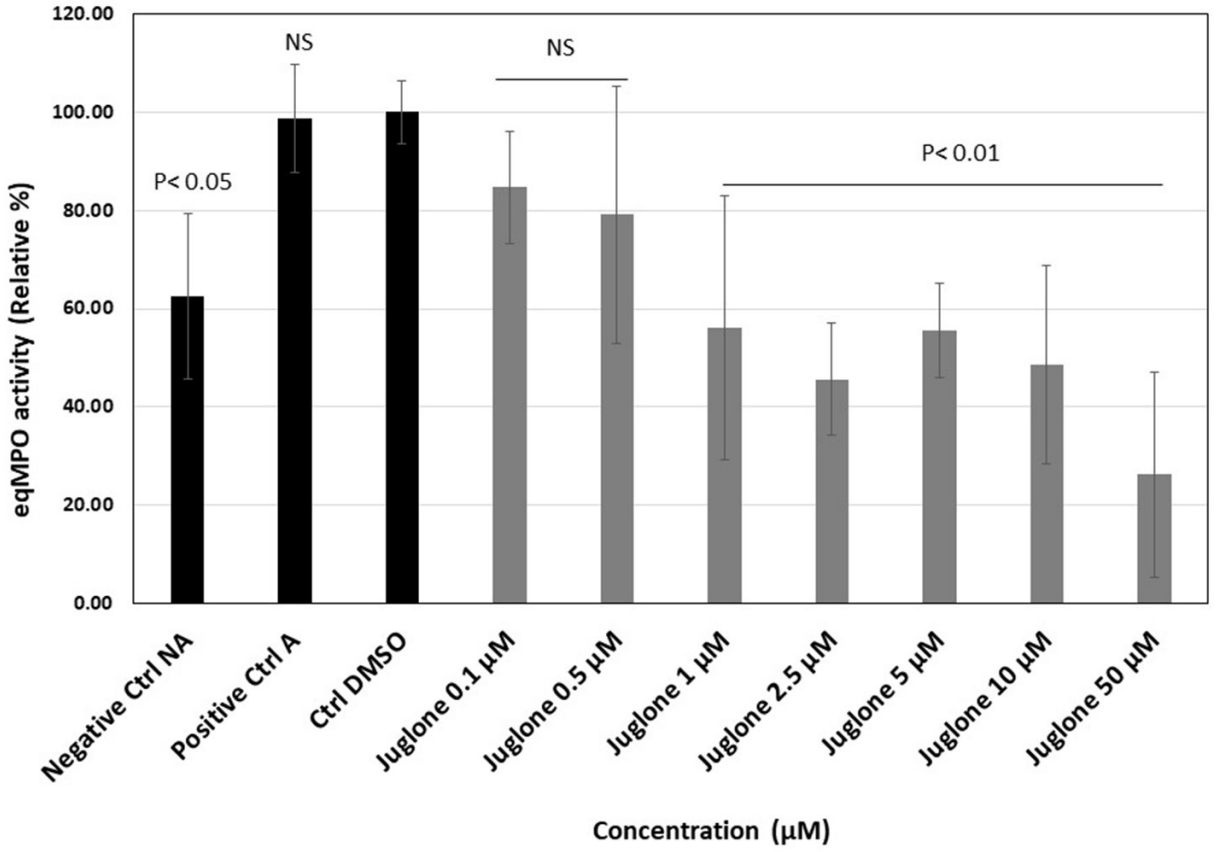

FIGURE 3 | Effect of juglone on the active myeloperoxidase (MPO) release during the degranulation of neutrophils in the absence or presence of increasing concentrations of juglone (from 0.1 to 50µM). Positive control (A), activated neutrophils alone; Ctrl DMSO, control with 1% dimethyl sulfoxide (DMSO). The results are presented as the mean value ± SD of independent experimental conditions (n = 6). The mean value obtained for the condition Ctrl DMSO was taken as 100%. $p < 0.05$ and $p < 0.01$ were considered as significant.

Myeloperoxidase Activity

Effects of Juglone on Equine MPO Activity

The classical assay showed that, upon addition of increasing concentrations of juglone, a dose-dependent decrease of MPO activity was observed (Figure 4A). With the highest concentrations, 25 and 50µM, the inhibition reached 85 and 95%, respectively. The inhibition values were significant ($p < 0.01$) vs. the DMSO control for the 10, 25, and 50µM juglone concentrations. Compared to gallic acid, taken as the reference molecule, the inhibition values obtained with juglone were less pronounced. The use of the solvent of juglone (1%DMSO) and of gallic acid (1% distilled water) did not significantly influence the MPO activity compared to the MPO in PBS (positive control).

The SIEFED assay, allowing the elimination of the excess of juglone before the measurement of the MPO activity, showed a significant ($p < 0.01$ vs. the DMSO control) dose-dependent inhibition for the three highest juglone concentrations of 10, 25, and 50µM (Figure 4B). But the inhibition was lower compared to similar concentrations of gallic acid. As for the classical assays, 1% DMSO and distilled water (dH2O, 1%) used to prepare gallic acid did not significantly influence the MPO activity compared to the PBS control.

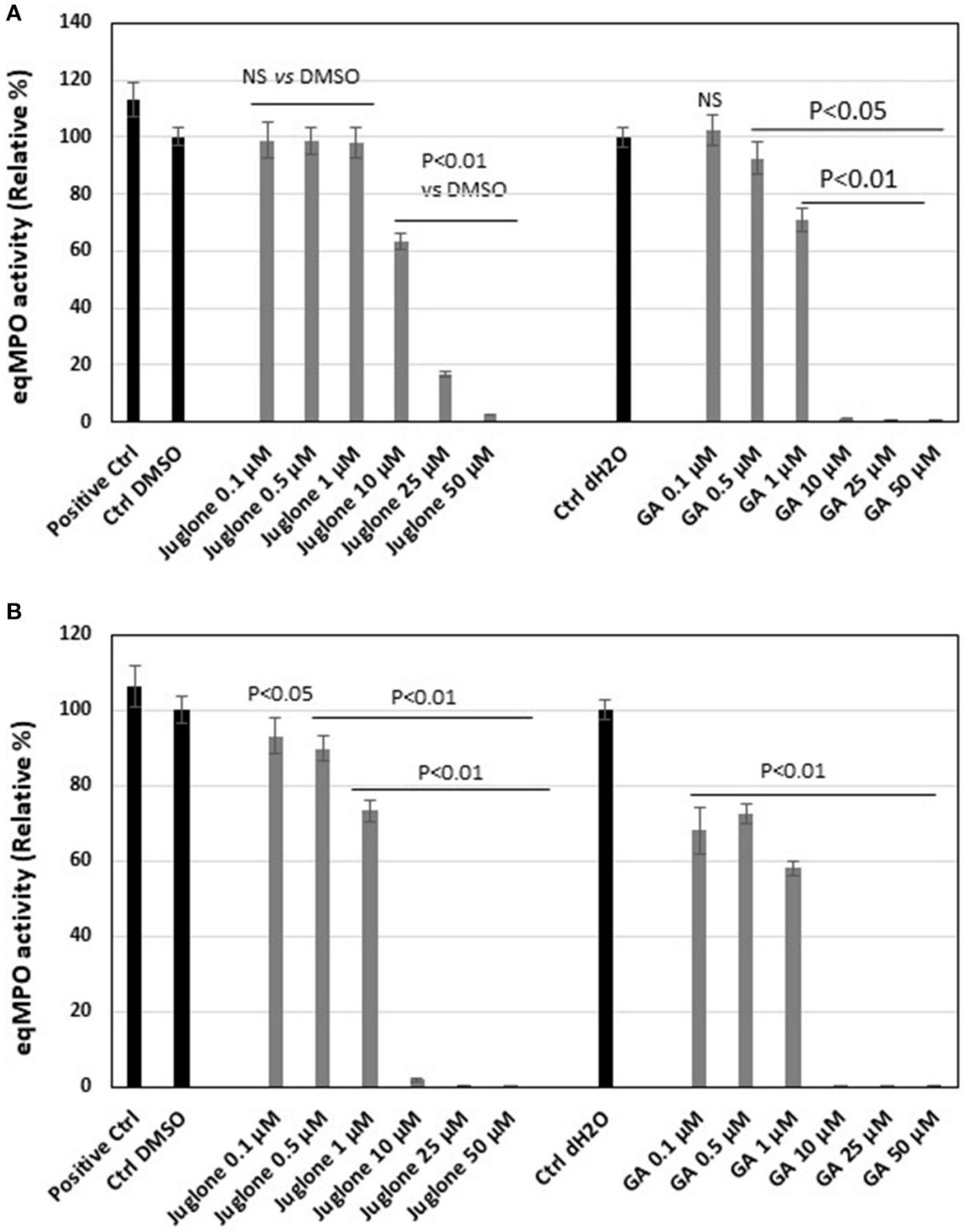

FIGURE 4 | Equine myeloperoxidase (MPO) activity and effect of increasing concentrations of juglone vs. gallic acid taken as the reference inhibitor. (A) Activity of MPO measured by a classical enzymatic assay. (B) Activity of MPO measured by the specific immunological extraction followed by enzymatic detection (SIEFED) assay. The results are the mean ± SD (n = 4). Positive Ctrl, PBS with MPO alone; Ctrl DMSO and Ctrl dH2O contained MPO + 1% dimethyl sulfoxide (DMSO) or distilled H2O (dH2O),

vehicles of juglone and gallic acid, respectively. Values of p are vs. DMSO or dH2O control taken as 100% activity of MPO.

Effects of Juglone on Human MPO Activity

As for the equine MPO, juglone exerted a significant inhibition on the human MPO activity (Figure 5). In the classical assay, the inhibition became significant ($p < 0.01$ vs. the DMSO control) starting from 7.5μM to the highest concentrations of 25 and 50μM (Figure 5A). In the SIEFED assay, the inhibition was significant for all the tested concentrations of juglone ($p < 0.01$ vs. the DMSO control). MPO activity was completely inhibited at 25 and 50μM (Figure 5B). As shown in Supplementary Figure 1, in the absence of MPO, juglone (25 or 50μM) did not react with H_2O_2 (no fluorescence). Likewise, no fluorescence was detected when H2O2 was not added in the complete system (MPO, juglone, Amplex Red, and nitrite) (negative Ctrl).

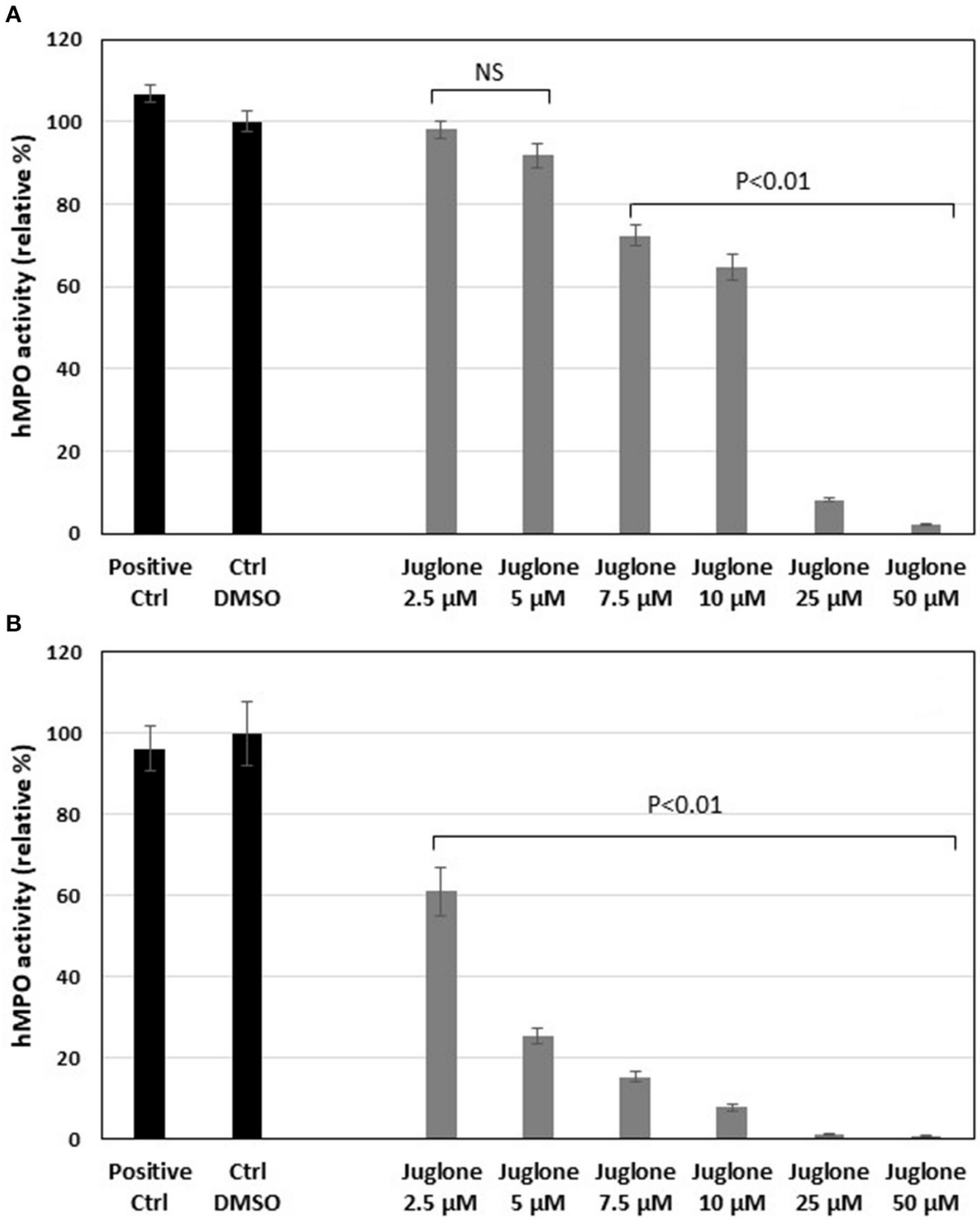

FIGURE 5 | Effects of increasing concentrations of juglone on human myeloperoxidase (MPO) measured by a classical enzymatic assay (A) or by the specific immunological extraction followed by enzymatic detection (SIEFED) assay (B). The results are the mean ± SD (n = 4). Positive Ctrl, PBS with MPO alone; Ctrl DMSO, control with 1% dimethyl sulfoxide (DMSO). Values of p are vs. DMSO control taken as 100% activity of MPO. NS, not significant.

Docking of Juglone on Human MPO

The docking of juglone was carried out on the well-known protein structure of human MPO (obtained by X-ray) (39). Figure 6 shows good stacking of the juglone structure on the MPO enzyme site, with a planar configuration of juglone. As shown, juglone was bound to Arg-239 by a 2.37-Å hydrogen bond between its ligand H atom and an N atom of the MPO Arg-239 residue (yellow dashed line). From this configuration, there is no interaction between the hydrogen atomof juglone and the heteroatom of His-95, another MPO residue present in the active site.

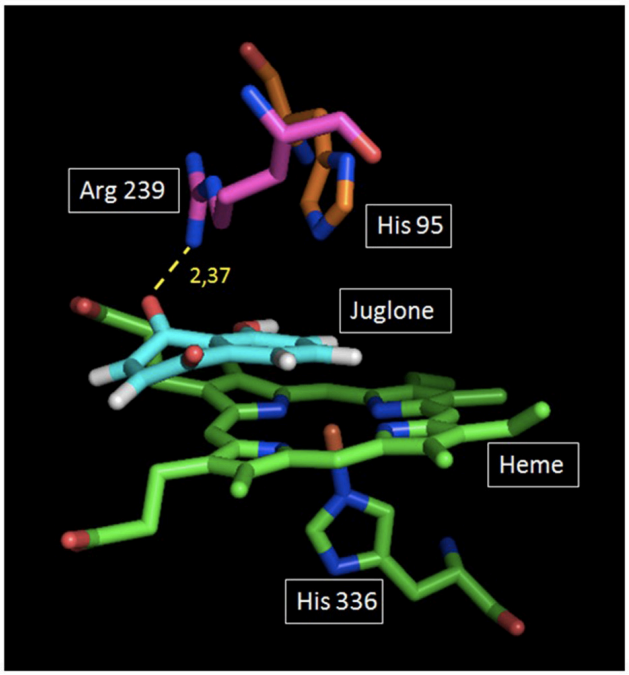

FIGURE 6 | Plausible docking solution of juglone and human myeloperoxidase (MPO) analyzed by GOLD. Green and blue atoms represent the heme and the amino acid (His-336) located below the heme. On top of the heme are two other amino acids of the apoprotein structure (Ar-239 and His-95). Juglone is in light blue color. The yellow dashed line represents the juglone–MPO Arg-239 bond.

Discussion

Laminitis is generally observed in horses when exposed to black walnut shavings. It was hypothesized that juglone, a compound found in all parts of plants of the walnut tree family (Juglans), was considered as an important toxic component involved in laminitis induction (Uhlinger 1989). Chiavaccini *et al.* (2011) demonstrated that oral administration of BWE induced an early leukocyte infiltration in the colonic mucosa of horses (McConnico *et al.* 2005). The changes in the colonic mucosa may allow the absorption of several molecules of intestinal origin, exacerbating systemic inflammation and possibly leading to distant tissue injury such as laminitis. Juglone was identified in the nuts and bark, but not in the heartwood of Juglans nigra mainly used for shavings (True and Lowe 1980, Minnick *et al.* 1987, Peroni 2017). It was concluded that BWE was "laminitogenic," but the exact implication of juglone remains unclear. However, other studies showed that juglone, when administrated per os to ponies, caused symptoms of laminitis inconsistently (Clark *et al.* 1990). Topical application of juglone to the equine digit caused local skin irritation, and intravenous administration caused acute pulmonary edema (True and Lowe 1980).

Although the role of juglone in laminitis remains controversial, a possible target of the molecule, through its naphthoquinone structure, is the modulation of the oxidant stress involved during the pathology. Indeed, during the early phase of laminitis, MPO, a pro-oxidant enzyme of neutrophils, and neutrophil elastase were present in plasma, skin, and laminar tissue, confirming the previously reported main role of neutrophils in the pathophysiology and in oxidative stress (Loftus *et al.* 2006, Riggs *et al.* 2007, de la Rebière de Pouyade *et al.* 2010, Laskoski *et al.* 2016).

Juglone, depending on the redox state in the cellular environment and the cell type, can behave as an antioxidant or a pro-oxidant agent (Ahmad and Suzuki 2019). Naphthoquinone compounds, like juglone, can inhibit oxidant reactions by quenching ROS, inhibiting ROS-producing enzymes, and chelating transition metal ions (like Fe2+) by a hydrogen atom transfer mechanism (Kappus and Sies 1981, Tejero *et al.* 2007, Tamafo Fouegue *et al.* 2016, Ahmad and Suzuki 2019). On the other hand, juglone is also cytotoxic and possesses antitumor and antimicrobial properties (Wulf *et al.* 2002, Jin 2010, Zakavi *et al.* 2013, Ahmad and Suzuki 2019). Like other quinones, the cytotoxicity of juglone includes redox cycling and reaction with glutathione (GSH), an endogenous antioxidant. Redox cycling represents a cyclic process of the reduction of a compound followed by the oxidation of the reaction product and the simultaneous generation of ROS (Tejero *et al.* 2007, Ahmad and Suzuki 2019). Juglone enhances lipid peroxidation through this process (Kumbhar *et al.* 1997). With GSH, juglone forms adducts, causing GSH depletion, interfering with endogenous antioxidant availability (Gant *et al.* 1988, Bruins *et al.* 2018). Taking into account both the oxidant and antioxidant properties of juglone, its protective and damaging effects can be expected.

In the present work, the effect of juglone was first studied on the ROS production by neutrophils. A significant reduction of ROS release by neutrophils was measured by CL (Figure 1) and EPR (Figure 2). The CL technique was used to measure the total ROS produced by neutrophils, which were activated by PMA, a stimulating agent active on NADPH oxidase, the enzyme responsible for the production of superoxide anion, the precursor of the other ROS (Derochette *et al.* 2013). Juglone strongly inhibited the total ROS production. EPR, combined with the DMPO spin trapping technique, is specific for superoxide anion (O^{-}_{2}) detection and confirms that juglone decreased and even suppressed the production of superoxide anion radicals by the neutrophils. This inhibitory effect was not due to a cytotoxic effect of the molecule toward neutrophils since the number of dead cells was not significant between the control and juglone-treated cells, as attested by the Trypan blue exclusion test. Moreover, the use of juglone instead of PMA to trigger the ROS production by PMNs did not induce light emission, suggesting the absence of oxidant properties in this neutrophil model.

These inhibitory activities can be related to the antioxidant effects of juglone described by Ahmad and Suzuki (2019). Antioxidants reduce ROS, and the balance between ROS and antioxidants defines oxidative stress. Accumulating evidences suggest that the antioxidant properties of juglone are useful in combating oxidative stress-related diseases (like Alzheimer's in human medicine) (Galas *et al.* 2006, Zhou *et al.* 2015, Ahmad and Suzuki 2019). Zhou *et al.* (2015)demonstrated that juglone increased the activity of superoxide dismutase (SOD) and decreased oxidative stress in the liver of rats. But juglone could act in an indirect manner at the level of NADPH oxidase (Nox2), the enzyme responsible for the production of superoxide anion, the first activated species in the ROS cascade. Nox2 becomes active when its cytosolic components are phosphorylated at the level of serine residues, translocated, and then could assemble with flavoprotein b in the plasmamembrane. A peptidyl prolyl cis/trans isomerase, Pin1, intervenes in the phosphorylation of the serine residues, and juglone has been reported to inhibit the pathway of Pin1, as demonstrated in CL097-induced priming of fMLP–neutrophil ROS production (Makni-Maalej *et al.* 2012) and lipopolysaccharide (LPS)-induced priming of ROS production by neutrophils (Liu *et al.* 2019). It may be suggested that, also in neutrophil stimulation by PMA, juglone could interfere with the Pin 1 pathway, reducing indirectly the Nox2 activity.

On the basis of the CL and EPR spin trapping results, confirming the ROS-scavenging activity of juglone, we decided to investigate its action on the degranulation of MPO by neutrophil activated using CB/fMLP and directly on the activity of this enzyme.

The degranulation of neutrophils was obtained upon stimulation with the CB/fMLP system: CB acts on the cytoskeleton at the level of the actin filaments and is used with fMLP to potentiate neutrophil degranulation (Saeki *et al.* 2001). In the supernatant of these activated neutrophils, the measurement of active MPO showed that juglone was effective at inhibiting the release of this enzyme, but with a variable effect from one neutrophil batch to another, as shown in Figure 3. Inhibition by juglone was

also observed on purified equine MPO by acting either on the enzyme itself or on the oxidant species released during enzyme activity (Figure 4A). But the SIEFED technique (Figure 4B) demonstrates that juglone can act directly at the level of the catalytic site of MPO. This technique allows binding of the MPO present in the sample by specific immobilized antibodies, then a washing up is done before revealing the enzymatic activity. If inhibition of MPO persists after the elimination of juglone, this means that the molecule remained bonded to the captured enzyme (Franck *et al.* 2006). Similar inhibitory effects were also observed with human MPO (Figures 5A,B), which allowed us to use human MPO for a docking study as the enzyme structure has been elucidated by X-ray crystallography (Carpena *et al.* 2009), which was not the case for equine MPO. This docking study showed a good stacking of juglone above the porphyrin ring of MPO and a hydrogen bond with Arg-239 (Figure 6). No interaction was found between a hydrogen atom of juglone and the heteroatom of His-95 on the apoprotein present in the active site. Nevertheless, the planar configuration of juglone and the link established with the Arg-239 residue confer a strong stability in the active site, hindering access to the iron-bearing catalytic site for H2O2, the normal MPO substrate, and inhibiting the important step of the formation of a p cation radical state on the porphyrin ring (Furtmüller *et al.* 2000). In this way, the formation of the highly oxidant HOCl molecule is impaired.

By generating potent oxidant molecules, MPO is a dangerous enzyme responsible for damage in acute and chronic inflammation pathologies, and its inhibition could be beneficial. Several natural compounds such as curcumin and resveratrol are candidates for MPO inhibition (Kohnen *et al.* 2007, Franck *et al.* 2019). Our study demonstrates that juglone, another natural compound, might be a useful candidate in inflammation pathologies.

Conclusion

The present work using an *in vitro* model of neutrophil degranulation and MPO activity indicates, for the first time, that juglone has anti-inflammatory effects, rather than pro-inflammatory ones, on equine neutrophils and MPO. Through its antioxidant properties, by scavenging the ROS produced by PMA-stimulated PMNs and by inhibiting the MPO activity, inhibitory effects confirmed by the docking study, juglone would be protective in the BWE model of equine laminitis rather than damaging, at least if we focus on the modulation of neutrophil activation. But concerning the oxidant neutrophil activity, another mode of action should be considered for juglone: a pathway involving NADPH oxidase. Finally, it should be taken into account that juglone, in *in vivo* conditions, could contribute with other compounds of BWE to intestinal inflammation and to the resorption of pathogen-associated molecular patterns (PAMPs) able to induce the systemic and local inflammation that characterizes the BWE model of equine laminitis.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

Author contributions

DS, AM-M, and NS: conceptualization. NS, AM-M, TF, and JC: methodology. AM-M, NS, and TF: investigation. AM-M, DS, GD-D, and NS: writing-original draft preparation. AM-M, DS, JC, TF, and GdR: writing-review and editing. DS and AM-M: supervision. All authors contributed to the article and approved the submitted version.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets. 2021.677675/full#supplementary-material

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Experimental section

Study 2

Presence of myeloperoxidase in lamellar tissue of horses induced by an euglycemic hyperinsulinemic clamp

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Abstract

Laminitis is a pathology of the equine digit leading to a failure of the dermo-epidermal interface. Neutrophil activation is recognized as a major factor in SIRS-associated laminitis. Less is known about the role of neutrophil activation in laminitis associated with metabolic disorders. The aim of this descriptive study was to observe whether myeloperoxidase is increased in the laminae during early stage laminitis in three horses subjected to a prolonged euglycemic hyperinsulinemic clamp (pEHC). After 48 h of pEHC-treatment, horses were subjected to euthanasia. Two healthy horses are used as control. Histological sections of lamellar tissue from all horses were immunohistochemically stained for myeloperoxidase and counterstained with hematoxylin-eosin. Histopathological changes that characterize insulin-induced laminitis and increased presence of myeloperoxidase, especially in the dermal lamellae, were increased in histologic sections of pEHC-treated horses. Neutrophil myeloperoxidase release may contribute to the pathophysiology of endocrinopathic laminitis.

Keywords: horse, myeloperoxidase, laminitis, insulin, neutrophils, metabolic disease

Introduction

Equine laminitis is a pathology of the digit resulting in severe lameness and ultimately displacement of the distal phalanx. This occurs due to structural failure with loss of integrity of the lamellae attaching the hoof wall to the distal phalanx (Leise 2018). Inflammation seems to play a central role in the pathogenesis of laminitis (de la Rebière de Pouyade and Serteyn 2011). In cases of sepsis, local infection is accompanied by systemic neutrophil activation. Systemic neutrophil activation is also encountered in equine laminitis, as demonstrated by the up-regulation of cytokine expression, the dynamic changes in blood neutrophil phenotype, the formation of neutrophil-platelet aggregates and the infiltration of inflammatory cells (Weiss *et al.* 1997, Black *et al.* 2006, Hurley *et al.* 2006, J. P. Loftus *et al.* 2007). The systemic and the local inflammatory responses have largely been described in previously developed laminitis models such as Carbohydrate Overload (CHO), Oligofructose (OF) and Black Walnut Heartwood Extract (BWHE). This underscores the major role of neutrophil activation (Johnson *et al.* 1998, Eps and Pollitt 2006, Riggs *et al.* 2007, de la Rebière de Pouyade *et al.* 2010). Using myeloperoxidase (MPO) as a marker of neutrophil activity, Riggs et al. (2007) confirmed neutrophil activation in blood, skin, and lamellar tissue starting from one and measured to 12 h following BWE administration. MPO is a hemic enzyme, responsible for the direct or indirect synthesis of many oxidizing species that participate in host defense mechanisms. MPO has dual peroxidase and chlorination activity, and their derived products (e.g., HOCl, nitrogen dioxide) are able to induce chlorination, nitration, and oxidation of protein residues. Hypochlorous acid (HOCl) produced via the chlorination activity of MPO, using hydrogen peroxide (H2O2) and a chloride anion is recognized as a powerful oxidizing agent necessary for the destruction of micro-organisms in the phagolysosome. When the inflammatory reaction becomes uncontrolled, excessive neutrophil degranulation or death induces significant MPO release into the extracellular environment, and the oxidant products derived from its activity can induce cell and tissue damage (Taurog and Dorris 1992, Podrez *et al.* 2000, Dunford 2000). Laminitis has also been associated with equine metabolic disorders, such as obesity, pituitary pars intermedia dysfunction, and equine metabolic syndrome (EMS). The primary characteristic of these pathologies is the development of insulin resistance, characterized by hyperinsulinemia with eu-or hyperglycemia and a subsequent chronic pro-inflammatory state (Johnson *et al.* 2004). As in the human metabolic syndrome where increased adipose tissue mass amplifies the secretion of proinflammatory adipokines that decrease insulin sensitivity, induce oxidative stress, and impair microvascular function; the EMS is associated with similar risk factors and characterized by regional adiposity, hyperinsulinemia, insulin resistance, hypertriglyceridemia, and recurrent laminitis (Geor and Frank 2009, Frank *et al.* 2010). An experimental model of laminitis showed that healthy Standardbred horses subjected to prolonged hyperinsulina-emia develop laminitis within 48 h (de Laat *et al.* 2010). Furthermore, natural cases of equine endocrinopathic laminitis are clearly associated with hyperinsulinemia (Karikoski *et al.* 2015). Inflammation was thought to be limited, with only moderate

neutrophil infiltration observed in hoof lamellae of horses with laminitis induced using a hyperinsulinemia model (McGowan and Patterson-Kane 2017). However, a study by Holbrook et al. (2012) showed a marked increase in neutrophil oxidative burst activity in obese hyperinsulinemic horses.

The objective of this study was to determine the potential implication of neutrophil activation evidenced by MPO release, following experimental laminitis induction using the prolonged euglycemic hyperinsulinemic clamp (pEHC) model. We hypothesized that as in the BWE model, an increased presence of MPO would be identified in the digital lamellae of horses undergoing pEHC laminitis induction.

Material and methods

Animals, Laminitis Induction and Sample Collection

Archived formalin fixed–paraffin embedded lamellar tissue samples from 5 horses from a previous experiment were used with approval from the Animal Ethics Committee, The University of Queensland (SVS/506/17). Laminitis was induced using the euglycemic hyperinsulinemic model as described by Asplin et al. (2007) in three horses. Two healthy horses were used as negative controls. An initial intravenous bolus (45 miu/kg) of recombinant human insulin (Humulin- R, Eli-Lilly Australia Pty Lfd) diluted in 50ml of 0.9% sodium chloride (Baxter Healthcare Pty Ltd) was administered via a 14- gauge catheter and was followed by a continuous intravenous infusion of insulin in 0.9% sodium chloride (Baxter Healthcare Pty Ltd) at a fixed rate of 6 miu/kg/min. Additionally, a continuous intravenous infusion of 50% glucose (Baxter Healthcare Pty Ltd) was administered, and the rate was adjusted to maintain euglycemia (4.0 ± 1.0 mmol/L). Blood glucose was measured using a portable glucometer (Accu- Chek Performa, Roche Diagnostics). The Obel scoring system was used to quantify the clinical symptoms (Menzies-Gow *et al.* 2010). After 48 h of pEHC-treatment, horses were subjected to euthanasia, and a minimum of 2 samples of mid-dorsal hoof lamellae were obtained immediately on each horse. Tissues were formalinfixed for 24 h and transferred to 70% ethanol for 48 h prior to paraffin embedding.

Immunohistochemical Staining

Histological sections were prepared at 4µm thickness and mounted on a glass slide following standard technique. 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 phosphate-buffered saline (PBS) (2 x 3min). Based on preliminary tests, no antigen retrieval protocol and hydrogen peroxide blocking steps were required. Immunostaining of MPO was performed with purified equine MPO and using a rabbit specific horseradish peroxidase/diaminobenzidine ABC detection immunohistochemistry kit (Abcam) (Franck *et al.* 2005). Sections were surrounded with a hydrophobic barrier pen prior to addition of protein block solution (PAP Pen, Abcam) for 10min at 22◦C to block nonspecific background staining. After one wash with PBS (1 x 3min), the primary anti-MPO antibody (rabbit antibody obtained against purified equine MPO) diluted 1:1000 in dilution buffer (20mM PBS pH 7.4 + 0.5% bovine serum albumin and 0.1% Tween 20) was applied for 1 h at 22◦C. Negative control sections were prepared by adding only the dilution buffer without primary antibody. After washing with PBS (3 x 3min), the anti-rabbit antibody conjugated with biotin (kit) was added to all sections for 15min at 22◦C. Slides were washed again with PBS (3 x 3min) and the streptavidin-peroxidase solution (kit) was added to all sections for 15min. After

rinsing with PBS (3 x 3min), the chromogen diaminobenzidine/substrate solution (kit) was added to all the sections and incubated for 5min. The appearance of MPO labeling (brown colouration) was monitored. The slides were then placed in water (3 x 3min), followed by addition of hematoxylin-eosin (HE) solution (Merck) for 90 s. The slides were rinsed under tap water for 2min, and water-soluble mounting media was added and left to dry 24 h in the dark. The sections were assessed using light microscopy with a Zeiss Axioskop microscope, and all photographs were obtained using the same light intensity and shutter speed. Slides of control and treated horses were prepared in parallel and stained simultaneously. Representative histological sections were selected for each animal and classified in 3 groups: no antibody slides (5); control group (7) and pEHC group (16). Six investigators blinded to the experimental groups semi-quantitatively assessed immunostaining outcomes. For each individual image, MPO labeling was scored by each investigator as follows: no brown staining, weak but localized staining; weak but diffuse staining; medium staining and strong staining. mean scores were calculated for each image (n = 28) and calculated for each group.

Results

Animals and Clinical Signs

All horses were adult Standardbred geldings retired from racing. The mean ± standard deviation of age and weight was 6 ± 2 years and 492 ± 45 kg, respectively. The pEHC treated horses developed a mild tachycardia, as well as increased digital pulses after 18 to 24 h of pEHC. All pEHC treated horses had clinical signs of Obel grade 1 lameness prior to euthanasia. The control horses did not display any clinical signs of laminitis.

Immunostaining Intensity

Differences were observed comparing the images coming from the no-antibody slides, the MPO-Stained slides from the healthy horses, and the MPO-Stained slides from the pEHC horses. The mean scores for the MPO labeling were 1,7 (+/– 0,5) for the control horses and 3,4 (+/– 0,5) for the pEHC-treated horses.

Histopathology and Immunohistochemical Staining

The Figure 1 shows photomicrographs (x100) of the lamellae of control horses HE staining without primary MPO antibody (Figures 1A,B) and HE staining with complete MPO immunohistochemical protocol (Figure 1C). Histologic sections of control horses show minimal, localized MPO labeling in the dermal lamellae. The secondary epidermal lamina have rounded tips in control horses. The nuclei of the epithelial basal cells are oval shaped in control horses The Figure 2 shows photomicrographs (x100) of the lamellae of pEHC-treated horses HE staining without primary MPO antibody (Figure 2A) and HE staining with complete MPO immunohistochemical protocol (Figures 2B,C). Secondary epidermal lamellae have tapered tips, appear elongated and narrow, and are acutely angled on primary epidermal lamellae. An intense diffuse MPO labeling is observed, especially in the interstitial tissue of the dermal lamellae (Figure 2B). A clear demarcation of MPO labeling is observed at the interface of the secondary epidermal and dermal lamellae with absence of MPO labeling on the epidermal side (Figure 2C). The absence of MPO labeling in negative control slides confirming the absence of non-specific binding of the primary anti-MPO antibody (Figures 1A,B, 2A). In the presence of anti-MPO antibody, MPO labeling is visible in all the samples (Figures 1C, 2B,C).

In the supplemental figure, images show reconstruction of photomicrographs of the dermo epidermal interface of a control horse and a pEHC-treated stained with HE and anti-MPO. The intensity of the MPO labeling decreases from the dermal side toward the epidermal side of the hoof, so that the MPO labeling is most intense at the base of the primary dermal lamellae and least discernible at their tips.

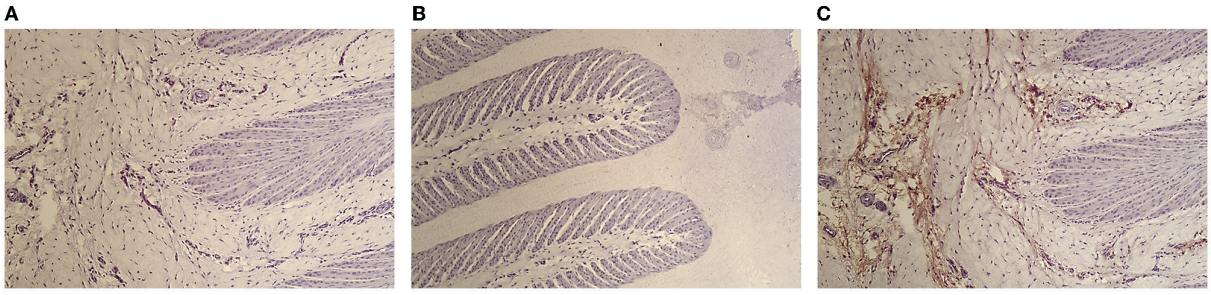


FIGURE 1 | Photomicrograph of the hoof lamellae of a control horse hematoxyllin staining without primary MPO antibody (A,B) and hematoxyllin staining with complete MPO immunohistochemical protocol (C) (x100).

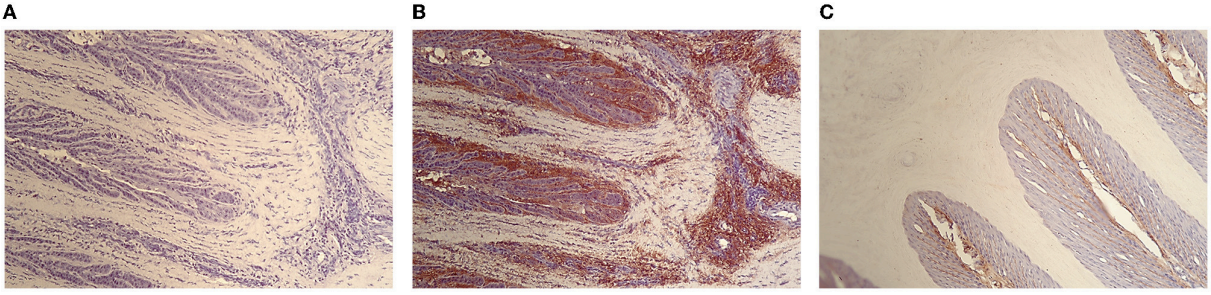
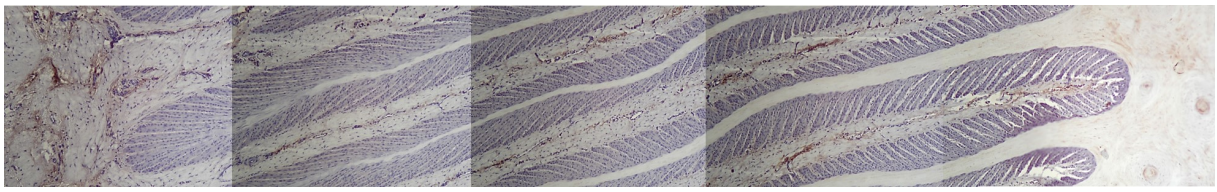
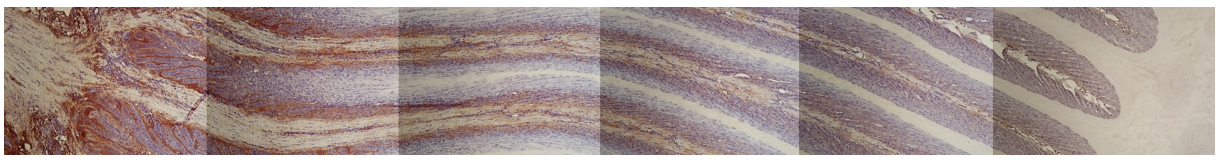


FIGURE 2 | Photomicrograph of the hoof lamellae of a pEHP treated-horse hematoxyllin staining without primary MPO antibody (A) and hematoxyllin staining with complete MPO immunohistochemical protocol (B,C) (x100).



Supplementary Figure S1 | Reconstruction of photomicrographs of the dermo-epidermal interface of a control horse stained with hematoxyllin and anti-MPO (x100). The dermal side is to the left and the epidermal side is to the right.



Supplementary Figure S2 | Reconstruction of photomicrographs of the dermo-epidermal interface of a pEHC-treated horse stained with hematoxyllin and anti-MPO (x100). The dermal side is to the left and the epidermal side is to the right.

Discussion

Laminitis occurring secondary to sepsis is known to result from a significant inflammatory response that includes leukocyte emigration, in particular the neutrophil into the lamellar tissue (de la Rebière de Pouyade and Serteyn 2011, Leise 2018). Horses administered BWE show clinical signs of laminitis and local inflammation with neutrophil activation and MPO release (Riggs *et al.* 2007, de la Rebière de Pouyade *et al.* 2010). This study highlights the presence of MPO in equine lamellae of horses with insulin-induced laminitis, suggesting that neutrophil activation is also present in this model. All histologic sections of the treated horses presented well described histological features of the hyperinsulinemia model such as elongated, narrowed secondary epidermal lamellae with tapered tips and acute-angle orientation, as well as rounded and centrally located nuclei (Asplin *et al.* 2007, de Laat *et al.* 2010, McGowan and Patterson-Kane 2017, Stokes *et al.* 2020). Together with the clinical signs of laminitis observed, this confirms that laminitis induction was successful.

Significant positive MPO labeling was observed in all sections of insulin-treated horses. During an excessive neutrophil stimulation, MPO can be released in the extracellular matrix or blood. Therefore, MPO is considered a marker of neutrophil activation or inflammation. Due to its dual activity, MPO can chlorinate, nitrate and oxidize most biological organic molecules, which results in severe tissue damage (Klebanoff 2005, Valadez-Cosmes *et al.* 2022). In addition, MPO can be taken up by endothelial cells, which can be subsequently damaged by its products (Mathy-Hartert *et al.* 1995, Benbarek *et al.* 2000, de la Rebière *et al.* 2008). Although the activity of the enzyme is not showed in this study, MPO and the oxidative species derived from its activity could play a role in establishing events leading to laminitis in a variety of ways. The presence of active MPO could perpetuate the lamellar injury.

In previous studies using the pEHC model, a limited number of neutrophils has been observed in lamellar tissue (de Laat *et al.* 2011). It was concluded that the extent and severity of inflammation in hyperinsulinemia-induced laminitis are less important than would be expected, when compared to other tissues subjected to similar levels of cellular stress and mechanical compromise (Asplin *et al.* 2007, de Laat *et al.* 2011). However, these conclusions are based on identification of the neutrophil degranulation itself. Our histologic sections agree, with the observation of only rare neutrophils. However, the increased presence of MPO supports the active involvement of neutrophils in the pathophysiology of laminitis. This may be explained by neutrophils degranulation in the bloodstream and MPO diffusion from the circulation to the dermal lamellae. Indeed, the presence of MPO in the bloodstream, skin, and lamellae after laminitis induction using a BWE model was confirmed by Riggs *et al.* (2007). Neutrophils, in addition to causing tissue injury when dysregulated, also appropriately respond to damage-associated molecular patterns (DAMPs) signals from other injured tissues. Monocytes/macrophages and endothelial cells can also release a minor quantity of MPO. Additional

immunohistochemical staining for calprotectin (using aMAC387 antibody) might be used to support the contribution of neutrophils and monocytes/macrophages in MPO release (de Laat *et al.* 2010). Another possibility is that neutrophil extracellular traps (NETs) are formed during laminitis, explaining the extensive MPO labeling with almost complete absence of neutrophils. NETs are typically formed to trap micro-organisms, but their formation has also been confirmed during non-infectious disease processes. In human patient, plasma NET parameters such as MPO-DNA complexes were higher in obese patients than in the control group and correlated with body weight, body mass index, waist and hip circumference, glucometabolic parameters, and systolic blood pressure (D'Abbondanza *et al.* 2019). Increased NETosis was also found in type II diabetes patients compared to healthy controls (Carestia *et al.* 2016). As in diabetic people where evidence indicates that insulin regulates neutrophil function and that this regulation is in turn related to increased neutrophil chemotaxis and oxidative burst, Holbrook and colleagues showed in horses a marked increase in neutrophil oxidative burst activity in hyperinsulinemic obese horses (Walrand *et al.* 2004, Holbrook *et al.* 2012). However, further experiments are needed to confirm the origin of the MPO presence in lamellar tissue and the possible toxic role that the enzyme could play.

The main limitation is the small number of horses included in the study. This precluded any statistical analysis to complete the histopathological description and qualitative assessment of the immunohistochemical staining. Future studies will focus on the presence of NETs and chlorination residues in lamellar tissues of affected horses as well on the evolution of systemic MPO concentrations.

Conclusion

This study highlights the presence of MPO in the lamellae of horses with insulin-induced laminitis, supporting a role for neutrophil activation in endocrinopathic forms of laminitis and justifying future research to confirm the link between hyperinsulinemia, neutrophil activation in equine laminitis.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Approval number: SVS/506/17. The project from which archived samples were used for these experiments was approved by the Universityof Queensland Animal Ethics Committee (AEC) that monitors compliance with the Animal Welfare Act (2001) and the Code of Practice for the care and use of animals for scientific purposes (current edition). All animals were monitored continuously by the investigators.

Author contributions

NS and DS: conceptualization. TF and NS: methodology. CM and AS: investigation. TF and DS: data curation and supervision. NS and GR: writing—original draft preparation. NS, GR, TF, and DS: writing—review and editing. DS: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets. 2022.846835/full#supplementary-material

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Experimental section

Study 3

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 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 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

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 *et al.* 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.

Material and methods

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.

Collection of lamellar tissue

Lamellar tissues were collected immediately after euthanasia. Laminitic 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.

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.

Extraction of MPO and NET in lamellar tissue

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).

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.

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.

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.

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.

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 (immunohistochemistry (IHC) kit) was applied for 10 min. The slides were then rinsed twice again with PBS for 2 min.

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.

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.

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 $p<0.05$.

Results

Signalment and history

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

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.

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).

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.

MPO and NET extraction from lamellar tissue

Tissue samples for extractions were available for all cases except two laminitic 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 laminitic 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 ($p=0.068$, $R=0.67$, $n=8$).

Histopathology and immunohistochemical staining for MPO and NET

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.

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).

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 etiologies, 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 etiologies, 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 etiology. 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 Equine herpes virus 1 (EHV-1) and renal insufficiency. Finally, a multifactorial etiology 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).

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.

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Tables

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)	Bioptis	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 1: Summary of chemicals and reagents.

	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 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.

	Clinical parameters											
Case	Heart rate (bpm)	Respiratory rate (rpm)	Rectal temperature (°C)	Growth lines	Increased hoof temperature	Increased digital pulse	Depression coronary groove	Convex sole hoof	Visibly thickened white line	Positive response to hoof tester	Obel grade of lameness (/4)	Posture
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 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.

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

	Histology	Immunohistochemsitry			Extraction analysis			
Case	Neutrophil inflammatory infiltrates	Negatve 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

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

Figures

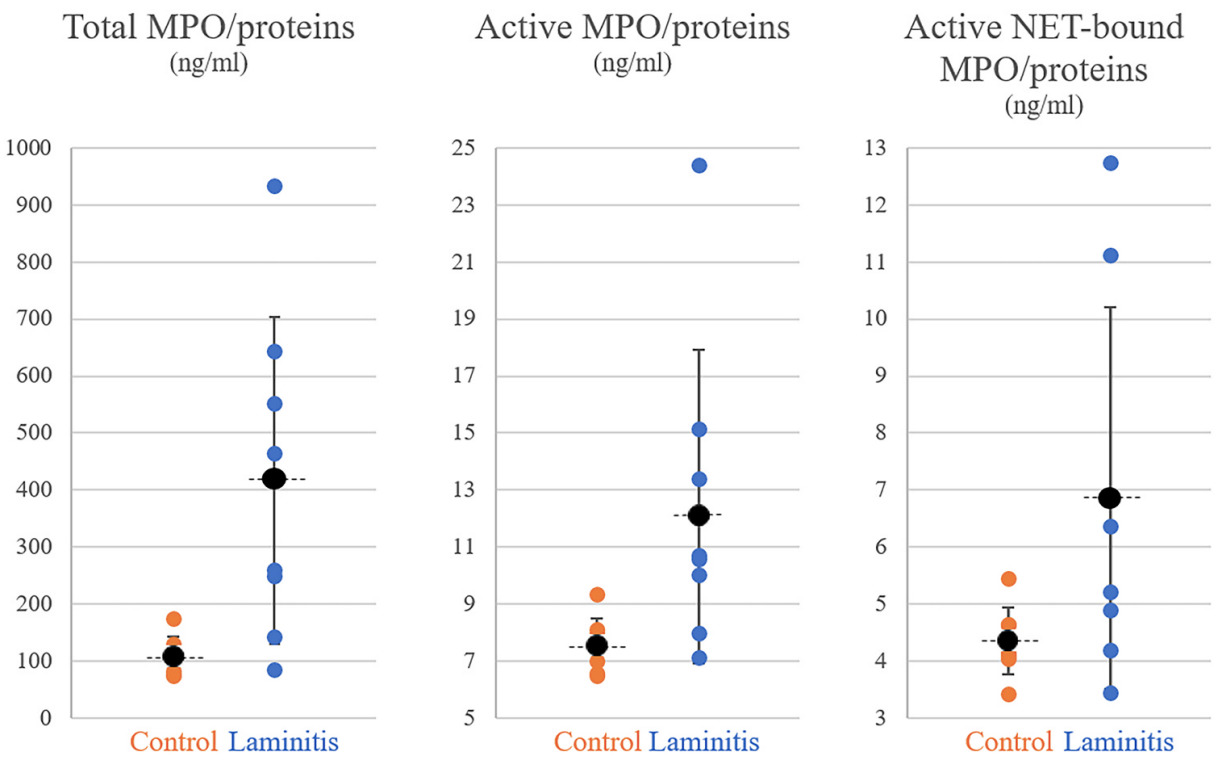

Figure 1 – Dot plots showing the total MPO, active MPO and active NET-bound MPO concentrations for control horses and laminitis cases. MPO: myeloperoxidase, NET: neutrophil extracellular traps, prot: total proteins.

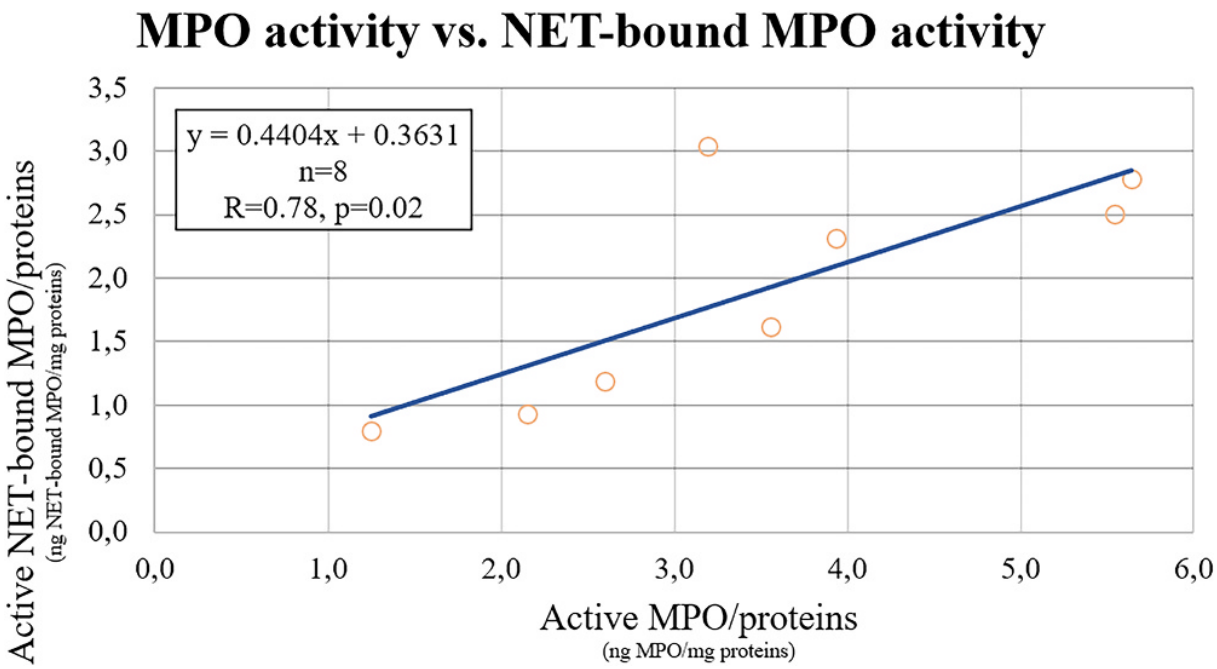

Figure 2– Correlation between MPO activity and NET-bound MPO activity.

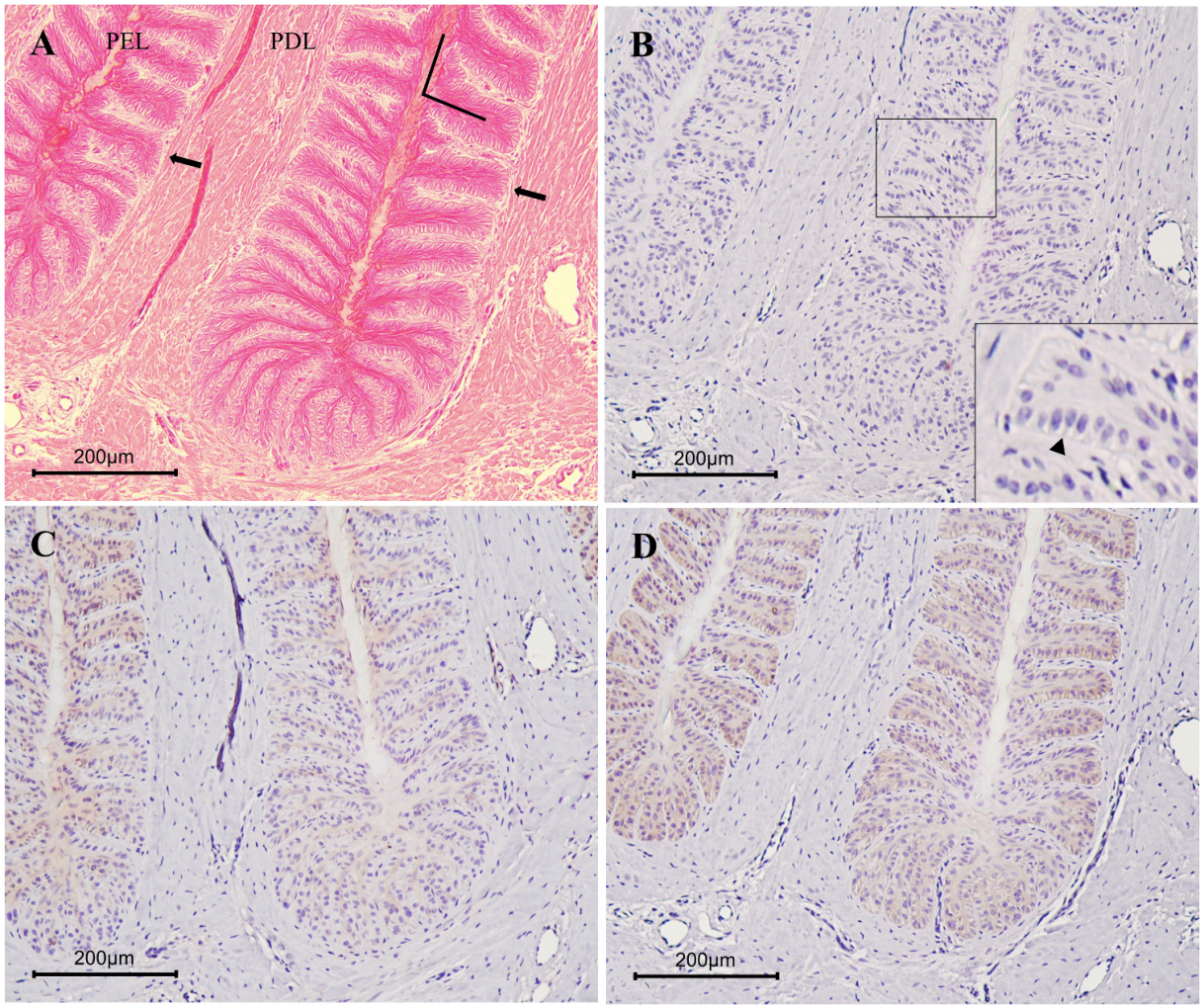

Figure 3 – Photomicrographs of a control horse stained with HE (A), negative control (B), MPO (C) and NET (D) immunohistochemical staining. The secondary epidermal and dermal lamellae are oriented perpendicularly on their primary axis (A, lines), have rounded tips (A, arrows) and the lamellar basal cells 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

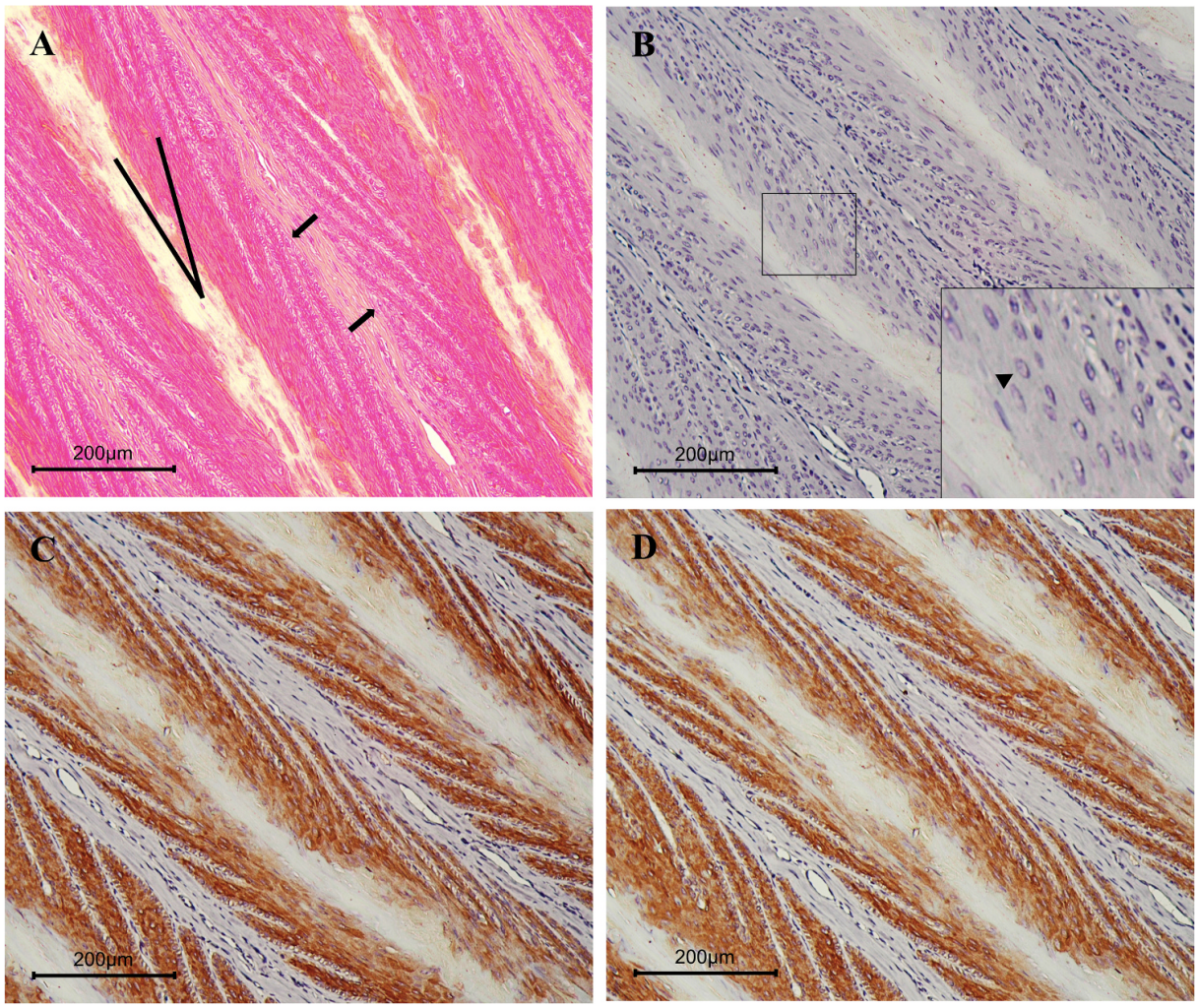

Figure 4 – Photomicrographs of a laminitic horse stained with HE (A), negative control (B), MPO (C) and NET (D) immunohistochemical staining. The secondary epidermal lamellae have tapered tips (A, arrows), appear elongated and narrow, and are acutely angled on primary epidermal lamellae (A, lines). The 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).

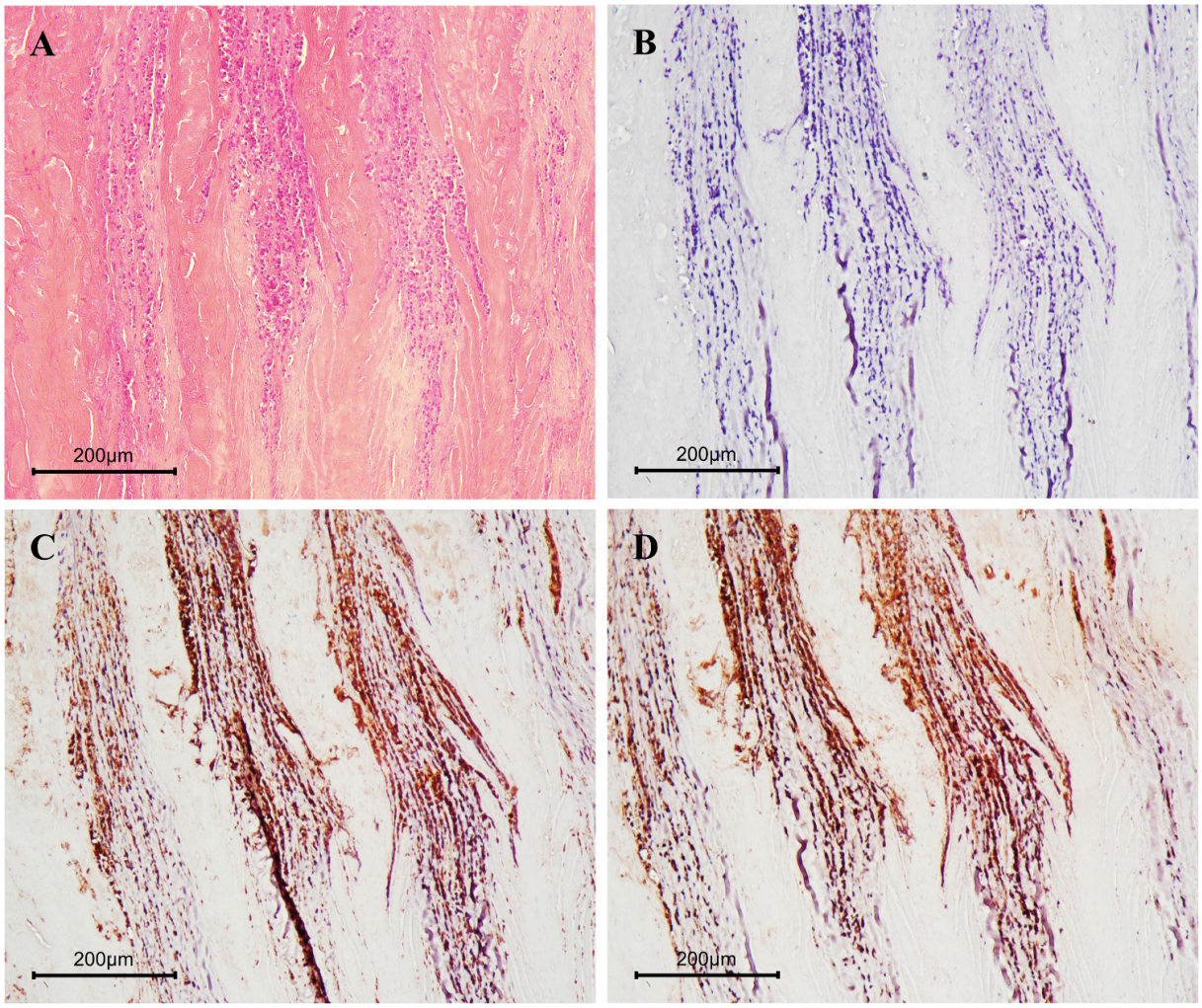

Figure 5 – Photomicrographs of a laminitic donkey stained with HE (A), negative control (B), MPO (C) and NET (D) immunohistochemical staining. Lamellar anatomy is almost unrecognisable due to severe tissue destruction. Intense MPO and NET labelling is observed in what looks like the remaining keratinocytes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

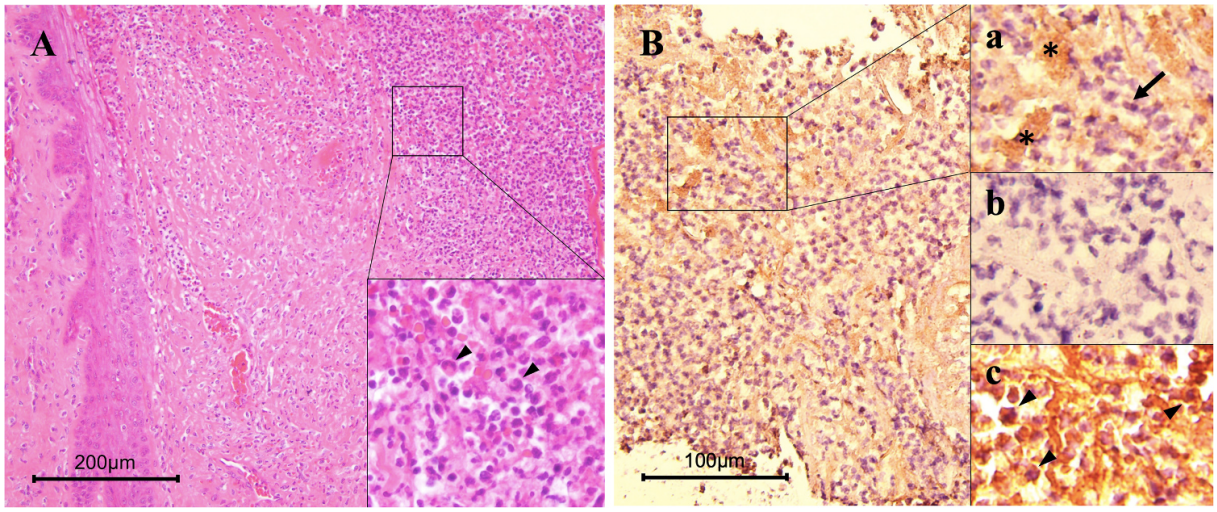

Figure 6 – Photomicrographs of a laminitic horse stained with HE (A) and NET (B) immunohistochemical staining. Figure 6A shows severe neutrophil infiltration (arrowheads) in the dermal tissue. Figure 6B shows NET labelling, visible as diffuse brown spots devoid of a nucleus and a cell wall, present around 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).

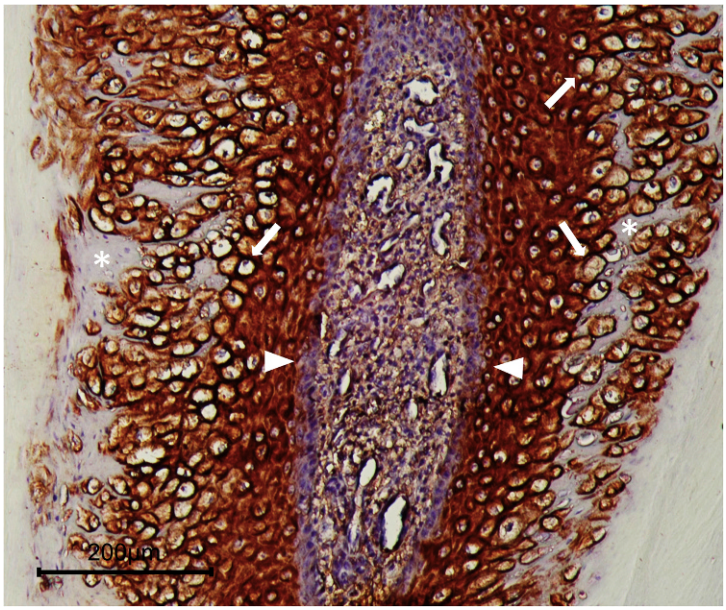

Figure 7 – Photomicrograph of a transverse section of the lamellae of a laminitic horse immunohistochemically stained for NET. The keratinocytes of the epidermal lamellae are strongly labelled for NET, whereas the lamellar basal cells are less intensely brown coloured (arrowheads). The dermal tissue 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

Experimental section

Study 4

Macroscopic and microscopic histopathological characteristics of hoof lamellae of horses and donkeys with severe naturally occurring laminitis

In preparation for submission in Veterinary Pathology, 2024.

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Abstract

Laminitis is a common, severe and multifactorial condition of the hoof lamellae of equids. The mechanisms involved in its pathophysiology have been investigated using histopathology in the developmental phase of the disease in equine models. However, information regarding lesions in naturally occurring laminitis is lacking in horses and donkeys. This study describes macroscopic and microscopic lesions in equids with naturally occurring end-stage laminitis.

Lamellar samples were collected in 9 horses and 5 donkeys euthanized due to severe laminitis and in 8 control horses and one control donkey. Samples were processed for histology and stained with haematoxylin-eosin, periodic acid-Schiff and Masson's trichrome before evaluation by light microscopy and analysis with a digital pathology software (QuPath).

Macroscopic analysis revealed overall thickening of the lamellar aera, haemorrhage, abnormal lamellar architecture, lamellar separation, exudate, focal abscesses. Histomorphometric analysis showed thickening of the area containing the lamellar tissue ($p<0.001$), an increased number of epidermal bridges ($p<0.001$), increased total primary epidermal lamellar lengths ($p=0.02$) and narrower primary dermal lamellae ($p=0.004$). The dermis showed fibrosis and the proportion of keratosis increased while the proportion of dermal tissue decreased with progressively worsening pathology. The descriptive analysis showed severely altered lamellar architecture, varying degrees of hyperkeratosis progressively developing into severe dyskeratosis, cellular degeneration and necrosis, basement membrane detachment, oedema and inflammatory cell infiltration. None of these abnormalities were seen in controls.

This study constitutes the first to provide a detailed description of macroscopic and microscopic lesions in horses and donkeys with naturally occurring laminitis, thereby providing additional insights in the progression of the disease.

Keywords: Horse – Donkey – Laminitis – Epidermal Lamellae – Dermal lamellae – Neutrophils

Introduction

Laminitis is a common and severe disease of the digit of horses and may account for 15% of lameness cases in the equine population (de Laat *et al.* 2013a). It is characterised by a structural failure of the dermal-epidermal interface, causing destabilization and ultimately displacement of the distal phalanx, thereby leading to severe lameness and dramatic pain, sometimes requiring euthanasia (Menzies-Gow *et al.* 2010, Katz and Bailey 2012, Leise 2018). Laminitis often has a multifactorial etiology and results from local consequences of an excessive systemic inflammatory reaction with decreased blood flow (ischemia) 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, Serteyn *et al.* 2014).

Histologic analysis has been used to study the pathogenesis of laminitis in a variety of models representing different etiologies. For example, the systemic inflammatory response syndrome (SIRS) was simulated using the black walnut heartwood extract model or by alimentary carbohydrate overload with starch or oligofructose (Garner *et al.* 1975, Van Eps and Pollitt 2010, Belknap 2010). A prolonged hyperinsulinemic clamp (pEHC) has provided a way to model endocrinopathic laminitis (Asplin *et al.* 2007). Using these different models, histological descriptions of experimentally-induced laminitis have enhanced appreciation of the pathophysiological processes involved by defining features of the laminitis lesions (Pollitt 1996, Morgan *et al.* 2003, Faleiros *et al.* 2004, Van Eps and Pollitt 2009, Asplin *et al.* 2010).

Reports include histological descriptions of lamellar morphology (Pollitt 1996, Morgan *et al.* 2003, de Laat *et al.* 2011, de Laat *et al.* 2013a), assessment of basement membrane integrity (Pollitt 1996, Asplin *et al.* 2010), evaluation of the degree of apoptosis (Faleiros *et al.* 2004), leucocytic infiltration using calprotectin immunohistochemistry (Faleiros *et al.* 2009, de Laat *et al.* 2011, Visser and Pollitt 2011) and presence of degranulation products of neutrophils like myeloperoxidase (Riggs *et al.* 2007, Storms *et al.* 2022) and elastase (de la Rebière de Pouyade *et al.* 2010). The reports of experimentally induced laminitis, usually describe early changes in the developmental phases of laminitis. However, information regarding histological changes during disease progression and in end-stage laminitis cases is scarce. Furthermore, studies including naturally-occurring laminitis cases are scant (Roberts *et al.* 1980, Wattle 2000, Karikoski *et al.* 2015, Karikoski *et al.* 2016, Cassimeris *et al.* 2019). Karikoski et al (2015) report a clinical study including 14 natural endocrinopathic laminitis cases. Another study describes lamellar abnormalities in 6 horses with PPID and laminitis, 10 horses with PPID without laminitis and 10 control horses (Karikoski *et al.* 2016). Interestingly this study showed that the lamellar architecture of PPID animals without laminitis was normal. Severity of lamellar pathology of PPID animals with laminitis was variable and seemed to be unrelated to the duration of laminitis. Cassimeris et al (2019) described lamellar lesions in 12 cases of endocrinopathic laminitis. However, none of these studies included

macroscopic and microscopic evaluation of the lesions in clinical cases with other etiologies than endocrinopathic disturbances. Furthermore, information on laminitis lesions in donkeys is lacking. An accurate description of various types of experimentally induced and naturally occurring cases of laminitis is essential in order to improve our understanding of the disease progression.

Besides being a common disease in the horse, laminitis is also a very common cause of lameness in donkeys, with a prevalence of 4.2% (Thiemann *et al.* 2021). Donkeys are usually considered small horses and are therefore treated in the same way. However, some anatomic differences are important to acknowledge. For example, donkeys have an upright boxy hoof with a solar surface that has an oval shape and the dorsal hoof wall is 5-10° more upright. The frog is well-developed in the palmar/plantar aspect but does not extend as far rostrally as in the horse (Thiemann *et al.* 2021). The third phalanx sits more distally in the hoof capsule and therefore palpation of a depression at the coronary band to indicate sinking of the phalanx is less straightforward and the founder distance is increased compared to the horse, with founder distance above 13 mm considered to indicate distal displacement of the third phalanx (Thiemann *et al.* 2021). The internal structure of the horn in the donkey hoof has a higher moisture content (Hopegood *et al.* 2004), easing the adaptation to the arid environment that donkeys originate from. These differences in anatomical and ultrastructural properties are likely to influence both the mechanical proprieties of the hoof and the development of foot pathology, particularly when donkeys are kept in wetter, temperate climates (Thiemann *et al.* 2021). Whether these anatomic differences influence lesions' histologic appearance and evolution in laminitis cases remains elusive.

The objectives of the study were to describe macroscopic and microscopic lesions in end-stage, naturally occurring laminitis in both horses and donkeys.

Material and methods

Animals

This study included a total of 24 animals. Fourteen equids (9 horses and 5 donkeys) presented at the Teaching Hospital of the University of Liège between June 2020 and August 2023 with naturally developed laminitis diagnosed based on clinical signs and radiographic examination. These animals were euthanized due to severe and uncontrolled laminitis. 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 9 horses from the slaughterhouse without any sign of acute laminitis or systemic inflammatory disease were used as controls. Lamellar samples of one control donkey were collected as well. Signalment and clinical examination findings were collected for these cases.

Collection of lamellar tissue and slide preparation

Lamellar tissues were collected immediately after euthanasia. The 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 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. (i) For light microscopy analysis the tissues were placed in 4% formaldehyde for 24h and then transferred to 70% ethanol for 48h before paraffin embedding. (ii) For transmission electron microscopy the samples were devided in small fragments, fixed in 2% glutaraldehyde in phosphate buffer and post-fixed in a 2% aqueous solution of osmium tetroxide. After slicing, the ulthrathin sections were contrasted with uranyl acetate and lead citrate.

Macroscopic analysis

Macroscopic analysis was performed on the isolated sections prior to shaping them for paraffine embedding and histologic processing. The sample was evaluated for the presence of haemorrhage, lamellar architecture, visible lamellar separation, exudate, abscess formation and softness of palpation.

Light microscopy

Histological sections of 4 µm of hoof wall were mounted onto glass slides and stained with Haematoxylin and eosin (HE), Masson's trichrome (MT, to highlight fibrosis) and periodic-acid Schiff (PAS, to highlight the basement membrane). The slides were scanned with an Axioscan 7 scanner (Zeiss, Germany). Whole slide images were analysed with an open-source automated software analysis program for digital pathology (QuPath version 0.4.3). The slides were analysed by two experienced pathologists and one PhD student trained in hoof pathology.

Morphometric measurements of the primary and secondary dermal and epidermal lamellae are shown in Figure 1 and are based on existing literature (de Laat *et al.* 2011, Karikoski *et al.* 2015). All measurements were performed at 8 consecutive primary epidermal lamellae (PEL) or primary dermal lamellae (PDL) and were performed using QuPath.

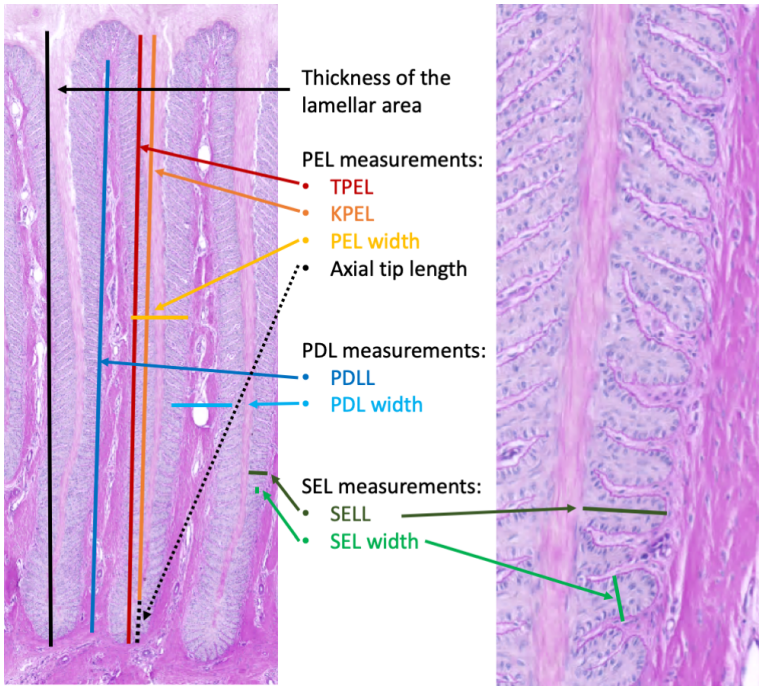

Figure 1 – Indication of the measurements performed on primary and secondary epidermal and dermal lamellae. PEL: primary epidermal lamellae, TPEL: total primary epidermal lamellar length, KPEL: keratinized primary epidermal lamellae length, PDL: primary dermal lamellae, PDLL primary dermal lamellae length, SEL: secondary epidermal lamellae, SELL: secondary epidermal lamellae length

To objectively determine keratosis and fibrosis, a region of interest of 0.3 cm^2 was drawn on each occasion and automated tissue detection was performed to correct for blank spaces. Thereafter, for keratosis and fibrosis quantification, built-in algorithms for pixel classification of QuPath and machine learning were used, and the percentage of area taken by keratin or collagen in the region of interest was calculated (Fig. 2).

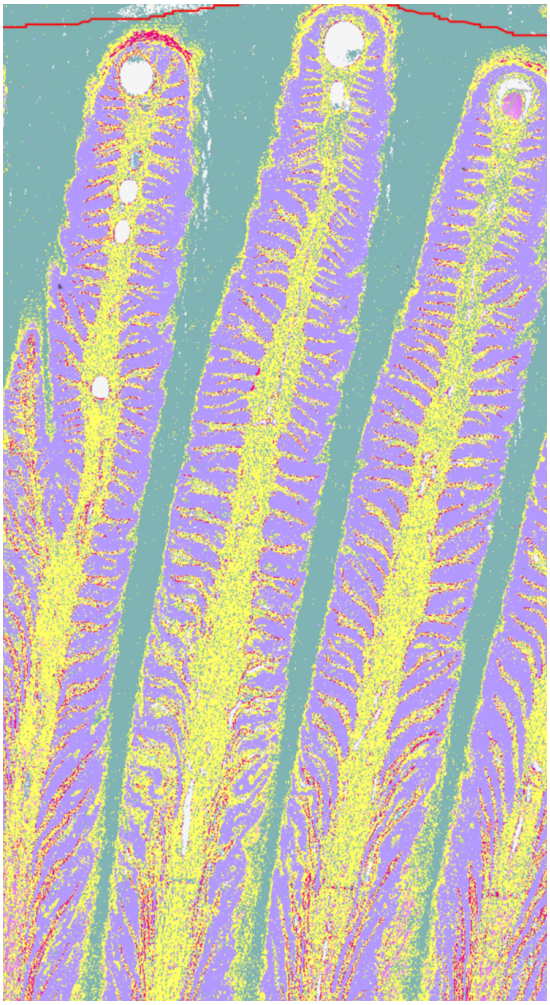

Figure 2 – Indication of the measurement of keratosis (green), epidermal tissue (purple), dermal tissue (yellow).

Further descriptive analysis included PEL and secondary epidermal lamellae (SEL) type, PEL and SEL tip shape, PEL-SEL angle, epidermal bridges (epidermal tissue bridging the gap from one PEL to another, usually in an abaxial location at PEL bases), keratin pearls, presence of epidermal islands (isolated epidermal tissue surrounded by dermis, usually located close to the SEL), keratocyte apoptosis, haemorrhage, basement membrane abnormalities, inflammatory cell infiltration, vascularization and oedema.

Transmission electron microscopy

Contrasted ultrathin sections were observed with a Zeiss EM910 electron microscopy.

Statistical analysis

Statistical analyses were performed with commercially available software (Medcalc 20.218, Ostend, Belgium). All clinicopathological parameters were normally distributed according to the Kolmogorov-

Smirnov test and expressed as mean ± standard deviation. A Student's unpaired t-test was performed to compare differences between the values of the different variables. Statistical significance was set at $p<0.05$.

Results

Signalment and history

Supplementary material 1 summarizes the details about the history and signalment of each case.

Laminitis cases

The mean age of the 14 laminitis cases was 14.0 ± 5.2 years old, with ten females and four geldings. There were five warmblood-type horses, two ponies, one paint horse, one fjord pony and five donkeys. The mean weight was 453.6 ± 121.5 kg with a mean body condition score of 6.9 ± 1.9/9 and a mean creasty neck score of 2.9 ± 1.4/5.

In most horses, the etiology of laminitis was multifactorial including endocrinopathic laminitis with equine metabolic syndrome or pars pituitary intermedia dysfunction which was sometimes combined with other pathologies like pneumonia, herpes virus myeloencephalopathy, renal insufficiency, hepatic lipidosis and endotoxemia (see supplementary material 1).

Control cases

Samples of the control horses were taken at the slaughterhouse therefore their individual history was unknown. Samples of the control donkey were taken at the pathology department of the faculty of Veterinary Medicine, University of Liège.

The mean age of the control cases was 19.6 ± 6.3 years old, with seven females and three geldings. There were 6 warmblood-type horses, one Appaloosa, one Haflinger, one pony and one donkey. The weight of the control horses was not available. The donkey weighed 150 kg. The mean body condition score of all control animals was 5.8 ± 0.9/9 and the mean cresty neck score was 1.4 ± 0.8/5.

Control horses were significantly older than laminitis cases (n=23, p=0.035). Laminitis cases had a significantly higher cresty neck score (n=23, p=0.005). There was no significant difference between the body condition scores of control and laminitis cases (n=24, p=0.06).

Clinical examination and radiographic findings

The clinical parameters for each case are shown in Supplementary material 2.

All but two laminitis cases presented with tachycardia (mean: 73.7 ± 24.4 bpm) and all but one laminitis case presented tachypnoea (mean: 40.7 ± 24.2 rpm). The rectal temperature was usually within normal limits except for two horses and one donkey (mean 38.0 ± 1.2°C). All laminitis cases had increased

digital pulses. Other clinical signs included a palpable depression at the coronary band (13/14), palpable increased hoof temperature (10/14), growth lines (9/14), visibly thickened white line (10/11), convex sole (9/13) and a painful response to palpation with a hoof tester (6/7). 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 (12/13) including saw-horse stance (11/12), lying down more frequently (4/12) and one horse was permanently 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 herpes virus myeloencephalopathy. The mean Obel grade of lameness was 3.5 $\pm$ 0.7/5.

All control horses and the control donkey did not present the clinical signs related to laminitis discussed here. Furthermore, they did not present any fever (mean temperature: 37.4 $\pm$ 0.5°C), indicating probable absence of systemic disease. Heart rate and respiratory rate were not assessed as they were not considered representative for their normal condition given the stressful environment (slaughterhouse) the animals were in. For the control donkey this information was not available.

Radiographs were performed in 12/14 laminitis cases and the individual case information can be found in Supplementary material 3. The most common radiographic finding was capsular rotation (11/12) followed by sinking of the third phalanx (10/12) and phalangeal rotation (7/12). Furthermore, some horses presented air opacities (6/12), osteitis of the third phalanx (5/12) and lipping (3/12). A convex sole was observed in 6/12, two of which also presented sole perforation.

No radiographs were taken of the feet of the control animals.

Macroscopic analysis

Macroscopic samples were available for all included cases. All laminitic horses and donkeys showed thickening of the area containing the lamellar tissue, between the inner horn and the dermis. Hemorrhage (13/14) was usually localized in the epidermal tissue. Macroscopic lamellar architecture revealed alterations such as wavy appearance of the PEL, irregular PEL lengths, elongation of the PEL that were observed also histologically (Fig. 3). Visible lamellar separation (13/14) usually occurred abaxially and was visible as regular, longitudinal spaces between the lamellae or as irregularly located focal spots of lamellar separation (Fig. 3). Exudate from the sample (11/14) mainly originated from the spaces between lamellar separation. Focal abscess formation was only observed in one horse. Palpation of the sample revealed a soft consistency.

Macroscopic analysis of the lamellar tissue of control cases did not show any of the abnormalities mentioned above. Interdigitation between the primary epidermal and dermal lamellar was macroscopically visible as regular, straight lines, all of similar lengths (Fig. 3). Compared to laminitis cases, the lamellar tissue of control animals was firmer.

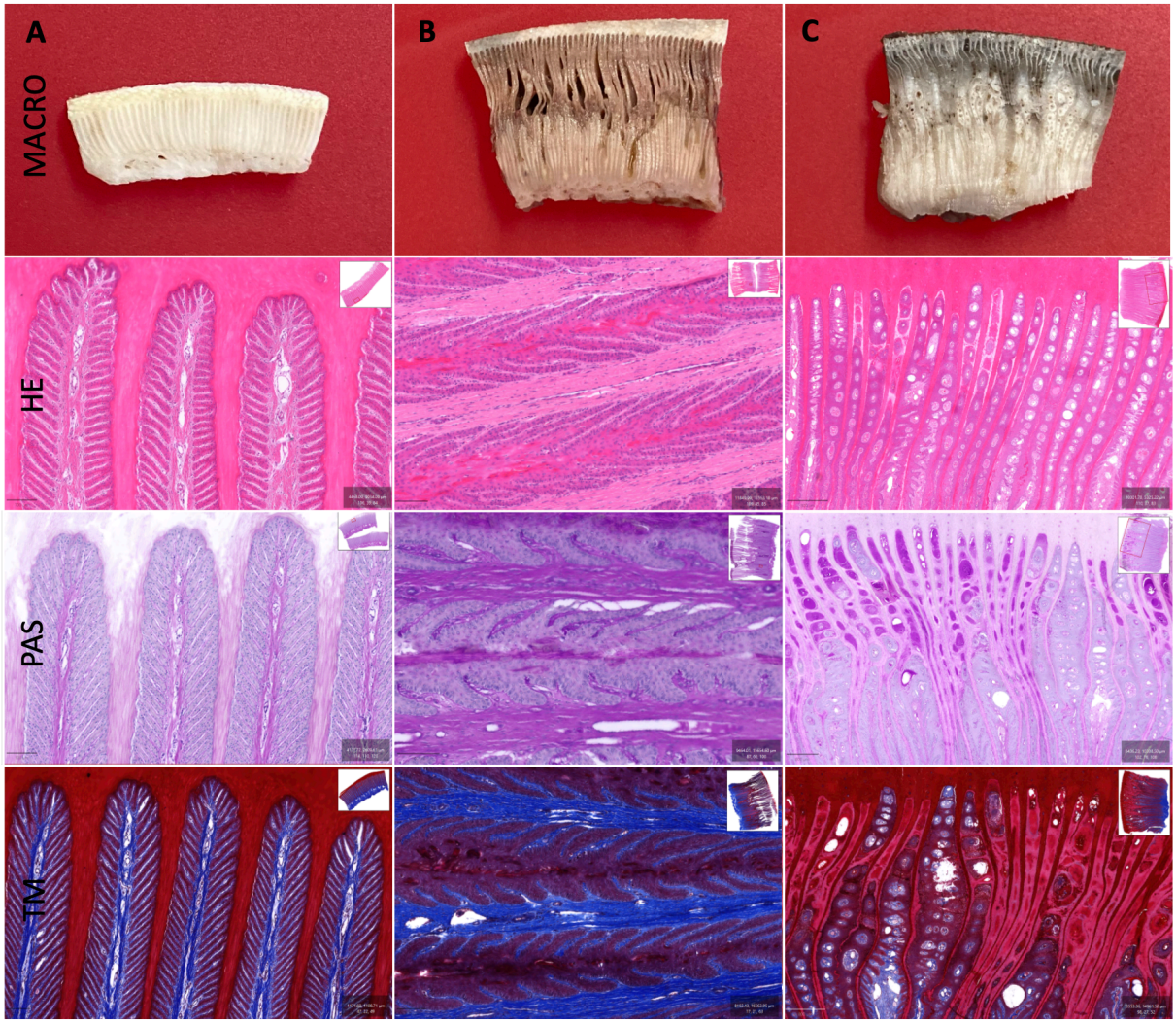

Figure 3 – Macroscopic pictures and photomicrographs of lamellar tissue with HE, PAS and MT staining of a control horse (A), laminitic donkey (B) and laminitic horse (C) showing increasing severity of lamellar pathology (A<B<C). A: shows normal macroscopic and microscopic anatomy. B: depicts significant macroscopic lamellar separation that is observed as well microscopically. C: indicates abnormal lamellar architecture with severe hyperkeratosis visible on the macroscopic and microscopic images.

Microscopic analysis

Histomorphometry

Histomorphometric parameters were measured when possible. In some cases, tissue destruction was too severe to adequately identify some anatomic structures, making measurements impossible. In these cases, the dermal and epidermal tissues were completely separated (2/5 donkeys, 7/9 horses). There were no significant differences between laminitic horses and laminitic donkeys, therefore the entire laminitis group was compared to the entire control group. A detailed analysis of the histomorphometric parameters is presented in Table 1.

The total sample thickness was greater in laminitis cases compared to controls. The number of epidermal bridges was higher in laminitis cases than in controls where no epidermal bridges were observed in any case. The TPEL and KPEL lengths were greater in laminitis cases compared to controls. The PDL were narrower in laminitis cases compared to control horses (n=18, p=0.004).

	Criteria	**Laminitis**			**Control**			**Student's t-test**
		Mean	SD	n/14	Mean	SD	n/10	p
	Thickness of the lamellar area (µm)	10886.14	3754.57	14	4140.00	819.91	10	**0.000***
PEL	Epidermal bridges (number)	2.14	0.95	14	0.00	0.00	10	**0.000***
	TPEL (µm)	8228.81	3399.80	6	3850.94	745.53	10	**0.025***
	KPEL (µm)	6326.85	2504.58	6	3598.63	718.52	10	**0.044***
	Axial tip length (µm)	1901.96	1671.64	6	252.31	62.60	10	0.060
	PEL width (µm)	238.75	93.11	8	244.73	55.33	10	0.876
SEL	SELL at PEL base (µm)	182.65	84.67	6	145.95	64.66	10	0.386
	SELL at PEL tip (µm)	257.48	171.71	6	98.44	39.87	10	0.073
	SEL width (µm)	27.35	6.48	5	25.11	3.55	10	0.502
PDL	PDL length (µm)	6093.82	2749.64	7	3632.63	697.38	10	0.056
	PDL width (µm)	154.63	80.20	8	269.84	49.95	10	**0.004***
	Keratin pearls (number)	4.21	7.16	14	0.63	1.77	3	0.094
	Islands of epidermal tissue (number)	1.50	1.70	14	0.20	0.42	2	**0.015***

Table 1: Results of the histomorphometric analysis of lamellar samples. *statistically significant ($p<0.05$). PEL: primary epidermal lamellae, TPEL: total primary epidermal lamellar length, KPEL:

keratinized primary epidermal lamellae length, PDL: primary dermal lamellae, PDLL primary dermal lamellae length, SEL: secondary epidermal lamellae, SELL: secondary epidermal lamellae length

Descriptive analysis

The main observations in tissue of laminitis cases included an altered lamellar architecture and epidermal dyskeratosis that was more severe in cases with severe anatomic abnormalities (total loss of structure). Varying degrees of hyperkeratosis (Fig. 4) progressively developed into severe dyskeratosis, present in most cases (Fig. 5). The dermis showed fibrosis and the proportion of keratosis increased while the proportion of dermis decreased with progressively worsening pathology. Cellular degeneration and necrosis were most advanced in areas of severe anatomical abnormalities. The basement membrane often showed detachment and this was usually observed macroscopically too. Other main characteristics included oedema formation and inflammatory cell infiltration, including mainly neutrophils (Fig. 6). None of these abnormalities were seen in controls.

A detailed comparison of the observations between laminitis cases and controls is provided in Table 2.

Criteria		Laminitis case	Control
Lamellar characteristics	PEL shape	Curving	Straight
	PEL lengths/ thickness	Variable	Similar
	PEL tips	Sharp (11/14), tapered (2/14) > bifurcated (1/14)	Standard
	SEL type	Variable between cases and in the same sample: usually mixed or tapered > fused > club shaped, bifurcated	Standard
	SEL tip	Tapered (10/14) > round (1/14), unrecognizable (3/14)	Round
	PEL-SEL angle	Sharp (<45°, 9/14) or 45-90° (4/14), unrecognizable (1/14)	45-90°
Epidermal tissue		Dyskeratosis (14/14) with hyperkeratosis (mainly orthokeratosis), islands of epidermal tissue, keratin pearls and epidermal bridges	Normal, occasionally a keratin pearl or epidermal island
Dermal tissue		Fibrosis with increased collagen deposit (14/14) and dermal cellularity (11/14).	Normal

Keratinocytes	Cellular characteristics	Degeneration and necrosis (14/14) with cytoplasmic vacuolisation, cellular swelling, cellular isolation and phantom cells	Normal
	Nucleus	Abnormalities (14/14) including varying degrees of nuclear swelling, picnosis, karyorrhexis and karyolysis	Oval shape and vertical orientation
Inflammation		Varying degrees of inflammatory infiltrates with mainly neutrophils (11/14) especially in destructed areas and dermis, some macrophages and lymphocytes	No
Basement membrane		Blebbing (14/14) mainly at SEL bases with total detachment (10/14) usually at PEL bases	Normal
Lymphatic system		Intercellular oedema (9/14) mainly in the dermis, most severe in severe cases, usually accompanied by enlarged lymphatic vessels (8/14); free fluid (13/14) mainly in areas where de dermis and epidermis appeared completely separated and in the dermal tissue (Fig. 7)	Normal
Vascular system		Prominent microvasculature (10/14), free red blood cells (12/14) mainly in dermis or separated tissue, no intravascular thrombi	Normal

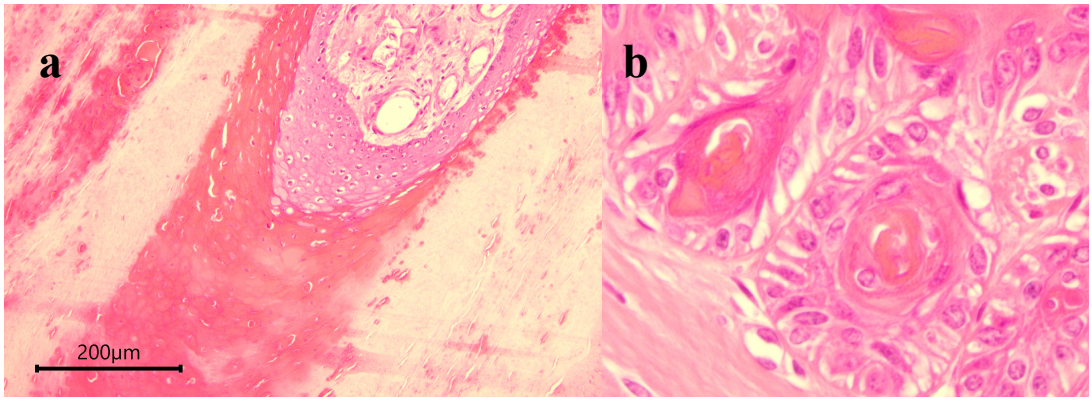

Figure 4 – Photomicrograph of lamellar tissue with HE stain showing severe hyperkeratosis with a para- and orthokeratotic component (a) and multiple keratin pearls (b).

Dermal tissue	39.15 %
Epithelium	35.50 %
Keratin	19.80 %

Dermal tissue	23.71 %
Epithelium	45.32 %
Keratin	14.72 %

Dermal tissue	18.80 %
Epithelium	17.86 %
Keratin	60.91 %

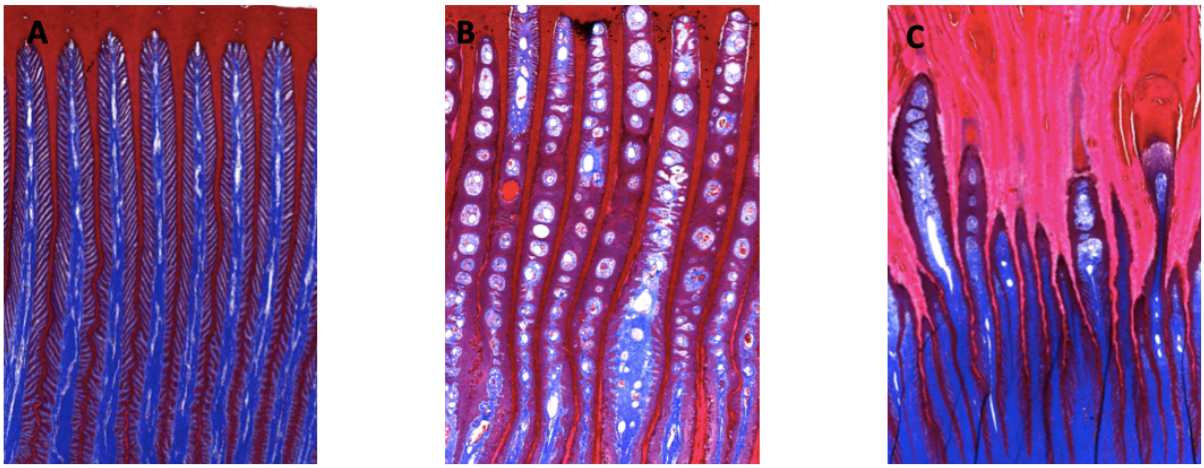

Figure 5 - Photomicrographs with MT stain showing the evolution of fibrosis and keratosis in control (A), moderate (B), and severe (C) laminitis cases varying from normal tissue to severe dyskeratosis. The proportion of keratin markedly increases while the proportion of dermal tissue decreases.

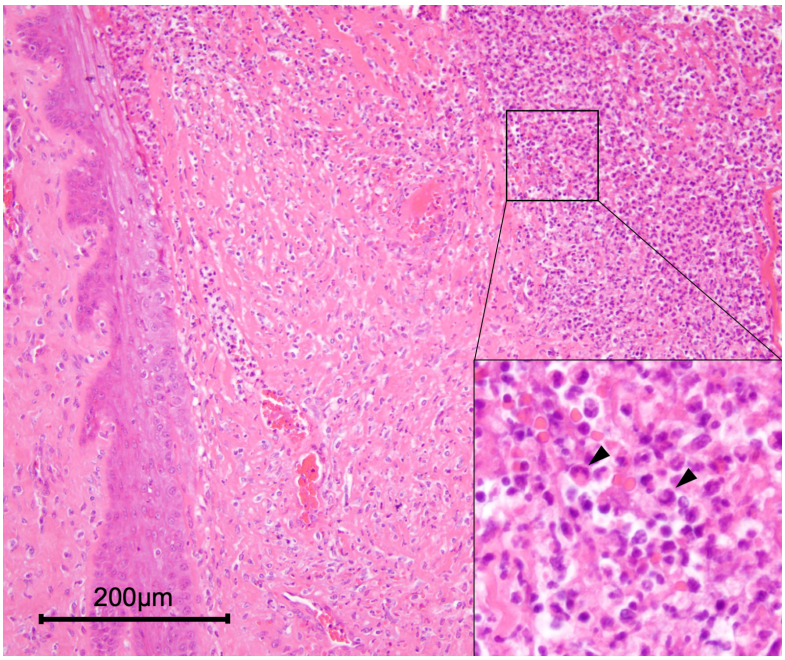

Figure 6 – Photomicrograph of lamellar tissue of a laminitic horse stained with HE showing severe neutrophilic infiltration in dermal tissue (arrows insert). Lamellar architecture is only recognizable on the left side of the image.

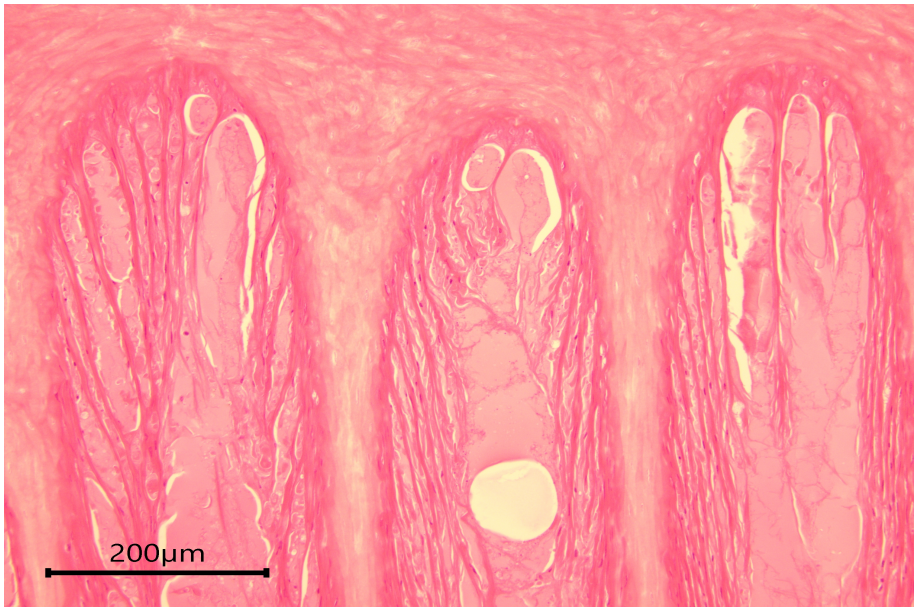

Figure 7 – Photomicrograph of lamellar tissue of a laminitic horse stained with HE showing the presence of free eosinophilic fluid in the empty spaces created by severe tissue retraction and separation around the keratinized axis.

Transmission electron microscopy

Images of transmission electron microscopy were available for 3 laminitic horses. They showed disappearance of hemidesmosomes with focal widening of the lamina densa and lamina lucida of the basement membrane (Fig. 8).

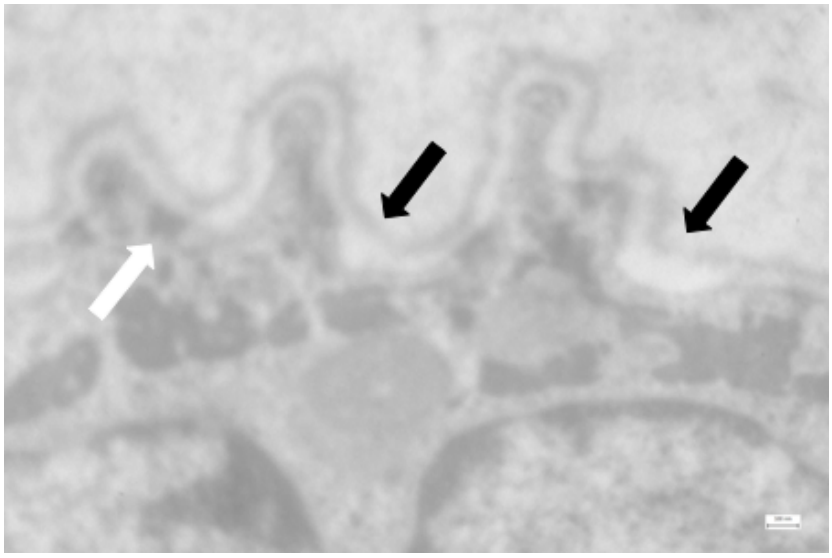

Figure 8 – Transmission electron microscopy image of a laminitic horse showing focal absence of hemidesmosomes causing widening of the lamina densa and lamina lucida (black arrows) near the remaining hemidesmoseomes (white arrow).

Discussion

This article is the first to describe both macroscopic and microscopic abnormalities observed in horses and donkeys with naturally occurring laminitis. Overall, the lamellar lesions observed in the present study were much more severe than those found in induced models of equine laminitis (Asplin *et al.* 2010, de Laat *et al.* 2011, de Laat *et al.* 2013a). This is due to the fact that horses were euthanized only after recurrent episodes of severe and uncontrolled laminitis. The main macroscopic and microscopic findings were the following: cellular degeneration, lamellar separation, loss of anatomic structure, and severe infiltration of neutrophils. Most of these lesions were so severe that they could even be observed macroscopically when looking at the sample before processing.

It was noted that the specimens in the present study were softer at palpation and often presented exudate on a macroscopic level, likely caused by the severe tissue degeneration and presence of oedema. Microscopically intradermal oedema was observed, as well as enlargement of the lymphatic vessels and the presence of free eosinophilic fluid mostly located in the empty spaces created by lamellar separation. This, combined with severe neutrophil infiltration and severely degenerated keratinocytes presenting picnosis, karyorrehxis, karyolysis and phantom cells, is evidence of necrosis.

Different mechanisms can compromise lamellar tissues and cause their degeneration. In the past, ischemia of the lamellar tissues due to the presence of intravascular thrombi, causing a lack of energy and blood flow to the lamellar tissues, has been evocated as a process causing laminitis to progress and caused severe lamellar damage with tissue necrosis as a consequence (Bailey 2017). However, this finding has been inconsistently described in literature (Weiss *et al.* 1994, Weiss *et al.* 1995, Asplin *et al.* 2010, de Laat *et al.* 2011), and this is enforced by the lack of intravascular thrombi observed in our study.

Studies on experimentally induced laminitis have demonstrated the activation of neutrophils early in the laminitis disease process (Riggs *et al.* 2007, de la Rebière de Pouyade *et al.* 2010). In addition, endothelial cell activation occurs in these early stages (Eades *et al.* 2002, John P. Loftus *et al.* 2007). Moreover, digital veins characterized by their high muscularity, low compliance, and high sensitivity to vasoconstrictive substances like endothelin-1(Allen *et al.* 1988, Baxter *et al.* 1989, Katwa *et al.* 1999, Eades *et al.* 2002), predispose the equine digit to elevated venous pressures, thereby increasing the hydrostatic pressure and therefore the escalating oedema formation (Eades *et al.* 2002). This phenomenon, together with the endothelial activation possibly causing an increase of endothelial permeability and neutrophil efflux, causes fluids to leave the vascular compartments and diffuse into the surrounding tissues, elucidating the oedema observed in our slides.

As the foot is enclosed in a non-elastic horn capsule, the presence of oedema elevates tissue pressure, causing the capillaries irrigating the lamellar tissue to collapse. This collapse deprives keratinocytes and other dermo-epidermal interface components, like hemidesmosomes, of vital nutrients, while waste product accumulation ensues, culminating in laminar necrosis and degeneration (Eades *et al.* 2002). Notably, signs of necrosis in keratinocytes, such as picnosis, karyorrhexis and karyolysis, along with phantom cells, become evident as observed in our slides. In addition, transmission electron microscopy images revealed the disappearance of hemidesmosomes, leading to basement membrane detachment and heightened lamellar instability, increasing the lamellar instability, a phenomenon seen as well in developmental phases of laminitis (French and Pollitt 2010). This necrotic tissue incites the inflammatory response with further neutrophil recruitment, as observed in our slides, thereby perpetuating the pathology in a vicious cycle.

The MPO released from activated neutrophils could also explain the relationship between neutrophils, endothelium and ischemia. The presence of MPO has been shown both in the early and late stages of laminitis (Riggs *et al.* 2007, Storms *et al.* 2022). During vascular inflammation, MPO is transcytosed across the endothelium and into the sub-endothelial extracellular matrix, where it promotes endothelial dysfunction by catalytically consuming nitric oxide (NO), for the production of free radicals through its peroxidase activity (Abu-Soud and Hazen 2000, Thai *et al.* 2021). Moreover, a recent study has shown that MPO increased vascular permeability, and that MPO modulates the overall charge of the vascular glycocalyx through interaction with glycosaminoglycans, causing modifications of its structure and thus affecting endothelial cell function (Kolářová *et al.* 2021). The production of free radicals that cause further local damage in combination with the endothelial dysfunction, could cause more leakage along the endothelial tissues. It would be interesting to study the presence of endothelial leakage in laminitis cases using Evans blue dye, for example.

In addition, MPO can be seen as a competitor for the consumption of NO, used by the endothelial cell to allow vasodilation. If vasodilation is then impaired, the blood flow to the foot is reduced, consequently causing a decreased influx of oxygen and nutrients and increased stasis of waste products. This can cause an energy deficit, resulting in cellular dysfunction and detachments of hemidesmosomes. Due to the lack of oxygen, mitochondrial dysfunction occurs, worsening the energy deficit and causing an increased production of ROS (Serteyn *et al.* 2014). Indeed, mitochondrial dysfunction has been shown to be present in the muscular tissue of horses presented with laminitis of varying etiologies (Serteyn *et al.* 2014). The combination of these abnormalities could cause local recruitment of neutrophils, which will then create more damage through this second possible vicious circle.

In our study, neutrophils were observed in many cases, and these neutrophil infiltrates were mainly localized in the dermis and sites of these severe tissue abnormalities. This raises the question of whether neutrophil activation occurs as a cause or a consequence of the disease process. The implication of

neutrophils and their byproducts like MPO and elastase has been confirmed early in the disease process in previous studies (Riggs *et al.* 2007, de la Rebière de Pouyade *et al.* 2010) using the BWE model and was confirmed in our research using the pEHC model (Storms *et al.* 2022), thereby indicating their involvement in the onset of the disease. However, it is recognized that neutrophils are attracted to sites of significant tissue damage. In our clinical end-stage cases, neutrophils were indeed observed in the most destructed parts of the tissue. It could be argued that they either contribute to this severe damage when inflammatory reactions become uncontrolled or that they are drawn to these sites due to the already existing damage. A combination of both these mechanisms seems likely. Given that MPO was present in the early stages of laminitis, it is conceivable that this inflammatory cycle becomes uncontrolled, causing or exacerbating local tissue damage. Subsequently, additional neutrophils may be attracted to these sites, perpetuating this cycle of cause and effect.

Our study included various cases, all diagnosed with laminitis, but having different inciting causes or even combinations of them. Interestingly at this advanced stage of laminitis, it was not possible to define specific lesions related to precise inciting causes; this prompts us to conclude that regardless of the inciting cause, once the pathology is installed, a similar evolution occurs for all cases and ultimately leads to desolidarisation of the dermo-epidermal interface with tissue separation as a consequence. Also, no notable differences were observed between the section of horses and donkeys; therefore, the disease progression is probably similar in both species regardless of their anatomic variations concerning the structure of their feet. Unfortunately, this study included only one control donkey; it was therefore not possible to draw any conclusions with regard to any histological differences between the lamellar tissue of healthy horses and donkeys.

This study has some limitations. First, histomorphometric results did not show significant differences for all parameters. However, it is important to notice that the measurements considered probably underestimate the true extent of the lesions, as the most severe cases were not measured because of the severity of tissue abnormalities making measurements impossible. Furthermore, the consequently reduced the sample size for those measurements, could have impacted the validity of the findings. These factors suggest that certain measurements may have differed significantly if they had been obtainable for all cases. In addition, most of the values were quite standard in control cases and similar in between the measured PEL, whereas in laminitis cases they showed more variability between lamellae of the same case and between cases (explaining the high standard deviations observed), as for example SEL lengthening was observed as well as SEL fusion.

Secondly, another limitation arises regarding the 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 disease episodes 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. Furthermore, it was possible to include only one control donkey; this made drawing conclusions about the normal histological appearance of the lamellar tissue of donkeys and its comparison to the tissue of horses impossible. Unfortunately, it proved very difficult to collect tissue for control donkeys as they are not allowed in the slaughterhouse, and many of the ones that are euthanised for clinical reasons are overweight or show signs of systemic inflammation and are therefore not suitable candidates for this paper.

Conclusion

In conclusion, our study is the first to provide a detailed description of the macroscopic and microscopic abnormalities observed in end-stage laminitis cases, including both horses and donkeys. Newly described elements include tissue oedema, increasing keratosis and decreasing dermal tissue proportions as well as severe inflammatory infiltrates. Thereby, we provide additional insights into the disease progression in equids and histologic abnormalities observed in donkeys.

Conflict of interest

No conflict of interest.

Acknowledgements

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Supplementary materials

See tables below.

Cases		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
Horse 8	First episode of laminitis worsening over 2 weeks, stric diet management since 1 month	Warmblood	F	15	570	8	3
Horse 7	Severe pneumonia and laminitis, tested positively for PPID, presented recurrent laminitis episodes	Pony	G	20	435	4	0
Horse 6	Severe laminitis progressively worsening over 1.5 months, tested positively for EMS	Warmblood	G	13	612	9	5
Horse 9	Recurrent laminitis episodes for 4 years, first hospitalization for laminitis 6 weeks prior with successful management, now severe relapse with sole perforation, tested positively for PPID	Pony	F	18	349	5	1

Controls							
Horse 1	/	Haflinger	F	21	/	6	2
Horse 2	/	Warmblood	F	15	/	8	3
Horse 3	/	Appaloosa	H	14	/	6	2
Horse 4	/	Warmblood	H	20	/	6	1
Horse 5	/	Warmblood	F	20	/	5	1
Horse 6	/	Warmblood	F	17	/	6	2
Horse 7	/	Warmblood	F	25	/	5	1
Horse 8	/	Warmblood	G	31	/	5	1
Horse 9	/	Pony	F	24	/	6	1
Donkey 1	Severe bleeding post castration	Donkey	G	9	150	5	0

Supplementary material 1: Table containing the details about the signalment and history of all included cases. EMS: Equine metabolic syndrome, PPID: pars pituitary intermedia dysfunction, BCS: body condition score, CNS: cresty neck score, G: gelding, F: female.

Cases	Heart rate at arrival (bpm)	Respiratory rate at arrival (rpm)	Rectal temperature (°C)	Growth lines	Increased hoof temperature hoof	Increased digital pulse	Depression coronary groove	Convex sole hoof	Visibly thickened white line	Positive response to hoof tester	Obel grade of lameness (/4)	Posture
Horse 1	84	36	38,3	No	FL, HL	FL > HL	FL, HL	FL	/	/	4	Saw-horse stance, often recumbent
Horse 2	60	16	38,1	FL, HL	No	FL > HL	FL, HL	No	FL (reddish color)	Fl, HL	4	Saw-horse stance, often recumbent
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	Solar 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
Horse 8	48	42	39,5	No	FL	FL	FL	FL	FL	FL	4	Saw-horse stance, often recumbent
Horse 7	80	40	37,2	No	FL	FL	FL	FL	FL	/	3	Saw-horse stance, often recumbent
Horse 6	88	44	38,8	FL, HL	FL, HL	FL,HL	FL	FL	FL,HL	/	4	Saw-horse stance
Horse 9	40	20	37,9	FL, HL	FL, HL	FL, HL	FL, HL	FL, HL solar perforation LF, RH	FL,HL	FL,HL	3	Saw-horse stance
Controls												
Horse 1	/	/	38,3	No	No	No	No	No	No	/	No	No
Horse 2	/	/	37,9	No	No	No	No	No	No	/	No	No
Horse 3	/	/	37,2	No	No	No	No	No	No	/	No	No
Horse 4	/	/	36,7	No	No	No	No	No	No	/	No	No
Horse 5	/	/	37,7	No	No	No	No	No	No	/	No	No
Horse 6	/	/	37,2	No	No	No	No	No	No	/	No	No
Horse 7	/	/	37,5	No	No	No	No	No	No	/	No	No
Horse 8	/	/	37,8	No	No	No	No	No	No	/	No	No

Horse 9	/	/	36,7	No	No	No	No	No	No	/	No	No
Donkey 1	/	/	/	No	No	No	No	No	No	/	No	No

Supplementary material 2: Table containing all information about all clinical parameters for each individual case. Bpm: beats per minute, rpm: respirations per minute, FL: Forelimb, HL: Hindlimb, LF: left forefoot, RF: right forefoot.

Cases	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
Horse 8	Yes	Yes	Yes	No	No	No	Yes
Horse 7	/	/	/	/	/	/	/
Horse 6	Yes	Yes	Yes	Yes	No	Yes	Yes
Horse 9	Yes	Yes	Yes	Sole perforation	Yes	Yes	Yes
Controls							
Horse 1	/	/	/	/	/	/	/
Horse 2	/	/	/	/	/	/	/
Horse 3	/	/	/	/	/	/	/
Horse 4	/	/	/	/	/	/	/
Horse 5	/	/	/	/	/	/	/
Horse 6	/	/	/	/	/	/	/

Horse 7	/	/	/	/	/	/	/
Horse 8	/	/	/	/	/	/	/
Horse 9	/	/	/	/	/	/	/
Donkey 1	/	/	/	/	/	/	/

Supplementary material 3: Table containing all radiographic information for each individual case. P3: third pha

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Experimental section

Study 5

Revealing the Therapeutic Potential of Muscle-Derived Mesenchymal Stem/Stromal Cells: An In Vitro Model for Equine Laminitis based on Activated Neutrophils, Anoxia-Reoxygenation and Myeloperoxidase

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Abstract

Laminitis in horses is a crippling condition marked by the deterioration of the dermal–epidermal interface, leading to intense lameness and discomfort, often necessitating euthanasia. This study aimed to establish an in vitro model of laminitis using a continuous keratinocyte cell line exposed to anoxia-reoxygenation and activated neutrophil supernatant. A significant decrease in keratinocytes metabolism was noted during the reoxygenation period, indicative of cellular stress. Adding muscle-derived mesenchymal stem/stromal cells during reoxygenation demonstrated a protective effect, restoring keratinocytes metabolic activity. Moreover, incubation of keratinocytes with activated neutrophil supernatant or myeloperoxidase alone induced increased keratinocyte myeloperoxidase activity, which was modulated by stem cells. These findings underscore the potential of muscle-derived mesenchymal stem/stromal cells in mitigating inflammation and restoring keratinocyte metabolism, offering insights for future cell therapy research in laminitis treatment.

Keywords: horse, laminitis, metabolism, keratinocyte, mesenchymal stem cell, mitochondria

Introduction

Laminitis, a prevalent condition in horses, is marked by the degradation of the dermal–epidermal junction, which can cause instability and potential displacement of the distal phalanx. This pathological process frequently results in severe lameness and acute pain, occasionally leading to euthanasia (Menzies-Gow *et al.* 2010, Katz and Bailey 2012, Leise 2018). Although the precise etiology remains incompletely understood, evidence suggests that laminitis arises from local manifestations of an exaggerated systemic inflammatory response, leading to compromised blood flow, foot inflammation, endothelial/vascular dysfunction, extracellular matrix degradation, and metabolic perturbations in keratinocytes (Peroni *et al.* 2005, Hurley *et al.* 2006, John P. Loftus *et al.* 2007).

The early involvement of polymorphonuclear neutrophils (PMNs) in the onset of laminitis is well established in the literature (de la Rebière de Pouyade and Serteyn 2011). In 2007, Riggs *et al.* demonstrated the presence of myeloperoxidase (MPO), a major protein from the alpha granules of neutrophils, in the bloodstream, skin, and lamellar tissue of horses with black walnut extract (BWE)-induced laminitis. Furthermore, recent evidence shows the presence of MPO in lamellar tissue of clinical cases suffering from severe laminitis (Storms *et al.* 2022). MPO is a heme-containing peroxidase expressed mainly in neutrophils and to a lesser degree in monocytes. In the presence of hydrogen peroxide and halides, MPO catalyzes the formation of reactive oxygen intermediates, including hypochlorous acid (HOCl). MPO has been demonstrated to be a local mediator of tissue damage and the resulting inflammation in various inflammatory diseases (Aratani 2018).

Even in cases where mitochondrial dysfunction has not been directly observed in the hoof, an energy deficiency has been proposed as a potential cause for the disruption of hemidesmosomes, which may contribute to the failure of the dermal-epidermal junction (French and Pollitt 2004). The relationships between inflammation, particularly neutrophil activation, either associated with ischemic reperfusion events or not, and mitochondrial dysfunction, are documented across various organs such as the heart, kidneys, and liver (Shepherd *et al.* 2022).

In 2014, a clinical study demonstrated a marked reduction in muscle mitochondrial oxidative phosphorylation in horses suffering from acute laminitis of various etiologies when compared to levels observed in both fit and obese healthy horses (Serteyn *et al.* 2014). Recently, He *et al.* (2023) demonstrated that Neutrophil Extracellular Traps (NETs) induced mitochondrial dysfunction in cardiomyocytes associated with an increase of reactive oxygen species (ROS) release.

Given that neutrophil activation is observed in the early stages of laminitis and that we have recently identified the presence of neutrophil extracellular traps (NETs) in severe clinical cases, we hypothesize

a potential correlation between neutrophil activation, MPO activity, NETs release and metabolism dysfunction in equine laminitis (Storms *et al.* 2024).

NETs are extracellular structures composed of decondensed DNA strands intertwined with histones and neutrophil granule proteins, such as MPO and elastase, which are released from neutrophils to trap and neutralize microbes. However, excessive NET formation during uncontrolled inflammatory responses has been implicated in the development of thrombosis and multiple organ failure in sepsis (Sørensen and Borregaard 2016, Delgado-Rizo *et al.* 2017, Li and Tablin 2018). In ischemic-reperfusion injury, Zhang *et al.* (2022) reported that MPO-DNA complexes were the more cited markers of NETs induction.

In sepsis, a recent metabolomic study confirmed that among patients with septic shock, non-survivors had deeper and more persistent dysregulation of protein analytes attributable to neutrophil activation and disruption of mitochondrial metabolism than the survivors (Jennaro *et al.* 2023).

Ischemic-reperfusion injury (IRI) takes place during reperfusion by activating inflammation and ROS production, causing mitochondrial damage and apoptosis of parenchymal cells (Miceli *et al.* 2023). Mesenchymal Stem/Stromal Cells (MSCs) for the treatment of multi-organ IRI is currently considered a valid approach to reducing the injury (Rowart *et al.* 2015). Moreover, extracellular vesicles (EVs) derived from human umbilical cord MSCs attenuate rat hepatic IRI by suppressing oxidative stress and the inflammatory response of neutrophils (Yao *et al.* 2019).

In 2017, Magana-Guerrero demonstrated that amniotic-derived MSCs could interfere with NETs release by neutrophil via a mitochondrial pathway. In our group, we described a minimally invasive technology to obtain muscle-derived MSCs (mdMSCs). These cells have similar properties as MSCs from other sources and showed potent immunomodulatory effects (Ceusters *et al.* 2017).

Preliminary clinical studies using MSCs in laminitis showed encouraging results. The therapeutic potential is attributed to the unique properties of the MSCs that target damaged tissues, inhibit the immune and inflammatory response, and facilitate repair (Barrett and MacDonald 2023). However, the mechanisms of action could be deeply investigated.

Considering the aforementioned points, the objectives s of this study were: to generate an in vitro model of laminitis using keratinocytes exposed to anoxia-reoxygenation (A/R) in conjunction with activated neutrophils supernatant and to reveal the potential therapeutic effect of mdMSCs on keratinocytes metabolism and the MPO activity.

Material and methods

1. Chemicals and Reagents

Analytical grade phosphate salts, sodium and potassium chloride, sodium hydroxide, sodium acetate, H2O2 (30%), dimethyl sulfoxyde (DMSO), Percoll (GE Healthcare), Hematoxylin solution modified acc. to Gill III (Merck) , T-flasks, conical bottom centrifuge tubes were purchased from Merck (VWR International, Leuven, Belgium). The bovine serum albumin fraction V (BSA) was obtained from Roche Diagnostics (Mannheim, Germany). Cytochalasin B (CB), N-formyl-methionyl-leucyl-phenylalanine (fMLP), sodium nitrite, cisplatin, Triton X-100 were purchased from Sigma-Aldrich (Bornem, Belgium). The 96-well microtiter plates (Combiplate 8 EB), Amplex red® (10-acetyl-3,7-dihydroxyphenoxazine) (Invitrogen), Paraformaldehyde (PAF) solution 4% in PBS (Thermo Scientific), trypsin TrypLE Express (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and Hank's balanced salt solution (HBSS) 1× (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and Fetal Bovine Serum (FBS) were purchased from Fischer Scientific (Merelbeke, Belgium). The Dulbecco's Modified Eagle Medium Ham's F12 (DMEM F12) culture medium with Hepes and glutamine, penicillin-streptomycin, amphotericin B and the Dulbecco's phosphate buffered saline (DPBS) were purchased from Lonza (Verviers, Belgium). The purified equine neutrophil MPO was characterized by a specific activity of 70.4 U/mg and a protein concentration of 3.38 mg/mL. Antibodies against equine MPO, derived from rabbits and guinea pigs, were sourced from Bioptis (Vielsalm, Belgium). Additionally, rabbit polyclonal antibodies targeting citrullinated Histone H3 (citrulline R2 + R8 + R17) were acquired from Abcam (Cambridge, UK).

2. Cells

Keratinocytes (HaCaT) and muscle-derived mesenchymal stem cells (mdMSCs) were purchased from ATCC (USA) and Revatis SA (Aye, Belgium) and cultured in DMEM high glucose with (10% FBS) and in DMEM-F-12 (20 % FBS), respectively.

3. Preparation and characterization of the activated neutrophil supernatant

3.1. Equine neutrophils were obtained from blood collected by jugular venipuncture on 5 healthy horses on EDTA tubes as described by Pycock et al. (1987). Briefly, the neutrophils were isolated at room temperature (18–22°C) by centrifugation (400 x g, 30 min at 20°C) on a discontinuous percoll density gradient. The polymorphonuclear fraction was collected in DPBS counted and diluted into DMEM high glucose + 10% FBS to obtain a suspension of 2 million neutrophils/ml. Neutrophils were stimulated by adding 1µl of cytochalasin B (5 mg/ml) per ml of cell suspension in the medium and incubating them for 30 min at 37°C. Thereafter, 10 µl of fMLP (10-4 M) per ml of cell suspension was added and cell were incubated for 30 min at 37°C. Control

conditions were performed in parallel with cell suspensions without the addition of CB and fMLP or with the replacement of the stimulating molecules by DMSO, the solvent used for their solubilisation (Ctrl DMSO). Finally, the cell suspensions were centrifuged for 5 minutes at 600 x g and the supernatants were collected and stored at -20°C for future experiments. Activated neutrophil and non-activated neutrophil supernatants were called ANS and NANS respectively.

3.2. Active free MPO and active MPO bound to the NET were measured in the supernatants (NANS and ANS) after neutrophil incubation and stimulation according to the techniques described by Storms *et al*. (2024).

3.3. Measurement of active MPO by SIEFED

The SIEFED (Specific Immuno-Extraction Followed by Enzymatic Detection) technique employs microplate wells coated with an immobilized primary antibody (polyclonal rabbit anti-MPO IgG). The undiluted supernatant was added to the wells and incubated for 2 hours at 37°C in the dark, facilitating the capture of MPO by the antibodies. Following sample removal and three washing steps, the substrate (H2O2) and co-substrates (nitrite and Amplex Red) were introduced to detect the peroxidase activity of MPO, which was indicated by the oxidation of Amplex Red to its fluorescent product, resorufin.In more detail, the peroxidase activity of MPO was monitored by adding 100 µL of a 40 µM Amplex red solution freshly prepared in 50 mM phosphate buffer, pH 7.4, supplemented with 10 µM H2O2 and 10 mM sodium nitrite. Fluorescence was measured at excitation and emission wavelengths of 544 nm and 590 nm, respectively, over 30 minutes at 37°C using a fluorescent plate reader (Fluoroskan Ascent, Fisher, Merelbeke, Belgium). The fluorescence intensity was directly proportional to the amount of active MPO present in the sample. MPO concentrations were determined by referencing a calibration curve generated with purified equine MPO, ranging from 2 to 140 ng/ml.

3.4. NET-bound-MPO activity MPO was measured in the supernatant after neutrophil incubation and stimulation according to the techniques described in Storms et al. (2024). Neutrophil extracellular traps (NETs) were captured using anti-histone H3 (citrulline R2 + R8 + R17; anti-H3Cit) antibodies. The presence of active MPO bound to the NETs was subsequently detected using the same method as in the SIEFED assay. This involved coating a transparent 96-well microplate with immobilized primary rabbit anti-H3Cit antibodies (0.5 µg/ml) diluted in 20 mM phosphate-buffered saline (PBS). Following removal of the coating solution, the plates were incubated for 150 minutes at 22°C with blocking buffer (PBS with 5 g/L of BSA) and then washed four times with PBS containing 0.1% Tween 20. The plates were dried for 3 hours at 22°C and stored in a dry environment at 4°C until use. Samples were loaded into the anti-H3Cit-coated wells in duplicate and incubated

for 2 hours at 37°C. After the supernatants were removed and the wells were washed four times with PBS containing 0.1% Tween 20, active MPO was measured. To reveal MPO's peroxidase activity bound to the NETs, sodium nitrite and Amplex Red were added as described in the SIEFED assay, and fluorescence was measured over 30 minutes using a fluorescent plate reader (Fluoroskan Ascent, Fisher, Merelbeke, Belgium). The level of active MPO bound to the NETs was evaluated using a calibration curve, ranging from 2 to 140 ng/ml, created with purified equine MPO in wells coated with polyclonal rabbit anti-MPO IgG antibodies.ith purified equine MPO but using wells coated with polyclonal rabbit anti-MPO IgG antibody.

4. Effect of NANS and ANS on HaCaT metabolism in normoxia and in anoxia.

HaCaT were seeded in 2 transparent 96 microwell plates (10,000 cells/well) for 24h in DMEM high glucose with (10% FBS) to let them adhere. Afterwards the medium was removed, the wells were washed once with HBSS then the plate was divided into 5 parts to add medium alone, medium containing cisplatin (1.10-3M) as cytotoxic control, medium prepared with non-activated (NANS) and activated (ANS) neutrophils. A control assay was performed with the supernatant obtained with neutrophils in presence of DMSO (Ctrl DMSO). In all tested conditions, the HaCaT medium was used without red phenol. One plate was exposed to anoxia with a controlled oxygen level at 0.2% and the other one to normoxia (18.6 % oxygen) for 48 h. After incubation, the plate incubated under anoxia was transferred to normoxia with the other plate and incubated during 24h. The medium from the wells of the two plates was removed and the wells were washed once with HBSS before the addition of 100 µl HBSS and 10 µl MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). MTS used as metabolic viability-based assays was from Promega (CellTiter 96 Aqueous non-radioactive cell proliferation assay®). Just after MTS addition a first reading of the absorbance at 490 nm was made with Multiskan Ascent spectrophotometer (Thermo labsystem, Finland) then every 1 h till 4 h to follow the evolution of the absorbance.

5. Effect of mdMSCs on HaCaT metabolism

HaCaT were seeded as above in two transparent plates for 24h but some wells were left empty. At cell adherence, the medium was removed, the cells were washed once with HBSS then the wells containing HaCaT were divided into 3 parts to add medium (without red phenol), medium containing cisplatin (1.10-3M) as cytotoxic control and the medium prepared with activated neutrophil (ANS). One plate was exposed to anoxia with controlled oxygen level at 0.2% and the other one to normoxia (18.6 % oxygen level) for 48 h. Afterwards, the medium from the wells was removed and the wells were washed once with HBSS before the addition of 20,000 mdMSCs/well in all wells containing HaCaT to achieve a 50% HaCaT medium and 50% mdMSCs medium ratio. In the wells previously let empty mdMSCs

were also added in the presence of the mixed medium to have a control with mdMSCs alone. The plates were then placed in normoxia for 24h. Subsequently, the medium from the wells of the two plates was removed and the wells were washed once with HBSS before the addition of 100 µl HBSS and 10 µl MTS for the measurement of cell metabolism. Just after MTS addition a first reading of the absorbance at 490 nm was made with Multiskan Ascent spectrophotometer (Thermo labsystem, Finland) then every 1h till 4h to follow the evolution of the absorbance. For result interpretation, the metabolic response of mdMSCs alone is subtracted from that of HaCaT + mdMSCs.

6. HaCaT-MPO activity and immunolocalization

6.1. HaCaT incubation with ANS

The experiment was performed with a 6-well cell culture plate (CellStar, Greiner bio-one) seeded with 100,000 HaCaT cells per well for 24 h in DMEM high glucose with (10% FBS) to let them adhere. After 24 h seeding, HaCaT cells form colonies that have a squamous appearance. ANS obtained after CB/fMLP was diluted 4 or 8 times with the HaCaT medium and then added into the wells to have a final volume of 2 ml. Each dilution was tested twice. In the two remaining wells no ANS was added. Following the addition of ANS, HaCaT cells were incubated for 2 hours at 37°C with 5% CO2. After incubation, the medium was removed, and the wells were rinsed three times with 1.5 mL DPBS before measuring the in situ MPO activity.

6.2. HaCaT incubation with MPO

The experiment was performed with a 6-well cell culture plate (CellStar, Greiner bio-one) seeded with 100,000 HaCaT cells per well for 24h. To HaCaT adherent cells, a 100 µg/ml stock solution of equine MPO was added to have in two wells 250 ng/ml MPO and in two other wells 500 ng/ml MPO. In the two remaining wells no MPO was added. After MPO addition, HaCaT were incubated for 2h in the incubator (37°C, 5 % CO2). After incubation, the medium was removed, and the wells were rinsed three times with 1.5 mL DPBS before measuring in situ MPO activity or performing immunological detection of MPO.

6.3. Measurement of the HaCaT-MPO activity.

After washing, the in-situ peroxidase activity of MPO was monitored by adding 1 mL of a 40 µM Amplex red solution freshly prepared in 50 mM phosphate buffer, pH 7.4, supplemented with 10 µM H2O2 and 10 mM sodium nitrite.The fluorescence development was monitored during 30 min (37 °C) with a Fluoroskan Ascent (Thermo Fisher Scientific, Waltham, MA, USA) set at 544 nm and 590 nm for the excitation and emission wavelengths, respectively. Total fluorescence was directly proportional to the amount of active MPO in the sample.

6.4. Detection of HaCaT-MPO by immunocytology

After incubating HaCaT cells with human MPO and/or mdMSCs, the medium was removed, the wells were washed three times with 1.5 mL DPBS, and the cells were fixed with cold 4% PFA solution (Thermo Scientific) for 10 minutes.Thereafter, a cell permeabilization was done by incubating the cell during 10 min with 0.1% Tritron X-100. After 3 washings with 1.5 ml DPBS, a circular zone at the bottom of the wells was delimited with a hydrophobic slide marker. This zone was immuno-stained for human MPO using the horseradish peroxidase/diaminobenzidine ABC detection immunohistochemistry (IHC) kit (Abcam) and according to the protocol with slight modifications. A positive staining to MPO was detected by a brown coloration. Successively the steps were: hydrogen peroxide block (10 min, RT), protein block (10 min, RT), incubation (1h, 37°C) with the rabbit primary antibody against human MPO (Abcam 9535, diluted 1:100 x with 20 mM PBS pH 7.4 + 0.5% bovine serum albumin and 0.1% Tween 20), biotinylated goat (15 min, RT), streptavidin peroxidase (15 min, RT), DAB chromogen substrate (10 min, RT), hematoxylin solution (45 sec). Between each step 3 washings with DPBS were performed (3 x 3 min). Two ml of DPBS buffer were added before the observation with a Carl Zeiss Axioskop 20 for transmitted light and incident-light fluorescence microscopy connected to a digital camera Nikon D70. All photographs were obtained using the same light intensity and shutter speed.

7. Effects of mdMSCs on HaCaT-MPO activity

As in point 5.2, the experiment was performed with a 6-well cell culture plate (CellStar, Greiner bio-one) seeded with 100,000 HaCaT per well. Adherent cells were preincubated with 250 ng/ml and 500 ng/ml equine MPO for 2h then the excess of MPO was removed by 3 washings with PBS. Following the washing step, a mix (v/v, 1/1) of DMEM high glucose and DMEM-F12 was added in the 3 upper wells, while in the 3 bottom ones 200,000 mdMSCs in 1 mL DMEM-F12 were added to HaCaT cells previously covered by 1 ml DMEM high glucose. Thereafter, the culture plate was incubated for 24 h in the incubator (37°C, 5 % CO2). After removal of the medium, the wells were washed 3 times with 1.5 mL DPBS before the measurement of the in situ MPO activity.

8. Statistical analysis

Six to fifteen independent experiments were conducted for statistical analysis and figure generation for the MTS assay and the MPO activity.. Since the data did not conform to a Gaussian distribution, Kruskal-Wallis tests followed by post-hoc tests were employed to evaluate the impact of different conditions (factors) on the MPO activity or MTS assay values (variable). A significance level was set at $p < 0.01$ (Medcalc software, Ghent, Belgium). The data in the figures are presented as medians with 95% confidence intervals and are expressed as relative values (%) compared to control groups, which were standardized to 100% for the MTS assays.

Results

1. Free MPO and NET-bound MPO released by neutrophils

The concentration of free active MPO and NET-bound MPO significantly increased in the ANS compared to the NANS. Additionally, active MPO appears to be primarily associated with NETs (Fig. 1)

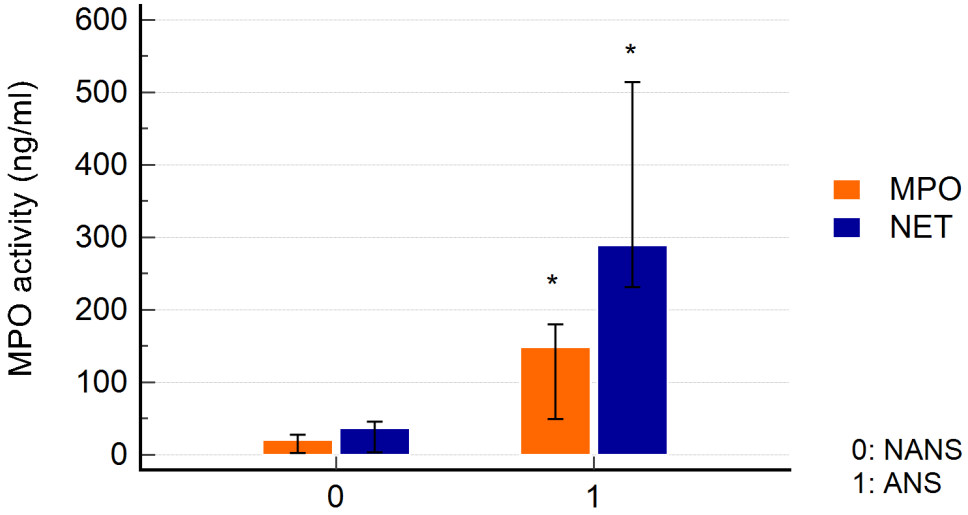

Figure 1. Measurement of free active MPO (MPO) and active MPO bound to the NET (NET) in the supernatant of non-activated (NANS) and activated (ANS) neutrophils with CB and fMLP. Results are presented as medians with 95% confidence intervals of 6 independent experiments. ($p < 0.01$ vs NANS).

2. Effect of normoxia and anoxia on HaCaT metabolism in the presence of neutrophil supernatants

HaCaT cells exposed to anoxia-reoxygenation, in the presence of ANS, exhibited a significant decrease in their metabolic activity compared to the control (normoxia) and the non-activated condition. Cisplatin used as a positive toxicity control showed a strong decrease in metabolic activity (Fig. 2).

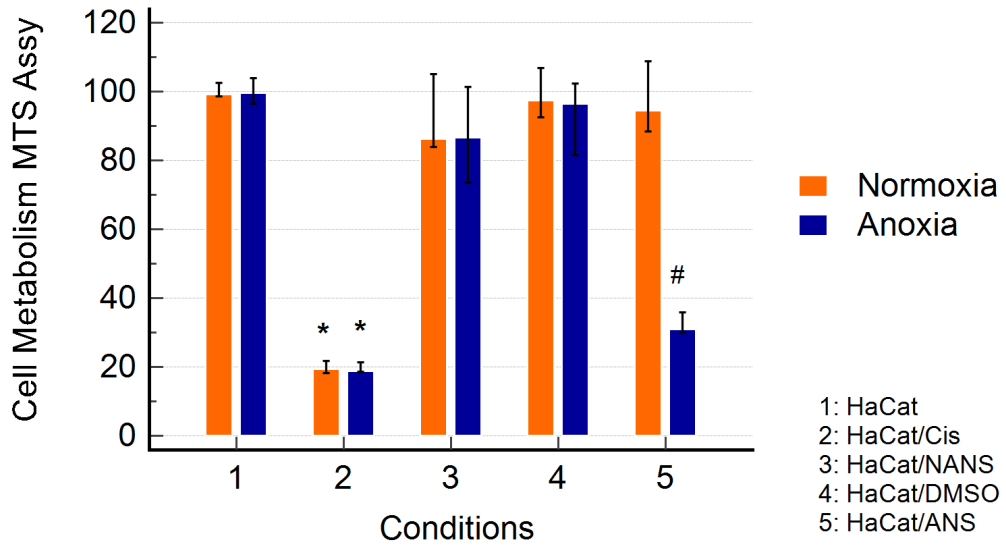

Figure 2. Effect of non-activated neutrophil supernatant (NANS) and CB/fMLP activated one (ANS) on the metabolism of HaCaT using MTS assay. HaCaT were cultured for 48 h in normoxia or anoxia (5% CO2) and then incubated for 24 h with fresh medium under normoxia followed by measurement of metabolic activity after 4 h (MTS assay). Ctrl DMSO: control with DMSO used for the solubilization of CB and fMLP. Results are presented as medians with 95% confidence intervals of 12 experiments and are expressed as relative values (%) compared to control groups, which were standardized to 100.. (* p<0.01 vs HaCat alone; # p<0.01 vs normoxia).

3. Effect of mdMSC on HaCaT metabolism submitted to normoxia or anoxia with ANS

MdMSCs co-cultured with HaCaT showed a significant increase of the HaCaT metabolism in normoxia and a protective effect against stress conditions induced by A/R in the presence of ANS (Fig. 3).

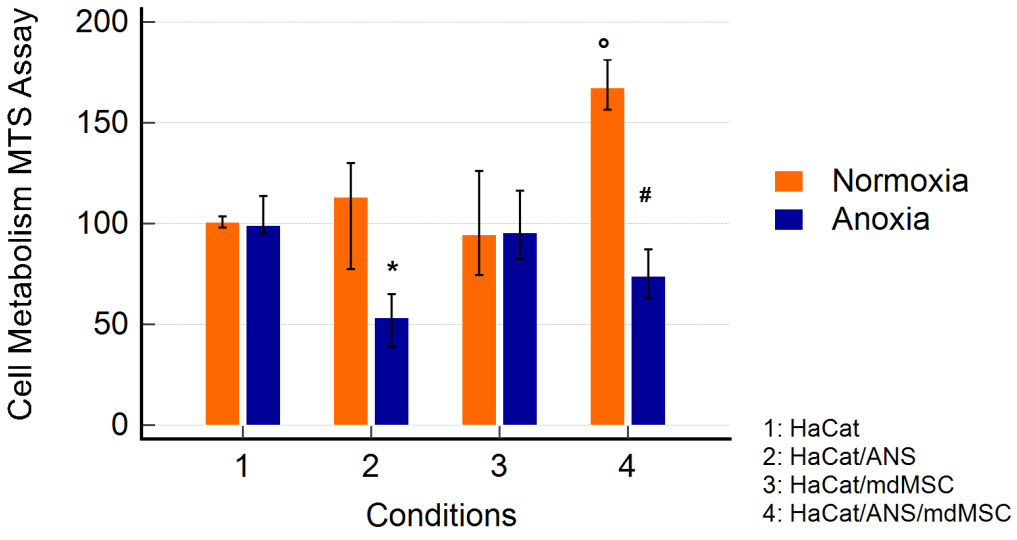

Figure 3. Effect of mdMSCs on HaCaT cultured in normoxia or anoxia for 48 h in the presence of activated neutrophil supernatant (ANS). MdMSCs were added at the reoxygenation period of 24h just after 48 h of normoxia or anoxia. After medium removal the MTS solution was added for the measurement of metabolic activity. Results consider the subtraction of the metabolic response due to mdMSC. Results are presented as medians with 95% confidence intervals of 15 experiments and expressed in relative % vs condition 1 set as 100 % response. (* $p<0.01$ vs normoxia. ° $p<0.01$ vs other conditions; # $p<0,01$ between condition 2 and 4 in anoxia).

4. Active MPO from ANS and purified equine MPO are captured by HaCaT

Despite washing of HaCaT cells exposed for 2h with ANS or purified MPO, an *in situ* peroxidase activity was measured. This activity appeared more dose-dependent for purified MPO than for ANS (Fig. 4). The capture of MPO by HaCaT was confirmed by IHC as shown in Figure 5. After the pretreatment of HaCaT cells by MPO and its immunolocalization by anti-MPO antibody and the Abcam detection IHC kit we observed an intense brown staining both in the cytosol and in the perinuclear zone of the cells (Figure 5 D). The perinuclear staining suggests that part of the enzyme has entered the cells. A slight brown coloration is however observed in cells non treated with MPO suggesting a slight unspecific staining (Figure 5 C). In absence of the anti-MPO antibody, no brown coloration was observed (Figure 5 A, B).

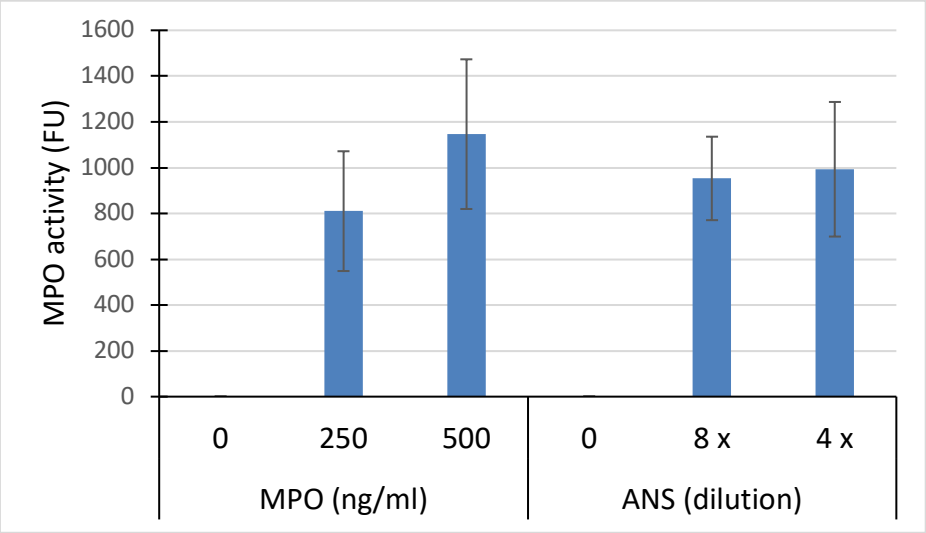

Figure 4. MPO activity measured on adherent HaCaT cells (100,000/ml) after their incubation with 250 ng/ml and 500 ng/ml of total equine MPO or with ANS diluted 4 and 8 times into the medium. After their incubation with MPO, cells were washed 3 times with PBS buffer before the in situ measurement of the peroxidase activity. Results are expressed in fluorescence units Mean +/- SD (n= 5 independent experiments).

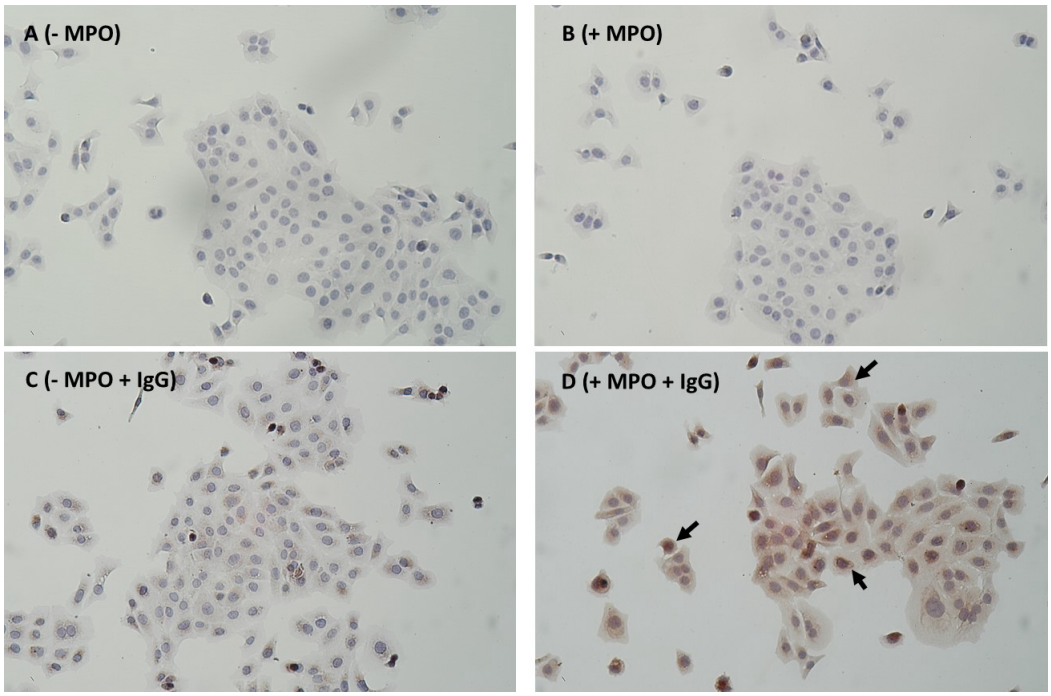

Figure 5. Photomicrograph of HaCaT pretreated (B, D) or not (A, C) for 2h with 500 ng/ml human MPO (+ MPO). After 3 washings with DPBS to remove the medium with MPO, cells were directly fixed and further stained following an immunohistochemical protocol. A and B without primary MPO antibody, C and D with primary MPO antibody (+ IgG) After 24 h seeding, HaCat cells form colonies that have a squamous appearance. Positive staining to MPO appeared in brown colour (see arrows for example). The nuclei were blue stained by counterstaining with hematoxylin. (×100).

5. MdMSC added to pre-treated MPO HaCaT cells inhibit the *in situ* activity of MPO

After the washing of HaCaT pre-treated 2h with MPO, mdMSCs were added to HaCaT and cultured for 24 h to let them adhere. After this incubation period, the mix of adherent cell populations (HaCaT + mdMSC) was washed 3 times with PBS and the *in situ* activity of MPO was revealed as above. First, we noticed a strong decrease in the *in situ* MPO activity 24h after their initial contact with MPO. This decrease appeared superior to 80% for HaCaT cells pre-treated with 500 ng/ml MPO (supplementary material). An additional decrease of MPO activity was observed when HaCaT were cocultured with mdMSCs compared to HaCaT cells alone. This decrease appeared significant ($p < 0.01$) when HaCaT cells were exposed to 500 ng/ml equine MPO (Table 1).

Table 1. MPO activity on adherent HaCaT cells (100,000/ml) preincubated with 250 ng/ml and 500 ng/ml equine MPO then after washing (3 x with PBS) put in contact 24h with or without mdMSCs (200,000/ml). After incubation and cell washing (3 x with PBS), the peroxidase activity was measured in situ. Mean +/- SD (n= 6).

	In situ peroxidase activity (fluorescence units)			
	HaCaT + MPO 250 ng/ml		HaCaT + MPO 500 ng/ml	
Horse	mdMSCs -	mdMSCs +	mdMSCs -	mdMSCs +
1	40.97	28.20	232.20	87.03
2	37.90	19.02	196.10	121.30
3	73.79	23.59	155.20	93.76
4	302.60	122.10	734.80	487.40
5	146.80	130.70	657.80	571.70
6	86.64	67.65	547.80	318.80
Mean	114.78	65.21	420.65	280.00
SD	100.13	50.53	255.93	212.94
p values		0.12		**<0.01**

Discussion

The primary objective of this study has been achieved. We have successfully developed a straightforward in vitro model to explore laminitis by subjecting a continuous-keratinocyte cell line (HaCaT) to ANS following a period of anoxia and subsequent reoxygenation. The MTS tetrazolium assay was used in this study to evaluate the activity of cellular metabolism by measuring the activities of mitochondrial NAD(P)H oxidoreductases or cytoplasmic esterases (Braissant *et al.* 2020, Ghasemi *et al.* 2021). This test is currently one of the most widely used methods to assess drug toxicity (Stockert *et al.* 2018). In our study cisplatin was used as cytotoxic molecule control to evaluate the stress intensity.

When cells were exposed to ANS and anoxia followed by reoxygenation a significant decrease of HaCaT metabolism was observed. Such decrease was not observed in cells staying in normoxic conditions. ANS was prepared using a combination of CB and fMLP to simulate a more physiological process for neutrophil stimulation and MPO degranulation. Our results showed that ANS contained an important concentration of free active MPO but also a more important NET bound active MPO in comparison to the supernatant obtained with NANS.

Herein, the inhibition of HaCaT metabolism upon exposure to ANS was observed specifically following a period of anoxia followed by reoxygenation. It is widely recognized that IRI arises from mitochondrial dysfunction, leading to the production of ROS such as superoxide anion, hydroxyl radical, and hydrogen peroxide (Marin *et al.* 2020, Huang *et al.* 2024). The latter serves as a substrate for MPO, generating additional potent oxidants that can contribute to exacerbate mitochondrial dysfunction. It is important to note that ANS likely contains various other products released by activated neutrophils, such as cytokines, lipid mediators, and other proteolytic enzymes able to participate in tissue injury (Burn *et al.* 2021). In addition, He *et al.* (2023) have indicated that NET release can also disrupt the mitochondrial function of targeted cells.

Interestingly, the addition of mdMSCs during the reoxygenation phase exhibited a protective effect, leading to a restoration of cellular metabolic activity in HaCaT cells treated with ANS and previously submitted to anoxia, suggesting a beneficial effect of mdMSCs in the restoration of mitochondrial function during stress condition as often described in the literature (Rodriguez *et al.* 2018). However, an increase in mitochondrial function was also observed in cell submitted to ANS without an anoxia period. Indeed, mitochondrial transfer was described under both physiological and pathological conditions. MSCs can transfer entire mitochondria or fragments via extracellular vesicles (EVs) or nanotubules (Liu *et al.* 2021). The mdMSCs mechanism of action may involve modulation of cellular respiratory function. Indeed, preliminary results presented at the international conference Targeting Mitochondria which took place in Berlin in 2023 suggest the ability of mdMSCs to transfer mitochondria

in HaCat cells (Franck *et al* 2023). However, the recovery of metabolic activity could also be due to the effect of mdMSCs on NETs present in the medium. Magana-Guerrero *et al.* (2017) demonstrated that amniotic-derived MSCs inhibited NET release by interfering with neutrophil mitochondria. Furthermore, their study showed that the inhibition of NETs, reduction in ROS levels, and loss of mitochondrial membrane potential were reversed by MSCs overexpressing TSG-6, an important factor that inhibits the TNF/NF-kB signalling pathway.

Additionally, to its pivotal role in the regulation of the energetic metabolism, the administration of MSCs is increasingly recognized as a valid strategy for treating multi-organ IRI, attenuating cytokine storms associated with acute respiratory distress syndrome (ARDS), systemic inflammatory response syndrome, and sepsis (Rowart *et al.* 2015). These beneficial effects are attributed in part to the immunomodulatory properties of mdMSCs. Our previous studies have demonstrated that in vitro, mdMSCs exhibit significant decrease of neutrophil modulation involving an inhibition of ROS production, active MPO release and NET-bound MPO activity (Franck *et al.* 2021).

Our findings suggested that active MPO played a significant role in the pathogenesis of laminitis. We observed its presence in lamellar tissue of horse hooves submitted to experimental laminitis and in clinical case where active NET-bound MPO was also measured and detected in lamellae (Storms *et al.* 2022, Storms *et al.* 2024). Thus, using an in vitro cell culture model we investigated whether MPO could enter into keratinocytes cells and if the supplementation of mdMSCs in this model could modulate the activity of MPO. We first demonstrated that equine MPO as well as MPO from ANS was captured by HaCaT cells and that this MPO remained active. IHC confirmed the presence of MPO intracellularly mainly in a perinuclear manner.

Remarkably, the coculture of HaCaT during 24h with mdMSCs induced a decrease of the activity of MPO captured compared to HaCaT cells alone. These effects are likely attributed to specific compounds released by mdMSCs and internalized by HaCaT cells to inhibit MPO activity intracellularly.

Our study has certain limitations that merit consideration. Firstly, HaCaT cells, being a continuous line of keratinocytes, may not fully represent the behaviour of primary keratinocytes. Therefore, it would be beneficial to validate our findings using primary keratinocytes to ensure the robustness of our model. Secondly, while our current model provides a simplified in vitro representation of laminitis, future advancements could involve the development of a more sophisticated model such as an OrganoPlate 3 line tissue chip as proposed by Mimetas company. This advanced model could incorporate additional components such as endothelial cells and a basement membrane, along with equine keratinocytes, to better mimic the complex physiological environment of the lamellar tissue.

Conclusion

Our study sheds light on the potential mechanisms underlying the recovery of HaCaT metabolic activity. While the transfer of mitochondria may play a role, our findings highlight the significant anti-inflammatory properties of mdMSCs, implicating the NETs, MPO activity, and ROS generation. These findings hint at a promising direction for future research in cell therapy for laminitis, where mdMSCs show promise in mitigating inflammation and potentially restoring keratinocyte metabolism. This work introduces a novel in vitro model that simulates laminitis in horses, aligning with the 3R principles (Replacement, Reduction, and Refinement) of animal research.

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Conflict of Interest

D. Serteyn and J. Ceusters are the coinventors of a patent about the muscle derived Stem Cells. The patent is licensed by the University to a Spin Off Company called Revatis where D. Serteyn and J. Ceusters are scientific advisors. The other co-authors declare no conflicts of interest.

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Discussion - Perspectives

1. MAIN FINDINGS

This PhD thesis investigated the implication of neutrophils in different types of laminitis at different time points and the potential treatment perspectives related to this inflammatory component. Our main findings included the following:

- First, we showed that juglone does not seem to be the responsible molecule for neutrophil activation in the BWE model.
- Secondly, we demonstrated that MPO was present in endocrinopathic laminitis using immunohistochemistry on lamellar sections of horses with laminitis induced using the pEHC model.
- Thirdly, we have shown that MPO and NET are both present and active in lamellar tissue of clinical cases of naturally occurring laminitis in horses and donkeys. Furthermore, part of the total MPO activity was due to NET-bound MPO. We have also described the lamellar damage in these advanced stages of laminitis pathology. In addition, the results of experimental models were confirmed in cases of naturally occurring laminitis of varying etiologies.
- Finally, we have shown that equine mdMSC have the ability to improve keratinocyte metabolism and have anti-inflammatory properties implicating the modulation of NET and MPO activity *in vitro*, thereby indicating that they may have a potential role in treating laminitis through mitigating inflammation.

2. REVISITING OUR RESEARCH OBJECTIVES

Building up on the existing evidence of neutrophil activation, evidenced by MPO and elastase, in the BWE model for laminitis induction (Riggs *et al.* 2007, de la Rebière de Pouyade *et al.* 2010), Study 1 aimed to investigated whether juglone could be responsible for this observation, through its cytotoxic properties, induction of oxidative stress with redox cycling, cell membrane damage, apoptosis, and necrotic cell death (Ollinger and Brunmark 1991, Aithal *et al.* 2009). Interestingly, we observed that juglone had a rather anti-inflammatory effect on equine neutrophils and MPO. This can be explained in different ways:

- First, juglone can behave as an antioxidant or a pro-oxidant agent depending on the redox state in the cellular environment and the cell type (Ahmad and Suzuki 2019). Naphthoquinone compounds, like juglone, have antioxidant properties, by inhibiting oxidant reactions by quenching ROS, inhibiting ROS-producing enzymes, and chelating transition metal ions (like Fe^{2+}) (Kappus and Sies 1981, Tejero *et al.* 2007, Tamado Fouegue *et al*. 2016, Ahmad and Suzuki 2019). Furthermore, juglone can also prevent the activation of NADPH oxidase by binding to the cytosolic proteins of

the NADPH oxydase thus inhibiting their phosphorylation that is essential for the assembly and activation of the enzyme. Juglone has also been reported to inhibit the LPS-induced priming of ROS production by neutrophils, by interfering with the pathway of the peptidyl-prolyl isomerase Pin1 protein (Liu *et al.* 2019). On the other hand, juglone is cytotoxic and possesses antitumor and antimicrobial properties (Wulf *et al.* 2002, Jin 2009, Zakavi *et al.* 2013, Ahmad and Suzuki 2019). Like other quinones, the cytotoxicity of juglone includes redox cycling and reaction with GSH, an endogenous antioxidant. Redox cycling represents a cyclic process of the reduction of a compound followed by the oxidation of the reaction product and the simultaneous generation of ROS (Tejero *et al.* 2007, Ahmad and Suzuki 2019). Juglone enhances lipid peroxidation through this process (Kumbhar *et al.* 1997). With GSH, juglone forms adducts, causing GSH depletion, interfering with endogenous antioxidant availability (Gant *et al.* 1988, Bruins *et al.* 2018). Considering both the oxidant and antioxidant properties of juglone, its protective and damaging effects can be expected. In our study, a predominant antioxidant activity was suspected.

- Secondly, our study showed, though the docking and SIEFED, that juglone was able to block the active site of the MPO enzyme offering another possible explanation for its protective effect.
- Finally, it is possible that our *in vitro* model was not able to represent the BWE model fully and that other mechanisms are at play. Potential pathways include the presence of other toxic principles in the BWE, the development of enteritis with resorption of LPS of bacteria or other mechanisms responsible for the development of systemic inflammation, endothelial abnormalities and cytotoxicity.

The primary goal of this thesis was to demonstrate that inflammation, evidenced by the presence of activated neutrophils and their byproducts, such as MPO, was not limited to SIRS-related laminitis but also extended to endocrinopathic laminitis and clinical cases regardless of the etiology. Addressing this gap two studies were conducted (Study 2 and Study 3).

Study 2 confirmed the presence of MPO in an *in vivo* pEHC model mimicking endocrinopathic laminitis, thereby expanding the importance of neutrophil activation to another large group of laminitis patients (Storms *et al.* 2022). This study established a link between neutrophil activation and endocrinopathic laminitis. The nature of this link needs further investigation, with multiple mechanisms potentially at play. Indeed, the interaction between neutrophil activation and insulin is complex and bidirectional. On one hand, evidence in humans and laboratory animals suggests that insulin regulates the neutrophil function and that this regulation is, in turn, related to increased neutrophil chemotaxis and oxidative burst (Safronova *et al.* 2001, Okouchi *et al.* 2002, Walrand *et al.* 2004, Walrand *et al.* 2006). Furthermore, diabetic individuals exhibit increased superoxide generation by neutrophils (Omori *et al.* 2008). In addition, diabetes primes neutrophils to undergo NETosis in a mouse model (Wong *et al.*

2015). Finally, a study by Holbrook *et al.* (2012) showed a marked increase in neutrophil oxidative burst activity in hyperinsulinemic obese horses. On the other hand, Wang *et al.* (2014) showed that MPO and its product HOCl might be significant contributors to the development of inflammation-induced insulin resistance and metabolic disease in *in vitro* and rodent models. Indeed, HOCl inhibits insulin-stimulated phosphorylation of insulin receptors through tyrosine nitration and inhibits insulin-induced binding of insulin receptor substrate-1 to the regulatory subunit P85 during insulin signalling. Finally, a nonspecific peroxidase inhibitor that blocked MPO activity in isolated neutrophils prevented diet-induced insulin resistance in obese wild-type mice (Wang *et al.* 2014). This evidence justifies further research towards MPO as a therapeutic target for preventing insulin resistance and the metabolic consequences of obesity (Heinecke and Goldberg 2014, Wang *et al.* 2014).

Another interesting element revealed in our pEHC study was the severe MPO labelling observed in the lamellar tissue of affected horses, in the presence of only rare neutrophils (Storms *et al.* 2022). Previous studies utilising the pEHC model similarly noted a limited number of neutrophils in lamellar tissue (Asplin *et al.* 2010). It was concluded that the extent and severity of inflammation in hyperinsulinemia-induced laminitis were less critical than would be expected when compared to other tissues subjected to similar levels of cellular stress and mechanical compromise (Asplin *et al.* 2010, McGowan and Patterson-Kane 2017). However, these conclusions were based on the identification of neutrophils themselves. Our histologic sections agree with the observation of sparse neutrophils. Nonetheless, the increased presence of MPO supports the involvement of neutrophils in the pathophysiology of laminitis.

The exact mechanism for this observation remained unknown. However, it may be explained in different ways: (i) Neutrophils could undergo degranulation in the very early stages of the disease process, with only degranulation products such as MPO being detectable at the time of clinical signs of laminitis. (ii) Alternatively, neutrophils might degranulate in the bloodstream, leading to the diffusion of MPO from the circulation to the dermal lamellae. Indeed, the presence of MPO in the bloodstream, skin and lamellae after laminitis induction using a BWE model was confirmed by Riggs *et al.* (2007). (iii) Another plausible explanation is the formation of NETs during laminitis, which could account for the extensive MPO staining despite the apparent absence of neutrophils, as NETs are composed of nuclear components and proteins from neutrophilic granules such as MPO (Mantovani *et al.* 2011). Furthermore, NET formation is involved in various disease processes associated with sepsis and endotoxemia as well as metabolic disturbances (Czaikoski *et al.* 2016, Carestia *et al.* 2016, Li and Tablin 2018, D'Abbondanza *et al.* 2019), making it the central hypothesis in our investigation. Indeed, NET induced thrombosis and multiple organ failure in murine sepsis models (Czaikoski *et al.* 2016, Li and Tablin 2018). Moreover, in murine models, endotoxemia triggers neutrophil activation, leading to NET formation consequently causing 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 observed during non-infectious diseases. In human patients, 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 2 diabetes patients exhibited increased NETosis compared to healthy controls (Carestia *et al.* 2016). Additionally, NETosis from isolated neutrophils is increased by high glucose levels *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). Therefore, NET seems also implicated in processes similar to those encountered in endocrinopathic laminitis.

Considering the elements above, Study 3 investigated the presence of neutrophil activation, evidenced by MPO and also by NET, in naturally occurring laminitis cases including both horses and donkeys (Storms *et al.* 2024). Our findings confirmed the presence of both MPO and NET in these cases. Moreover, we discovered a strong positive correlation between the NET-bound MPO activity and the total MPO activity, indicating that part of the total MPO activity was attributable to the MPO associated with the NET. Both MPO and NET can cause substantial damage when an inflammatory reaction becomes uncontrolled. Due to its dual activity, MPO can chlorinate, nitrate and oxidise most biological organic molecules, which results in severe tissue damage (Klebanoff 2005, Valadez-Cosmes *et al*. 2022). In addition, MPO can be taken up by endothelial cells, which can be subsequently damaged by its products (Mathy-Hartert *et al.* 1995, Benbarek *et al.* 2000, de la Rebière de Pouyade *et al.* 2008). Hence, MPO could play a role in establishing events leading to laminitis in various ways and could perpetuate the lamellar injury. While we have demonstrated the presence and activity of MPO in lamellar samples, it would be interesting to investigate the presence of its oxidation, chlorination, or nitration products in lamellar tissue to confirm its active role in causing tissue damage. In addition to the free MPO and the MPO associated with the NET, NET can also induce significant damage by itself. The cell-free DNA and histones present on NET are activators of the coagulation system (Gould *et al.* 2015), inducing thrombosis and multiple organ failure in murine sepsis models (Czaikoski *et al.* 2016, Li and Tablin 2018). Moreover, NET formation causes significant endothelial cell damage in endotoxemic mice (Clark *et al.* 2007, Sørensen and Borregaard 2016).

Together, the findings of our pEHC study and our clinical study highlight the presence of neutrophil activation as a common pathway in laminitis. This, along with the potential detrimental effects of excessive NET and MPO production, suggest that developing treatment options targeting MPO and NET could be beneficial for our laminitis patients.

In addition to confirming the presence of MPO and NET, our clinical study also aimed to describe the evolution of the lamellar lesions in severe laminitis cases. Multiple lesions have been observed, and severe anatomic abnormalities give an idea about the gravity of the condition in some of our patients. Notably, observations not necessarily seen in less advanced stages of laminitis included the presence of free fluid and tissue oedema, total loss of anatomic structure, tissue separation, and increasing keratin proportions and decreasing dermal tissue proportions. Interestingly, at this stage, neutrophils were observed in many cases, and these neutrophil infiltrates were mainly localised in the dermis and sites of these severe tissue destruction. This raises the question of whether neutrophil activation occurs as a cause or a consequence of the disease process. The implication of neutrophils and their byproducts like MPO and elastase has been confirmed early in the disease process in previous studies using the BWE model (Riggs *et al.* 2007, de la Rebière de Pouyade *et al.* 2010) and was confirmed in our research using the pEHC model (Storms *et al.* 2022), thereby indicating their involvement in the onset of the disease. However, it is also recognised that neutrophils are attracted to sites of significant tissue damage. In the presented clinical end-stage cases, neutrophils were observed in the most destructed tissue parts. It could be argued that they either contribute to this severe damage when inflammatory reactions become uncontrolled or that they are drawn to these sites due to the already existing damage. A combination of both these mechanisms seems likely. Given that MPO was present in the early stages of laminitis, it is conceivable that this inflammatory cycle becomes a vicious loop, thereby causing or exacerbating local tissue damage. Subsequently, additional neutrophils may be attracted to these sites, perpetuating this cycle of cause and effect.

Another research objective of the clinical study was to shed light on the representativeness of models of laminitis like the pEHC (Storms *et al.* 2022) and the BWE model (Riggs *et al.* 2007) by investigating whether MPO and NET were present in cases of naturally occurring laminitis. Indeed, naturally occurring laminitis often involves complex and multifactorial etiologies with multiple parameters to consider. Therefore, it raises questions about whether interactions between these diverse disease processes could impact the pathophysiology, necessitating adaptations in treatment plans. Our study successfully confirmed the presence of both MPO and NET in all cases regardless of the etiology of the laminitis pathology, thereby enforcing our hypothesis that inflammation with neutrophil activation represents a common pathway in the pathophysiology of laminitis irrespective of the inciting cause and indicating that our models accurately reflect the clinical manifestation of the disease, at least from the neutrophil activation point of view.

Finally, the presented clinical study attempted to address the scarcity of information regarding donkeys. Donkeys are usually treated the same way as horses, whereas multiple anatomic (Thiemann *et al.* 2021) and metabolic differences exist (Burden and Thiemann 2015). While we included multiple laminitic

donkeys in the study, securing a control group of sound donkeys proved challenging for various reasons including the high prevalence of obesity and metabolic disease among donkeys in our region, their rare admission to the slaughterhouse, and instances of euthanasia typically involving underlying inflammatory disease. Combining these elements made most cases unsuitable for inclusion in the control group. Even though anatomical lesions of the laminitic horses and donkeys appeared quite similar, and both groups exhibited MPO and NET-bound MPO in the same way, future research analysing lamellar tissue from donkeys not affected by the disease would be valuable in establishing baseline anatomical information and uncovering possible microscopic differences between horses and donkeys.

The results of these first parts of this PhD thesis (mainly Study 2 and Study 3), in addition to existing literature, support the importance of neutrophil activation as a general element in the pathophysiology of laminitis. Besides the consequences of neutrophil activation on insulin signalling systems, through the potential direct effect of MPO and its product HOCl, neutrophil-mediated mitochondrial dysfunction should also be considered. Neutrophils can mediate mitochondrial dysfunction, thereby worsening the pathology. The relationships between inflammation, particularly neutrophil activation, whether associated with ischemic reperfusion events or not, and mitochondrial dysfunction have been documented across various organs such as the heart, kidneys, and liver (Kaminski *et al.* 2002, Jaeschke and Hasegawa 2006, Zmijewski *et al.* 2008, Zhang *et al.* 2019, Hepokoski and Singh 2022). Although mitochondrial dysfunction has not been directly demonstrated in the foot, energy deficiency has been suggested as a mechanism for the disruption of the hemidesmosomes, leading to the failure of the dermal-epidermal interface (French and Pollitt 2004). The notion of neutrophil-mediated mitochondrial dysfunction has been previously suggested in muscle micro-biopsies from horses after strenuous exercise, where an increase in MPO activity was associated with a decrease of mitochondrial complex I activity and an increase in creatinine kinase level considered as a marker of muscle pain (Franck *et al.* 2010). Moreover, in cultured muscle cell lines, it was demonstrated that the potent neutrophil-derived oxidative enzyme MPO could enter the cell and disturb the electron transfer system, resulting in altered ATP production (Ceusters *et al.* 2013). Recent research by He *et al.* (2023) demonstrated that NET-induced mitochondrial dysfunction in cardiomyocytes is associated with increased ROS release. In 2014, a clinical study showed a significant decrease in muscle mitochondrial oxidative phosphorylation in horses affected by acute laminitis from diverse origins when compared to the values obtained from fit or obese healthy horses (Serteyn *et al.* 2014). In addition, mitochondrial dysfunction has been confirmed in human patients suffering from metabolic disorders related to insulin resistance (Lowell and Shulman 2005, Kim *et al.* 2008, Cheng *et al.* 2010). These findings highlight the complex interaction between neutrophil activation, mitochondrial dysfunction and insulin resistance.

Given that neutrophil activation is observed in the early stages of laminitis and that we have recently identified the presence of MPO and NET in severe clinical cases (Storms *et al.* 2024), the following study aimed to investigate the potential correlation between neutrophil activation, MPO activity, NET release and keratinocyte metabolism in equine laminitis and to propose a new treatment approach. A straightforward *in vitro* model was developed to explore laminitis by subjecting cultured keratinocytes to an ANS following a period of anoxia and subsequent reoxygenation. We observed that ANS contained an important concentration of free active MPO but also a more important concentration of NET-bound active MPO in comparison to NANS. The inhibition of the HaCat metabolism upon exposure to ANS was observed specifically following a period of anoxia followed by reoxygenation. It is widely recongised that ischemic reperfusion injury (IRI) arises from mitochondrial dysfunction, resulting in the production of ROS such as the superoxide anion, hydroxyl radical, and hydrogen peroxide (Marin *et al.* 2020, Huang *et al.* 2024). The latter serves as a substrate for MPO, generating additional potent oxidants that exacerbate mitochondrial dysfunction. Furthermore, recent studies have indicated that NET release can also disrupt the mitochondrial function of targeted cells (He *et al.* 2023). Together, this indicates that the observed decrease in metabolism could originate from additional intracellular oxidant activity leading to keratinocyte mitochondrial dysfunction.

The next step was to investigate a novel treatment approach considering the presence of neutrophil activation, as evidenced by the presence and activity of MPO and NET, alongside observed decreased metabolic activity of keratinocytes in our *in vitro* laminitis model. Based on their immunomodulatory properties, particularly their capacity to inhibit MPO and NET formation, it was investigated whether mdMSC could mitigate the damage associated with neutrophil activation and restore keratinocyte metabolic activity. MSCs exhibit anti-inflammatory properties in addition to their well-known regenerative properties, which have led to their expanding therapeutic use (Saeedi *et al.* 2019, Regmi *et al.* 2019). In a murine model of acute endotoxin-induced lung inflammation, MSC were found to reduce inflammation and inhibit NET formation (Pedrazza *et al.* 2017). Magana-Guerrero *et al.* (2017) demonstrated that amniotic-derived MSCs inhibited the detrimental effect of NET on mitochondria. Moreover, our group recently showed a significant inhibition of the NET-bound-MPO activity by mdMSC and a decrease of ROS production by activated neutrophils *in vitro* (Franck *et al.* 2021).

Consequently, the aim of the final part of this PhD thesis was to explore the therapeutic potential of mdMSCs in an *in vitro* equine laminitis model involving activated neutrophils, anoxia-reoxygenation and MPO. First of all, mdMSCs exerted a protective effect against stress conditions induced by anoxia-reoxygenation in the presence of ANS, improving cellular metabolic activity. The mdMCS mechanism of action may involve modulation of cellular respiratory function as previous studies have demonstrated that MSCs can transfer entire mitochondria or fragments via extracellular vesicles (EVs) or nanotubules

(Liu *et al.* 2021). Interestingly, the recovery of metabolic activity could also be explained by an mdMSCs effect on NET, as observed by Magana-Guerrero *et al.* (2017), who demonstrated that amniotic-derived MSCs inhibited NET release by interfereing with neutrophil mitochondrial function. Secondly, we observed a notable reduction in MPO activity attributable to the presence of mdMSCs. This beneficial effect is attributed in part to the immunomodulatory properties of mdMSCs. Our previous studies have demonstrated that *in vitro*, mdMSCs exhibit significant effects on neutrophil activation, such as modulation of ROS, inhibition of MPO activity, and inhibition of NET-bound MPO activity (Franck *et al.* 2021). These findings highlight the significant anti-inflammatory properties of mdMSCs, implicating their effect on NET and MPO activity. They suggest a promising direction for future research in cell therapy for laminitis, with mdMSCs showing promise in mitigating inflammation and potentially restoring keratinocyte metabolism.

Although this thesis mainly investigated the pathophysiology of laminitis as well as novel treatment options, some of the insights can be usefull in laminitis prevention. As neutrophil activation was present in all clinical cases regardless of the inciting cause (Storms *et al.* 2024) and as it has also been observed as an early event in the onset of laminitis (Riggs *et al.* 2007, de la Rebière de Pouyade *et al.* 2010), controlling this neutrophil activation can be considered a preventive strategy. ACP possesses antioxidant and anti-inflammatory properties and has been shown to decrease the ROS production in subsequently isolated neutrophils (Péters *et al.* 2009). Furthermore, an *ex vivo* study demonstrated the modulating effects of ACP on the production of ROS produced by stimulated neutrophils (Sandersen *et al.* 2011). LMWH inhibited MPO-associated damage of endothelial cells *in vitro* (de la Rebière de Pouyade *et al.* 2008) and its administration reduced the prevalence and severity of laminitis as a postoperative complication of colic surgery (de la Rebière de Pouyade *et al.* 2009). Furthermore, cryotherapy has been widely recognized for its preventive (van Eps and Pollitt 2009, van Eps *et al.* 2012, Kullmann *et al.* 2014) and therapeutic use (van Eps *et al.* 2014) in equine laminitis management, by reducing lamellar inflammation, lamellar oxygen and nutrient requirements and inhibiting destructive enzymes and oxidative reactions (Divers 2017). Finally, the use of MSCs in a setting of laminitis prevention could be considered based on their anti-inflammatory and regenerative properties. However, to the authors knowledge, this has not been investigated yet.

3. PROSPECTS FOR FUTURE RESEARCH

Our study confirmed the presence of neutrophil activation in endocrinopathic laminitis and clinical cases. Together with the existing literature about SIRS-associated laminitis (Riggs *et al.* 2007, de la Rebière de Pouyade *et al.* 2010), this covers the most prevalent causes of laminitis, suggesting that neutrophil activation could indeed be a central element in the laminitis pathophysiology. However, a

third, less common type of laminitis, the supporting limb laminitis, was not included in this PhD thesis and might therefore need further investigation.

In addition, future research, possibly with larger sample sizes, could help to relate the severity of the clinical signs to the extent of the histologic abnormalities and the degree of MPO and NET concentration in lamellar tissues. Correlating these values to MPO and NET concentrations in blood samples may confirm that the local inflammation comes from systemic disease and could provide a prognostic indicator in clinical cases. Indeed, elevated MPO values predict mortality in intensive care patients with SIRS, (Schrijver *et al.* 2017) and indicate a worse prognosis in patients with acute coronary syndrome (Kolodziej *et al.* 2019). Circulating NETs have proven to be of prognostic value as markers of neutrophil activation in humans with terminal cancer (Rosell *et al.* 2021).

We obtained encouraging results concerning the effect of mdMSC on keratinocyte metabolism and neutrophil activation. However, while *in vitro* models represent a simplified version of the live situation, they are essential to examine the effect of new therapeutic substances. In our model, we used HaCat cells, a continuous line of keratinocytes that do not fully represent the behaviour of primary keratinocytes. Therefore, it would be beneficial to validate our findings using primary keratinocytes to ensure the robustness of this model. Furthermore, while our current model provides a simplified, straightforward *in vitro* representation of laminitis, future advancements should involve creating models that more closely mimic the complex physiological environment of lamellar tissue, such as the Organ-on-chip technique, mixing different cell type populations like equine keratinocytes, endothelial cells and basement membrane. However, these methods are more complex to manage and highly sensitive to minor variations in experimental conditions and need an appropriate and specific device (Danku *et al.* 2022).

Based on our preliminary results and existing literature, MSC therapy presents a promising approach as a new treatment perspective for laminitis cases. Further research is imperative to unravel the underlying mechanisms driving MSC-mediated laminitis treatment and refine therapeutic strategies for this debilitating condition. For research purposes, it would be valuable to conduct studies under standardized conditions provided by various *in vivo* laminitis models. However, the induction of laminitis poses ethical concerns, limiting the number of cases and the severity of induced lesions. Clinical studies on more advanced lamellar lesions would be valuable to evaluate the potential regenerative effects of MSCs, in addition to our demonstrated impact on MPO and NET. Nevertheless, clinical studies face different challenges such as the variability in case presentations regarding severity and chronicity. Furthermore, envisioning this type of clinical trial, questions remain regarding the exact mechanism (i), the ideal time point of administration (ii), the administration route (iii, intravenous, intra-arterial, intra-

dermal), the administration regimen (iv, dosage, number of treatments, interval) and the origin of the MSCs (v).

(i) Regarding the mechanism, both the immunomodulatory properties and the possible transfer of mitochondria can be considered. To confirm the latter, an investigative approach could involve assessing baseline mitochondrial function in lamellar tissue of healthy horses and horses with laminitis, employing techniques like high-resolution respirometry.

(ii) Regarding the ideal time of administration, neutrophil activation has been shown in the early stages of laminitis (Riggs *et al.* 2007, de la Rebière de Pouyade *et al.* 2010, Storms *et al.* 2022) as well as in the end-stage cases (Storms *et al.* 2024). While administration of MSC at any time point might confer benefits based on these parameters, early initiation could offer superior clinical outcomes, as usually, the earlier the treatment is installed, the better the chances are for a satisfactory outcome.

(iii) Multiple administration routes merit consideration. The ultrasound-guided arterial perfusion of MSC has been shown to deliver high doses of MSC to the hoof in a more homogenous pattern compared to intravenous administration (Trela *et al.* 2014). However, the intra-arterial technique is more challenging with arterial thrombi being a described risk and deleterious especially in laminitis cases (Sole *et al.* 2012). Spriet *et al.* (2015) described and ultrasound guided-technique where the use of a needle limited the risk of arterial spasm compared to catheterisation.

(iv) To conclude the administration regimen, dose-response studies clarifying the optimal MSC number to be administered would have to be performed. For soft tissue injuries dosages usually range between 10-30 x 10^6 cells (Barrachina *et al.* 2018). To the authors knowledge, no studies are available specifically investigating the required amount of cells for laminitis treatment, but examples of regimens showing promising results were obtained in a study reporting the contribution of adipose tissue-derived MSC in combination with platelet-rich plasma (15 x 10^6 cells in 15 ml platelet-rich plasma) injected intravenously three times at one-month intervals to treat nine chronic laminitis cases (Angelone *et al.* 2017). Another study reported improved hoof growth and vascular perfusion in six horses with bilateral laminitis treated with 20 x 10^6 adipose-derived MSC three consecutive times with two weeks intervals (Oliveira *et al.* 2021).

(v) Another question arises regarding the origin of the MSCs that could be used. Our group has described a minimally invasive technology to obtain mdMSC. These cells have properties similar to MSCs from other sources and have potent immunomodulatory effects (Ceusters *et al.* 2017). However, the 6-week cultivation period presents a drawback for prompt treatment with autologous stem cells in laminitis cases. Interestingly, it was noted that adipose-derived stem cells from horses with endocrinopathic

disturbances like EMS, have altered properties and are therefore not ideal for treatment purposes (Marycz *et al.* 2016). It was proposed that the oxidative stress accumulated in the adipose tissue of these horses may affect their proregenerative potential. Epidermal progenitor stem cells have been successfully isolated from equine coronary corium and cultured (Da Silva *et al.* 2020, Marycz *et al.* 2021). A recent study showed that hoof progenitor stem cells resisted better to hyperinsulinemia than adipose-derived MSC and therefore showed more promise as a pool of stem cells for treating laminitis (Pielok *et al.* 2023). However, these studies harvested hoof progenitor cells on cadavers only, it would be interesting to investigate the potential of mdMSC harvested using a minimally invasive technique on live horses with similar symptoms. Finally, allogeneic stem cells have a greater and more rapid availability, thereby overcoming the delay related to the culture of autologous stem cells. However, concerns exist about their elimination by the immune system (Barrachina *et al.* 2018). Compatibility tests are underway, assessing donor suitability for specific laminitis cases alongside investigations into the properties of donor-derived stem cells to ensure alignment with therapeutic goals.

Alternatively, the use of MSC-exosomes can be considered. MSC-exosomes have the advantage of having a reduced risk of immune rejection and tumorigenicity compared to cellular therapy (Wei *et al.* 2021). Furthemore, an increasing body of evidence exist that MSC-exosomes have similar anti-inflammatory properties to MSCs themselves (Harrell *et al.* 2019). In addition, Feng *et al.* (2024) showed that exosome treatment mitigated IRI in mice by reducing neutrophil infiltration and NET formation. However, the application of exosome treatment still faces challenges in their production and separation and lack of standardized quality assurance assays (Wei *et al.* 2021).

It is noteworthy that besides the use of MSC, alternative treatment approaches targeting NET formation exist, including 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 non-anticoagulant heparin (Wildhagen *et al.* 2014) or by blocking citH3 (Li *et al.* 2014). However, these options have so far only been tested *in vitro* or in mouse models and therefore warrant further investigation before considering clinical application.

4. CONCLUSION

In conclusion, this PhD thesis contributed to the increasing body of evidence demonstrating that neutrophil activation, characterised by the presence and activity of MPO and NET, is present in the major types of laminitis, including endocrinopathic laminitis, and in clinical cases, irrespective of the inciting cause. Furthremore, this finding was consistent across different equine species, including both

horses and donkeys. The presence of these markers underscores the implication of neutrophil activation in the pathophysiology of laminitis, providing new insights in the mechanisms involved.

Our *in vitro* study suggests that mdMSC can inhibit the deleterious effects of inflammation and restore keratinocyte metabolism. These findings open a promising direction for developing innovative, regenerative treatment options in equine laminitis. The anti-inflammatory properties of mdMSC, coupled with their capacity to promote tissue repair, present an interesting opportunity to explore novel therapeutic strategies that could improve outcomes for animals suffering from laminitis. However, future studies are needed to further develop these strategies and define the optimal and most efficient use of mdMSC in clinical settings.

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