Equine laminitis. New insights into the pathogenesis. A review

PAOLO STEFANO MARCATO[1], ANTONELLA PERILLO[2]

[1] Departement of Veterinary Medical Sciences. Alma Mater Studiorum - University of Bologna, Italy
[2] Departement of Veterinary Medicine. University of Bari Aldo Moro, Italy

SUMMARY

Laminitis is typical of Ungulates but it is especially significant in horses. The most prevalent clinical signs (≥70%) are difficulty turning and a short/stilted or lame walk. Very severe, highly acute laminitis cause the third phalanx to detach from the hoof wall and to drop ventrally inside the hoof, favoured both by the destruction of the dermal-epidermal junction, as well as by a hyperplasia of the epidermal laminae that produce a horny growth (keraphylocele) acting as a wedge. Four main risk factors correspond to four types of laminitis: 1) sepsis /SIRS (systemic inflammatory response syndrome) related laminitis; 2) endocrinopatic laminitis; 3) pasture-associated laminitis; 4) supporting limb laminitis, experimentally linked to hoof lamellar hypoxia. Type 1 correlates with sepsis from Gram-negative polymicrobial bacteria (postpartum metritis with retained placenta, colic, proximal enteritis, volvulus and enterocolitis). The systemic inflammatory events that occur in type 1 laminitis coincide with marked increase in digital lamellar expression of a variety of inflammatory mediators and with activation of extracellular matrix metalloproteinases (MMPs). The most common form of laminitis, endocrinopatic laminitis (type 2), may occurr secondary to metabolic diseases (equine metabolic syndrome, Cushing's disease), usually in obese horses and ponies, and is exacerbated in animals that graze lush pastures (type 3 laminitis). The unifying pathogenic factor in types 2 and 3 laminitis, is hyperinsulinemia with insulin toxicity. The main failure in these cases is the loss of the adherence of the basal epithelial cells in the epidermal lamellae to the underlying dermal lamellae through the separation of the dermo-epidermal attachment at the basement membrane (BM) level. The early and key cytomorphological pathology points to lamellar cell stretching, suggesting cytoskeletal deformation with weakening and elongation of the lamellar epithelial cells, which translate into alteration of their tensegrity. These events may well induce some secondary alteration in the structure and/or elasticity of the BM, with cytoskeletal disengagement and loss of the hemidesmosomes and thus a further relaxation of the lamellae followed by loss of adhesion of the layer of basal epithelial lamellar cells with the underlying BM.
The lamellae are sparsely populated with insulin receptors (insR), whereas IGF-1 receptors (IGF-1R) are abundant. However insulin is unlikely to directly bind and to activate equine IGF-1R in vivo, even at high physiological concentrations. An indirect mechanism through which insulin could activate IGF-1R should be envisaged in the displacing IGF-1 from IGF-binding proteins (IGFBPs) such as IGFBP7 or fragments of IGFBP3, thereby increasing free IGF-1 concentrations, or in a direct action on a very small population of lamellar InsR.

KEY WORDS

Endotoxaemic laminitis, endocrinopatic laminitis, hyperinsulinemia, lamellar cell stretching.,cytoskeletal mechanics.

INTRODUCTION

The equine foot is a miracle of bioengineering produced by the forces of evolution, but becomes a common site of disease and injury when subjected to the demands of human domestication, being mainly the site of a significant and predominant disorder called pododermatitis[1]. A widespread, sporadic, aseptic pododermatitis, is typical of Ungulates, and can affect cattle, sheep and goats, rarely pigs, but it is especially significant in horses, the species to which the present review refers. This pododermatitis is called laminitis (founder, fourbure, infosura) and is a specific condition of the foot that can produce lameness. The terms «laminitis» and «founder» are used interchangeably. However, in horses, founder usually refers to a chronic (long-term) condition associated with rotation of the third (distal) phalanx (coffin bone), the catastrophic result of laminitis[3], whereas acute laminitis refers to symptoms associated with a sudden initial attack, including pain and inflammation of the dermal lamellae (lamellar dermis). Dermal lamellae form, together with the epidermal/insensitive lamellae of the inner hoof wall with which they interlock, the suspensory apparatus of the third phalanx[1], in other words where the horny wall, by its vertical keratophyl laminae, is fused with the podophyl laminae of the keratogenous layer.
Laminitis has been a recognized disease, since early Greek times, by Xenophon of Athens (380 BCE)[2] .
The main site of this process is the inner layer of the wall (stratum lamellatum) or the cornifying laminar epithelium (hard ker-

Corresponding Author:
Paolo Stefano Marcato (paolostefano.marcato@unibo.it)

atin or hard horn) of the hoof, i.e. the laminar or lamellar corium (dermis) or dermal lamellae (Figure 1). It consists of a lesion involving the complex interdigitation system of the keratinized lamellae which ensure that there is a firm bond between the epidermal hoof wall and the bone of the third phalanx. Damage to the lamellae can cause this interdigitation to fail and the underlying third phalanx to separate from the wall[3 4 5]. At the base, there is damage to the dermo-epidermal interface of the lamellae that causes failure of the attachment of epidermal lamellae, connected to the hoof wall, to the dermal lamellae, attached to the distal phalanx.

This lamellar separation, with failure of the attachment between the inner hoof wall and the distal phalanx, usually occurs in horses as a sequela to gastrointestinal disorders resulting from carbohydrate overload, colic, enterotoxaemia, systemic and metabolic diseases, especially equine metabolic syndrome[*6].

The pathogenetic mechanisms involved in the onset of the laminitis are different on the basis of theories that propose inflammatory, vascular, enzymatic, metabolic or traumatic factors[7]. With regard to two mechanisms that in the past have enjoyed much favour, i.e. inflammation and digital vascular dysfunction, there is debate over which is the primary or whether they are interdependent and have a simultaneous onset, bearing in mind that digital blood flow always plays a crucial role in triggering laminitis.

In the USA, it has been estimated that 15% of horses develop laminitis during their lifetime and 75% of affected horses that are admitted to university referral hospitals are eventually euthanatized. Moreover, in the USA the annual incidence of equine laminitis is 2% but rises to around 5% in spring-summer[8]. Almost half of all cases occur in animals at pasture. Foot lameness in the horse is the most prevalent and frequent medical issue, affecting about 11% of the general equine population in the UK in 2011[9].

Major insights have been gained into the pathophysiology of the condition only in the last 40 years, thanks to the development of several experimental models of laminitis[10].

Table 1 shows different possible causes or precipitating factors in equine laminitis[7]. Although much is known about equine laminitis, more is being discovered from cellular and molecular studies about the equine limbs and the disorders they are prone to.

Table 1 - Possible causes or precipitating factors in equine laminitis (from Baxter G.M.[7]).

1. *Carbohydrate overload*
a. excess grain intake
b. lush pasture (grass laminitis)
c. feed change to high energy legume

2. *Endotoxemia, sepsis, shock*
a. colitis
b. proximal enteritis
c. small intestinal strangulation/obstruction
d. retained placenta, metritis, abortion
e. septicemia or toxemia from any cause

3. *Excessive unilateral weight bearing (support laminitis)*
a. severe lameness
b. rehabilitation of fracture repair

4. *Management*
a. ingestion of cold water by overheated horse
b. unconditioned horse worked on hard surface (concussion or road laminitis)
c. overweight horses or ponies
d. trimming horses too short
e. black walnut wood shavings

5. *Miscellaneous*
a. treatment with corticosteroids
b. hypothyroidism
c. high estrogen plants
d. continuous estrus in mares
e. allergic-type reactions to certain medications.

CLINICAL SIGNS AND PATHOLOGY

Acute laminitis. In acute laminitis, there is a significant local increase in hoof wall temperature, a sharp pain in the hooves on hammer testing, and hypertension of the digital and collateral arteries[11]. Laminitis can involve the front or hind or all four limbs. In horses, it is found mainly in the forelimbs, while in cattle it affects the hind limbs and inner hooves. At a behavioural level (Figure 2), affected animals show prolonged leg resting and are reluctant to move at all, weight-bearing exclusively on the heel of the affected hoof so as to provide relief for the anterior parts where the pain is more intense. In addition, the animals exhibit hyperpnea

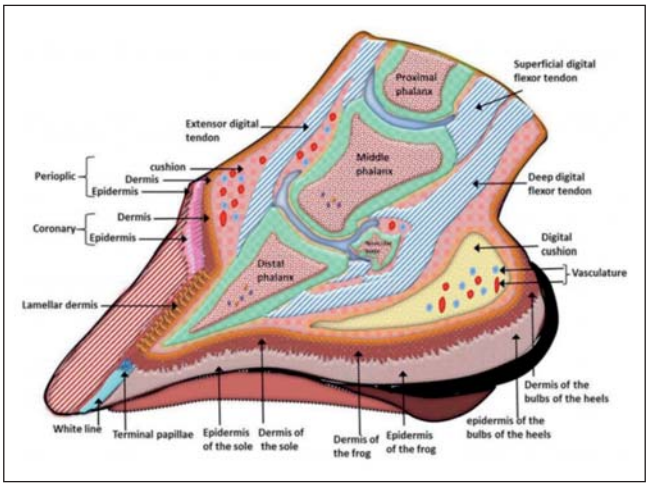

Figure 1 - Drawing of a sagittal section of equine hoof. Schematic diagram illustrating the entire structure of the horse hoof. (Figure from Al-Agele R. et al.[9]).

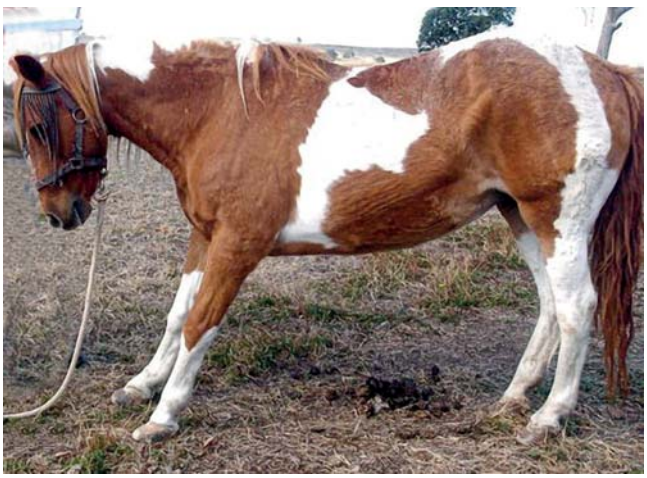

Figure 2 - Acute laminitis. Typical stance of a horse with laminitis of both forelimbs. The horse, when standing, may well lean back on to its hind feet in order to relieve the pressure on its front feet.

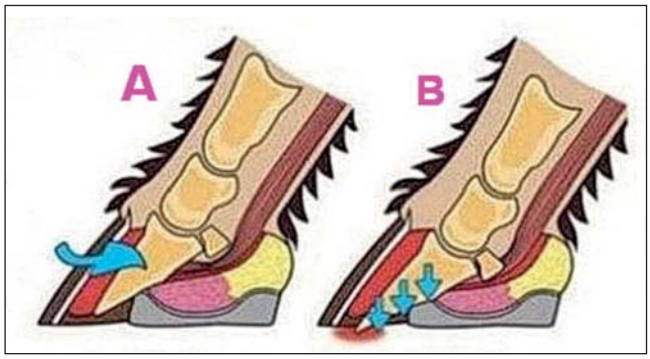

Figure 3 - Schematic drawings of laminitic hoofs. (A) The detached pedal bone (third phalanx) can rotate (arrow). (B) Pedal bone is forced downwards to protrude through the sole (arrows).

and tachycardia, and sometimes symptoms of toxic shock. The most prevalent owner reported clinical signs (≥70%) are difficulty turning and a short/stilted or lame walk[12].

Affected horses may have recurrent episodes and sometimes have to be euthanized due to permanent damage occurring in the hoof. Indeed, once the devastating pathological cascade of laminitis is under way, the anatomical dislocations are so overwhelming that there is little hope that the foundered foot can be restored to normal.

Examining a section of a horse's hoof shows only considerable congestion of the dermal lamellar interface and sometimes hemorrhagic phenomena. The skin of the coronary sulcus may be swollen by edema. In very severe, highly acute cases the disintegration of the dermal-epidermal junction can cause the third phalanx to detach from the hoof wall and to drop ventrally inside the hoof (rotation of the third phalanx in the opposite direction to the dorsal wall of the hoof or distal dislocation of the third phalanx), while a distinct depression and hemorrhagic effusion appear in the coronary sulcus[13].

In the horse, the distance between the dorsal aspect of the third phalanx and the outer wall of the hoof must be less than 18 mm. An increase in this distance is caused by an inflammatory swelling of the laminae with hemorrhage and oedema.

The lamellar tissue which holds the pedal bone in place and properly aligned, starts to lose consistency, then fails as the flexor tendon pulls on the phalanges and the downward rotation of the pedal bone begins, its tip tending to wedge itself under the sole. Deformation of the hoof occurs mainly under the sole that bulges from concave to convex. If rotation of the third phalanx continues, its tip can eventually penetrate the sole of the foot. Sinking is less common and much more severe. It results when a significant failure of the interdigitation between the sensitive and insensitive laminae around a significant portion of the hoof occurs.

Chronic laminitis. Long-term cases usually are the consequence of one or more attacks of the acute form. In chronic laminitis, the horse rests on the back of the hoof of affected limbs thus deforming the hoof, raising the heel together with a sometimes conspicuous elongation (known as a «Turkish slipper») and deformation of the front part of the hoof wall[14]. The sole, too, is deformed and can show extensive avulsion at the white line. Moreover, in severe cases, there may be a continuous solution in the anterior portion of the sole into which the sole margin of the third phalanx has moved. This is due to the fact that when the third (or distal) phalanx is lowered towards the sole, it compresses it, thinning it, deforming it and, in severe cases, even perforating it (Figure 3).

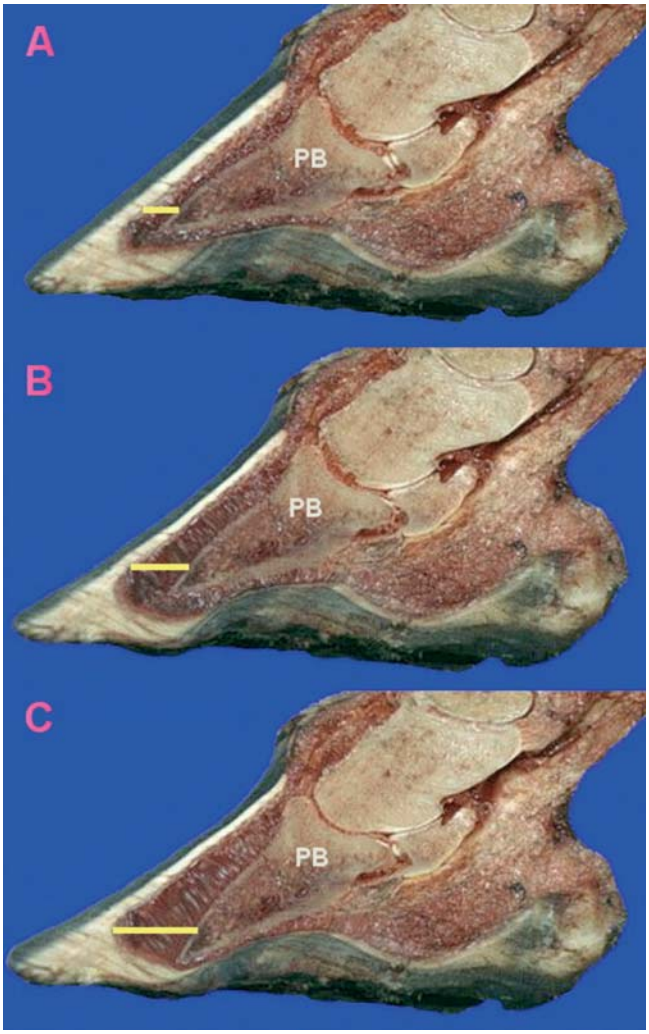

Figure 4 - Three parasagittal sections of equine hoofs, normal (A) and laminitic (B, C). B-C: Sequence of the rotation and sinking process. B. The term rotation has commonly been used when the dorsal surface of the pedal bone (third phalanx) stretches or separates its distal (bottom) attachment from the hoof capsule and appears to rotate downward. C. Sinking. This condition occurs when the pedal bone loses most of its attachment to the hoof capsule and moves distally (downward) in the hoof capsule. If rotation of the third phalanx continues, its tip can eventually penetrate the sole of the foot. PB = Pedal bone (coffin bone, third phalanx). Yellow line = Indicates the extension of displacement of pedal bone and of hyperplasia of lamellar dermis forming a wedge in C. (Figure from ELPO, modified).

Ventral deviation, in a flexor direction, of the distal phalanx, is favoured both by the destruction of the dermal-epidermal junction, as well as by a hyperplasia of the epidermal laminae that produce a horny growth (keraphylocele), which, by progressively filling the residual space between the third phalanx and the inextensible hoof wall, acts as a wedge between these two parts (Figures 4 and 13). In the hoof, irregular strips of horny growth are also characteristic (so-called laminitic or founder rings) (Figure 14).

In order to explain chronic laminitis, it is believed that certain hyperreactive subjects suffer exacerbations, including subclinical ones, of acute laminitic episodes following exposure to antigenic stimulation from vaccinations or environmental allergens (hypothesis of immunological hyperreactivity). Where such hyperreactivity is not linked to autoimmune components, in reference to the strong induction of chemokines for neutrophils and to the persistent presence of neutrophils, these cells cause the per-

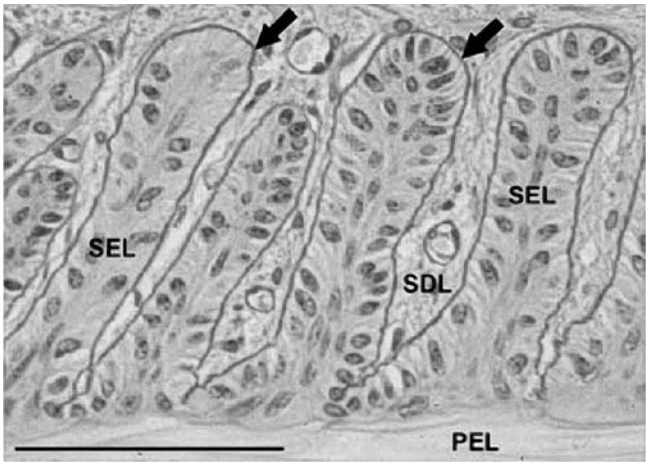

Figure 5 - PAS stained histological section of normal hoof lamellae highlighting the basement membrane (arrows). Closely adherent to the SEL (Secondary epidermal lamella) basal cells, basement membrane (arrows) shows as a dark unbroken line. Connective tissue fills the secondary dermal lamellae (SDL). PEL = Primary epidermal lamella, anuclear, keratinised. Bar = 10 µm.

petuation of the inflammatory state and tissue alteration[60].

MAIN RISK FACTORS

There are four main risk factors for laminitis[15]: 1) diseases involving sepsis or endotoxemia; 2) metabolic diseases from endocrine origin; 3) lush pastures (pasture-associated laminitis) especially in ponies; 4) protracted trauma (load-bearing laminitis, supporting limb laminitis).
Cases of laminitis during sepsis or endo (entero) toxaemia correlate with sepsis from Gram-negative polymicrobial bacteria, and include postpartum metritis with retained placenta, colic (proximal enteritis, volvulus) and enterocolitis. The systemic inflammatory events that occur in sepsis /SIRS (systemic inflammatory response syndrome) related laminitis coincide with marked increase in lamellar expression of a variety of inflammatory mediators including the cyclooxygenase (COX) enzyme, which catalyses the biosynthesis of prostanoids (prostaglandins, prostacyclin and thromboxane) from arachidonic acid. These results suggest that COX 2 and its metabolites are involved in the

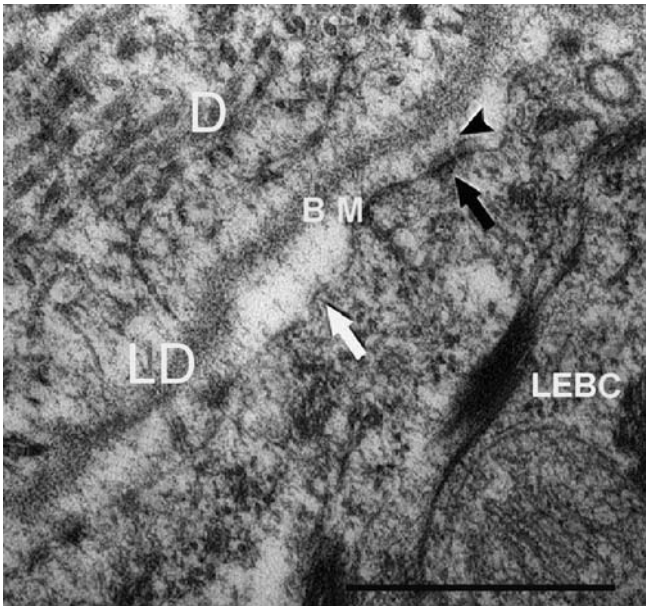

Figure 6 - Laminitic hoof. Ultrastructure of the onset of acute laminitis in a secondary epidermal lamella. Early separation of the basement membrane (BM) by disconnection of anchoring filaments of hemidesmosomes. Dark arrow points to normal looking hemidesmosomes still having anchoring filaments (arrowhead) attaching the lamellar epidermal basal cell (LEBC) to the lamina densa (LD) of the basement membrane (BM). White arrow points to fading and disappearing hemidesmosomes as laminitis starts. D = Dermis. Transmission electron micrograph. Bar = 200 nm. (Figure from de Laat M.A. and Pollitt C.C.[62], modified).

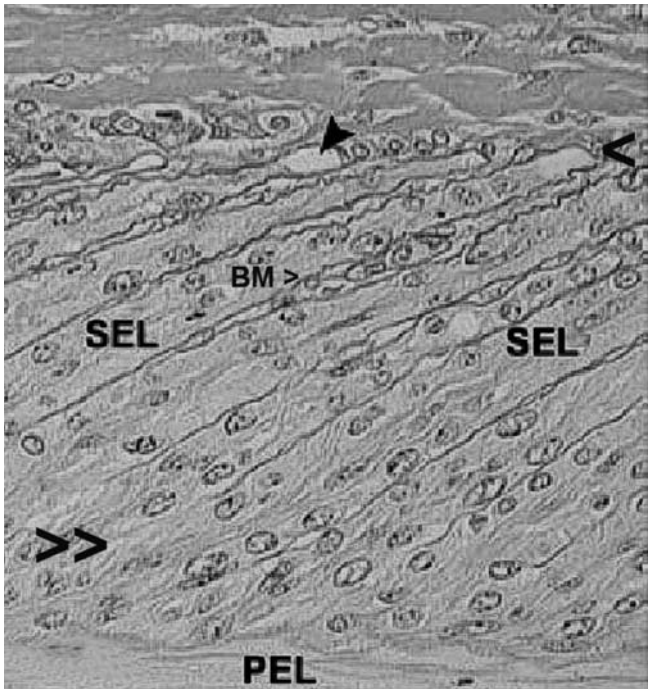

Figure 7 - PAS stained histological section of grade 2 (moderate) laminitis. The basement membrane (BM) appears as a partially continuous line stained dark grey (compare with the dark unbroken line of the preceding figure 5 of control normal lamellar tissue). At the tips of the pointed secondary epidermal lamellae (SEL) the BM has continued the process seen in figure 6 by lifting from the underlying basal cells to form empty, teat-shaped caps (single arrowheads). The BM has disappeared from the crypts between the SEL bases (double arrowheads). There is a reduced amount of connective tissue between the SELs that clump together to form amorphous BM-free masses, on either side of the lamellar axis. PEL = Primary epidermal lamella. (Figure from Pollitt C.C.[64], modified).

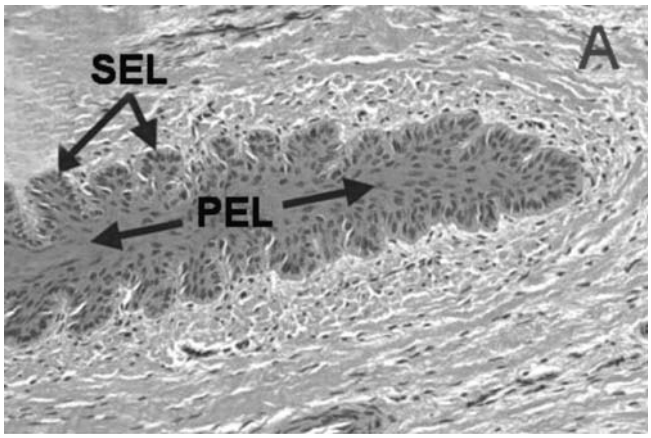

Figure 8 - (A) Photomicrograph of hoof lamellar dermis histology from normal horse. SEL = Secondary epidermal lamellae. PEL = Primary epidermal lamella. EE.

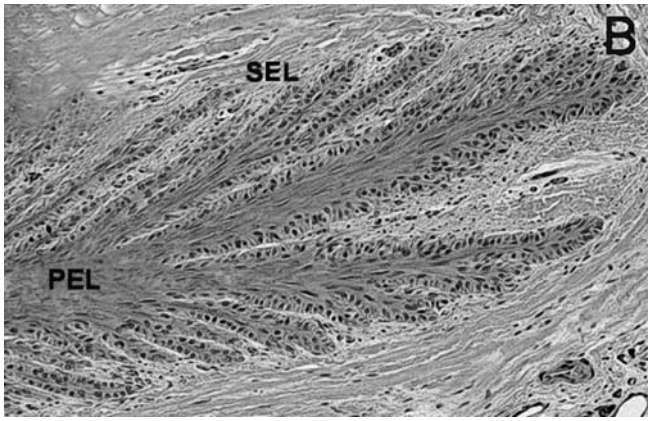

Figure 9 - (B) Photomicrograph of hoof lamellar dermis histology from laminitic horse. Acute lamellar pathology 48h post-induction of euglycaemic hyperinsulinaemia. Cell stretching: SELs (Secondary epidermal lamellae) are lengthened, attenuated and distorted. PEL = Primary epidermal lamella. The s.c. cell stretching is also due to simultaneus acceleration of cellular necrosis-proliferation cycle. Cellular pathology precedes leucocyte infiltration and basement membrane pathology (fig. 6 and 7), indicating that the latter changes may be secondary or downstream events in hyperinsulinaemic laminitis. EE. (Figure from de Laat et al[23], modified).

initiation of pathological changes seen in sepsis associated events such as sepsis related laminitis[16].

Endocrinopathic and grazing-associated laminites are the most common forms. Hormonal disorders, with their consequent metabolic alterations, are responsible for up to 90% of the laminitis cases occurring in animals at pasture.

Most cases of laminitis appear in horses and especially ponies feeding on grass (hence the term pasture laminitis)[19] and usually occur in obese horses and ponies and are exacerbated in animals that graze lush pastures. The risk of developing pasture laminitis lies in the dynamic interaction between predisposing factors (equine metabolic syndrome*, Cushing's disease) and environmental conditions, particularly the abundance of non-structural carbohydrates contained in pasture fodder.

Through analysis of High density single nucleotide polymorphism (SNP) genotype data it was determined that eight measured biochemical traits associated with equine metabolic syndrome (EMS) were moderately to highly heritable in Welsh ponies and Morgan horses[17].

Laminitis cases subsequent to protracted or biomechanical traumas are mainly linked to bearing excessive weight on a limb for long periods due to an inability to use the contralateral limb where it has been immobilized due to severe lameness, orthopedic procedures or paralysis.

PATHOGENESIS OF LAMINITIS

The main failure in laminitis cases is the disruption of the structural integrity of the dermo-epidermal bond of the digital lamellae, i.e. loss of the adherence of the basal epithelial cells in the epidermal lamellae to the underlying dermal lamellae[19]. But this stage in the pathology of the digit can be reached through vascular, enzymatic, inflammatory or mechanical mechanisms, or by a combination thereof.

According to the histopathology of submural tissues[20 21 22 23 24 25 26], some have focused on the structural alterations to the lamellar interface, such as the bevelling and the alterations to the axis of the secondary lamellae, attributed to a mechanical disorder of the hoof as a sequela to disease, while others have focussed on the lamellar and sublamellar regions with findings of hemorrhage, perivasal infiltrates, oedema, alterations of the vascular endothelium, the presence of platelets and fibrin emboli. Cytoplasmic vacuolations, nuclear pyknosis and the loss of cellular integrity, being obvious expressions of necrosis[27], and the separation between dermal and epidermal components at the basement membrane (Figure 6), in the early stages of laminitis, are all commonly regarded as changes to the lamellar epidermis. During the acute phase of laminitis, there is a tendency for the basement membrane to lose stainability, especially at the base of the secondary epidermal lamellar cells[26] (Figure 7). Moreover, at the top of these there are bistratified bullous residues of the detached basement membranes, while as the lesion progresses, detachment occurs with total loss of connection, leaving just a few bullous lesions.

Immunohistochemistry has shown the loss of key lamellar basement membrane components such as collagen IV and laminin. Key factors in laminitis are the loss of the anchoring filament protein laminin-332 (Ln-332, one of the main laminin isoforms of the epithelial basement membrane) and the numerical reduction in laminin-associated hemidesmosomes connecting the basement membrane to epithelial cells [47 48] (Figure 6).

In some cases of hyperacute laminitis, no separation of basal cells from the basement membrane is observed, but the key histopathological event is epidermal necrosis[27].

Lesions of the lamellae during the acute phase of laminitis manifest as a sequela to a pathology unconnected with the hoof. In fact, laminitis is associated, as an epiphenomenon, with a variety of predisposing conditions - excessive intake of cereals or fodder rich in non-structural carbohydrates (simple sugars, starch and fructan, a polymer of fructose molecules), i.e. highly digestible carbohydrates, consumption of black walnut wood shavings, colitis, pneumonia, retained placenta, metritis. Over half of the cases of laminitis are associated with alterations of the distal part of the intestine.

Researchers have used these pathological findings on the one hand to correlate them to the severity of lameness and to disease duration, and on the other to support, reject or advance etiopathogenetic hypotheses.

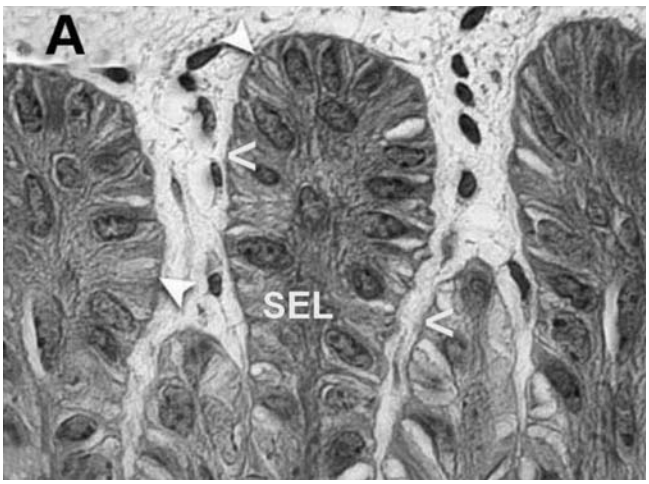

Figure 10 - (A) High magnification histology of hoof normal lamellar dermis. The secondary epidermal lamellae (SEL) are uniform in length and have rounded tips. The lamellar basement membrane is closely apposed to the SEL perimeter (white arrowheads). EE.

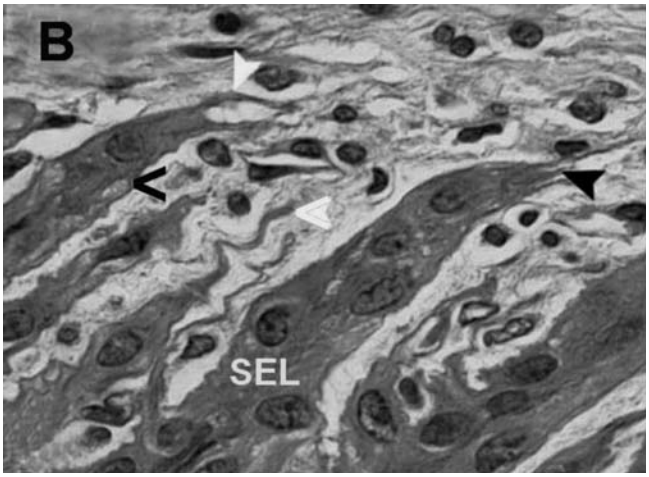

Figure 11 - (B) High magnification histology of lamellar dermis in laminitic horse. Severe lengthening and distortion of lamellar architecture. SELs (Secondary epidermal lamellae) are lengthened, attenuated with pointed tips, s.c. cell stretching (black arrowheads). Wavy strands of basement membrane, no longer apposed to basal cells, are present at SEL tips (white arrowheads). Many basal cell nuclei are oval-shaped but oriented parallel to the SEL axis instead of perpendicular. EE. (Figure from Walsh D.M. et al.[37], modified).

Metabolic-toxic hypothesis

The metabolic-toxic pathogenetic hypothesis proposes that the initial insult to the hoof is directed against the cornifying epithelium of the lamellar interface. In this context, one or more factors of haematic origin (circulating toxins or enzymes associated with a primary disease not originating in the hoof) could act by altering a biochemical step that is critical for the vitality of the lamellar epidermis or for normal cornification.

Supporters of this metabolic-toxic hypothesis[8] claim the severity of dermo-epidermal alterations is evidence that epidermal cells are the target of haematogenous triggers. They also suggest that the other epidermal and dermal changes observed in horses with laminitis are secondary consequences of inflammatory processes or depend on mechanical defects of the affected digits. The haematogenous triggering factors and their role in the onset of laminitis would converge in the dysfunctional regulation of the enzymes responsible for maintaining the attachment of the basement membrane to the basal epithelial cells. The morphological evidence of splitting of the basement membrane is seen as supporting this hypothesis and the consequent dysadesion between the basement membrane and the basal cells of the epidermal lamellae (Figure 6) are regarded as the effect of activating extracellular matrix metalloproteinases (MMPs)[28 29].

Vascular hypothesis

The other hypothesis, of a vascular pathogenesis of laminitis (the vascular theory, based on a model of ischaemia-reperfusion injury), is based on the histopathological data in which epidermal changes during the onset of laminitis are considered to be consequences of a blood flow disorder occurring before the onset of lameness. The resulting phenomenon of ischaemia/hypoperfusion followed by reperfusion could result in damage to epidermal cells and an inflammatory reaction with secondary onset of hyperemia and secondary structural impairment due to alteration (destruction and displacement) of the lamellar basement membrane. The damage to the cells of the basal layer, considered to be necrotic[27], is also documented by an increase in apoptotic and regenerative mitotic phenomena in these cells[8].

Evidence for the idea that reperfusion injury could also contribute to the development of laminitis comes with a biphasic episode of lamellar ischaemia interrupted by a period of hyperemia in the early stages of experimentally-induced laminitis in horses treated with black walnut extract (Juglans nigra), that mimics a systemic inflammatory reaction syndrome[30]. Endothelial vascular changes, the presence of platelets, fibrin thrombi and hemorrhage are considered to be signs of injury to digital blood flow. In essence, the dysadhesion of the lamellar basement membrane is regarded as a later step in the pathogenesis of acute laminitis, while the primary cause of laminitis becomes vascular hypoperfusion with regressive alterations of lamellar epidermal cells. The discovery of a new neurotransmitter system mediated by the neuromedin U peptide, which effectively and potently induces constriction of the equine common digital vein without affecting arterial tone, contributed to an understanding of the factors regulating digital blood flow. And this confirms existing knowledge that digital veins are more sensitive and reactive than digital arteries to vasoconstrictor agents (noradrenaline, 5-hydroxytryptamine, endothelin-1) and vasodilators (β-adrenergic agonists) and is consistent with the regional haemodynamic regulation of the hoof which is mainly controlled by the venous compartment. The vasculature of the hoof wall displays specific angio-adaptations to high pressure and tensile load[31]. However, his unique structural and functional features, such as high hydrostatic pressure and numerous arteriovenous anastomoses, make digital blood flow in the horse more susceptible and vulnerable to dysfunction. Haemodynamic disorders responsible for regional hypoxia-ischemia can also induce an upregulation-activation of destructive proteinases such as extracellular matrix metalloproteinases (MMP-2, -9 and other MMPs) in digital laminae. Damage to the basement membrane and consequent lamellar separation may result. Such damage can also lead in the hoof to vessel hyperpermeability, to diapedesis of neutrophils and to inflammation.

Hormonal hypothesis (Endocrinopathic laminitis)

As mentioned above, laminitis is a pathological syndrome in which the effects of hormonal dysfunctions (endocrinopathic

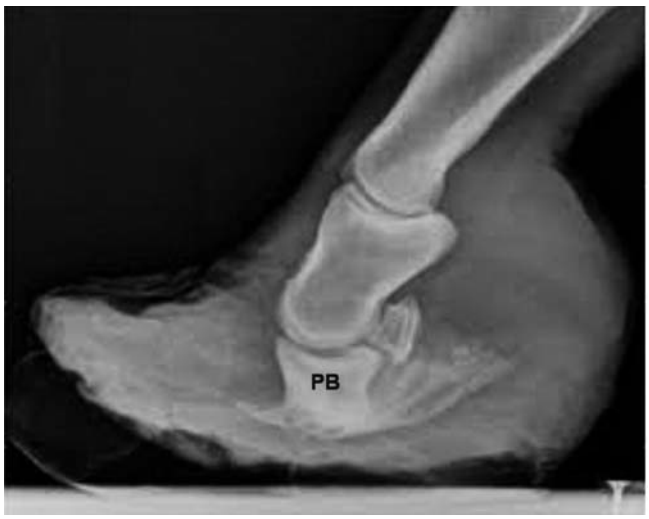

Figure 12 - Horse's foot with chronic laminitis. X-rays (radiograph) showing rotated pedal bone (PB) and chronic remodeling of the pedal bone and hoof' sole. (Figure from canberraequinehospital.com.au/).

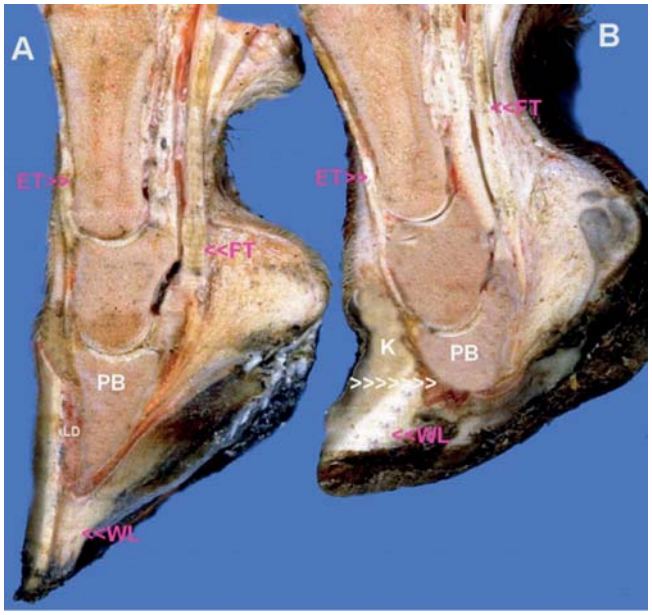

Figure 13 - (A) Parasagittal section of normal hoof. (B) Parasagittal section of severe chronic laminitic hoof. Pedal bone rotated and forced downwards and the sole has become convex. The pedal bone shows remodelling and atrophy. K = keraphylocele forming wedge as the distal pedal bone rotates and displaces distally. PB = Pedal bone (third, distal phalanx). >>>> = Direction of rotation of pedal bone. ET = Extensor tendon. FT = Flexor tendon. WL = White line. LD = Lamellar Dermis. (Figure from Marcato P.S.[63], modified).

laminitis) are great. Equine endocrinopathic laminitis, associated with conditions such as equine metabolic syndrome* (EMS), pituitary pars intermedia dysfunction (PPID), and exogenous corticosteroid administration, is the most common type of laminitis encountered in equine veterinary practice[32]. Common to these disorders appears to be disturbed glucose and insulin regulation, now termed insulin dysregulation (ID).

In a research on horses and ponies with acute laminitis, an endocrinopathy was identified in 94% of the cases. Of these endocrinopathies, equine metabolic syndrome (EMS) was the most prevalent underlying disease occurring in 82% of cases, whereas pituitary pars intermedia dysfunction (PPID) was present in ~38% of the animals[33].

Pasture-associated endocrinopathic laminitis is common, and occurs most frequently in spring and summer, suggesting that particular pasture conditions may exacerbate the syndrome. In horses predisposed to laminitis, although the triggering factor is represented mostly by pasture[34], there is a background endocrinopathy in up to 90% of cases and usually in overweight animals.

The unifying pathogenic factor, in endocrinopathic laminitis (including equine metabolic syndrome* and Cushing's disease) and in so-called pasture laminitis, is hyperinsulinemia[35,36]. When horses digest sugar and starch in the gastrointestinal tract, and absorb glucose, they also release insulin from the pancreas to aid glucose uptake into the cells, which makes the cells sensitive to insulin. When horses eat large amounts of non-structural carbohydrates [such as those in the CHO experiment (administering a CarboHydrate Overload) and in certain lush pastures], the cells become insulin resistant. In such cases, the horse is said to have insulin resistance (IR). This decreases glucose uptake from the blood by the cells, normally enhanced by insulin. And this depletes the supply of glucose, or its metabolization in cells, including those of the foot.

Insulin toxicity appeared to be a key factor in triggering equine laminitis. The onset of laminitis is associated with plasma insulin that exceeds 100 μIU/ml [normal range = 8 to 30 μIU /ml][37]. Insulin has vasoregulatory effects, which is considered one of the plausible links between IR and laminitis. In response to insulin, vasodilation normally occurs due to increased synthesis of nitric oxide (NO) by endothelial cells. However, insulin can also induce vasoconstriction by stimulating endothelin-1 (ET-1) synthesis and activating the sympathetic nervous system. Activation of IGF-1Rc [the insulin-like growth factor (IGF)-1 (tyrosine kinase) receptor] stimulates at least two different signaling pathways in vascular endothelial cells[23]. NO is secreted when the phosphatidylinositol 3-kinase (PI3K) pathway is activated, whereas activation of the mitogen-activated protein kinase (MAPK) pathway causes the release of ET-1. It is known that IR states involve inhibition of the NO synthesis pathway by PI3K, while the MAPK pathway may be overstimulated due to compensatory hyperinsulinemia, leading to an increase in ET-1 synthesis. Therefore, as NO production decreases, vasoconstriction can be promoted in the insulin-resistant animal and this alters the ability of the vessels to respond to stimulation. Histological examination in horses in the pre-clinical phase of insulin-induced laminitis (Figure 9) have shown that significant changes in the lamellae occur before the onset of clinical symptoms.

Gene expression and immunohistochemistry studies have indicated that the lamellae are sparsely populated with insulin receptors, whereas IGF-1 receptors (IGF-1R) are abundant, suggesting that the action of insulin may be mediated by insulin binding to the IGF-1R. Radioligand-binding studies using 125I-IGF-1 and 125I-insulin confirmed an abundance of high-affinity IGF-1R in lamellae (KD 0.16 nM, Bmax 243 fmol/mg protein)[46]. However, the affinity of insulin for binding to the lamellar IGF-1R (Ki 934 nM) was >5,800 fold less than that of IGF-1, suggesting that insulin is unlikely to bind and to activate equine IGF-1R in vivo, even at high physiological concentrations. Moreover there was no evidence to support the presence of insulin/IGF-1 hybrid receptors in lamellae. These findings suggest that insulin does not act directly through IGF-1 receptors and that an alternative theory is required to explain the mechanism of insulin action in laminitis.

In conclusion, insulin is capable of binding to equine IGF-1 re-

Figure 14 - A mare's hoof with chronic endocrinopathic laminitis resulting from repeated episodes of acute laminitis, as evidenced by multiple "founder rings" (white arrowheads) around the hoof wall, which generally indicates a laminitis of long duration. (Figure from alleghenyequine.net).

ceptors at very high concentrations, but these concentrations exceed the levels reached in horses and ponies in vivo, even in the most severely insulin-dysregulated animals.
According to Nanayakkara et al.[43] the mechanism by which insulin causes laminitis remains still unknown, but an indirect action via stimulation of the IGF-1 receptor appears to be highly unlikely.
However, Patterson-Kane et al.[15] suggest that one indirect mechanism through which insulin could activate IGF-1R should be envisaged in the displacing IGF-1 from IGF-binding proteins (IGFBPs) such as IGFBP7 or fragments of IGFBP3, thereby increasing free IGF-1 concentrations, or in a direct action on a very small population of lamellar InsR. This would explain the apparent causal link between hyperinsulinaemia and laminitis and it would be consistent with the presence of mitotic figures in the epithelial cells examined from the tissues of horses exposed to supra-physiological intravenous infusions of insulin.
Future work will determine how IGF-1 receptor-mediated cell stimulation might lead to weakening and elongation of the secondary epidermal lamellae (lamellar epithelial cell stretching). Whether there is a direct hormonal influence on cellular cytoskeletal mechanics will likely necessitate in vitro experimentation with superior lamellar cell/explant culture models.

EXPERIMENTAL MODELS OF LAMINITIS

The pathogenetic mechanisms of laminitis struggle to find unequivocal explanations, but there is little doubt that experimental models of laminitis have led to significant progress in understanding.
Sepsis /SIRS (systemic inflammatory response syndrome) related laminitis. In well-tested experimental models reproducing laminitis, oral administration of either carbohydrate (starch or oligofructose) or black walnut heartwood extract is used[8]. In carbohydrate overload, a disturbance of the intestinal bacterial flora can be detected along with an alteration of intestinal permeability, a sign of structural tissue alteration. Furthermore, many feel that laminitis results in the release of biologically active products, derived both from bacteria and from the host. Development of laminitis as a result of starch overload, suggests that changes in intestinal microbiota may also influence diseases outside of the equine gastro-intestinal tissue[61].
The experimental model of laminitis that involves administering a carbohydrate overload, known as the CHO model, is the one that most realistically simulates clinical cases of sepsis-related laminitis (e.g. enterocolitis, occlusion of the large intestine, acute metritis) and also those resulting from so-called pasture laminitis[50]. Indeed, carbohydrate overload causes a reduction in caecum pH, an increase in bacteria which produce lactic acid, destroy Gram-negative bacteria which release vasoactive endotoxins and a substantial alteration of the cecal mucosal barrier resulting in absorption of pathogenic bacterial molecules, including endotoxins.
Bacterial endotoxins are capable of causing haemodynamic alterations in the vascular bed of the hoof by modifying the local quali-quantitative balance of vasoactive substances or the responsiveness to them of the vessels or variously interfering with the functionality of receptor systems that regulate vasomotility. This can occur through endothelium-dependent increase of digital artery relaxation mediated by β-adrenergic agonist[51].
It is also believed that the rapid development of bacteria of the genus *Streptococcus* in the cecum and colon, observed in parallel with laminitis induced with carbohydrate overload, can directly cause laminitis through the production of exotoxins capable of activating matrix metalloproteinases (MMPs) present in the lamellar structure. Activated MMPs can break down hemidesmosomes, which are essential components of the basement membrane, ultimately causing detachment of the basement membrane from the basal epidermal cells[33].
Thus, the experiment confirms that nutrition can simulate the laminitis that occurs as a result of systemic inflammation usually as a sequela to inflammatory processes and destruction of microbial populations in the large intestine (so-called inflammatory laminitis).
Administering more starch than the small intestine can digest causes indigestible materials to pass through to the large intestine. Moreover, fructans, although partly subject to acid hydrolysis or fermentation in the anterior intestine, are thought to still pass in considerable quantities and relatively unmodified into the large intestine where, due to intense proliferation of amylolytic and saccharolytic bacteria producing lactic acid, pH is greatly reduced, leading to mucosal damage.
So-called inflammatory laminitis is also characterized by activation of neutrophilic granulocytes and their migration from the blood into the tissues of the lamellae where they make a significant contribution to the alteration of these structures[52]. The importance of neutrophils in the laminitis development phase and the systemic nature of the inflammatory process are confirmed by the presence of neutrophil elastase (a protease released by azurophilic granules) in the plasma, skin and lamellar tissues. Furthermore, granulocyte elastase is an enzyme that acts by disintegrating the basement membrane of the lamellae. Pro-inflammatory cytokines (chemokines CXCL1, CXCL6 and CXCL8) play a key role in granulocyte activation, migration and degranulation[53]. Maximum granulocyte infiltration and epithelial alteration occur at the onset of laminitis. However, in experimental inflammatory laminitis, the increase in concentration of chemokines in the lamellae comes before the accumulation of granulocytes and lesion of the lamellae have reached their peak. This shows that the intervention of granulocytes is the cause of, and not a reaction to, the degradation of the basement membrane and the structural alteration of the lamellar tissue.
Also in the experimental Black walnut extract (BWE) model of laminitis, the inflammatory process in the lamellae is very evident and documented by the increase in proinflammatory cytokines, chemokines, cyclooxygenase (COX)-2[54] and the endothelial adhesion molecules needed for the adhesion and diapedesis of leukocytes [30]. In these experimental conditions of systemic inflammation, several affected organs, such as the stomach and skin, are made up of epithelial tissues and therefore it is plausible that extensive degradation of the components of their basement membranes is also required. However the pathology is more manifest and harmful in the equine foot as the dysadhesion-dislocation of the lamellar basement membrane, a phenomenon that is only detected in the hoof in the case of laminitis, is favoured by the weight-bearing on the hoof and by the locomotor forces that under normal conditions the hoof would be able to withstand without damage.
Endocrinopatic laminitis. Digital lamellae from insulin treated ponies were attenuated and elongated with many epidermal basal cells (EBC) in mitosis[49]. Unlike carbohydrate induced laminitis in horses there was no global separation at the lamel-

lar dermal/epidermal interface among ponies. Sporadic EBC basement membrane (BM) separation was associated with the proximity of infiltrating leucocytes. Lamellar proliferation may be an insulin effect through its mitogenic pathway. Aberrant lamellar mitosis may lengthen and weaken the lamellar, distal phalanx attachment apparatus and contribute to the clinical signs that developed. Examination by TEM showed excessive waviness of the basement membrane zone and pointed tips of some secondary epidermal lamellae, an ultrastructural lesion typical of laminitis. The average number of hemidesmosomes/microm of basement membrane was decreased and their distance from the centre of the lamina densa of the basement membrane was increased[49].

Insulin weakens the structural integrity of equine lamellar explants and an ex vivo model for evaluation of hyperinsulinaemia induced lamellar failure was established. The histological evaluation of explants of digital lamellae incubated with insulin validated the structural failure that occurs through the separation of the dermo-epidermal attachment at the basement membrane (BM) level[38 39]. Ultrastructural study[62] reveals that in the acute phase of insulin-induced laminitis the BM zone is extensively disorganized with loss of uniformity of the lamina lucida and lamina densa, reduction, fragmentation and disorientation of hemidesmosomes (HDs), and cytoskeletal disengagement of the HDs contributing to the weakening of the dermoepidermal junction and lamellar failure (Figure 6). HD loss is also accounted by disassembly of HDs during excessive cellular proliferation, secondary to hyperinsulinemia.

In experimentally-induced laminitis with prolonged insulin infusion, leukocyte infiltration of the lamellae is very low or absent, but there is nevertheless a link between hyperinsulinemia and inflammation as the MAPK (mitogen-activated protein kinase)-dependent signaling pathway is implicated in neutrophil migration/diapedesis[40]. As mentioned above, MAPK is stimulated by insulin and correlated with insulin-stimulated cell proliferation in various tissues, and is also activated by various cytokines and inflammatory mediators.

Other studies[32] also provide evidence of inflammatory signaling (pro-inflammatory cytokines and chemokines detected) within the digital lamellae of horses experiencing prolonged supraphysiologic hyperinsulinemia by euglycemic hyperinsulinemic clamp (EHC) technique. However, it seems unlikely that this signaling, possibly elicited through exposure to increased concentrations of glucose, insulin (acting through insulin receptor and/or IGF-1 receptor), leptin, or other metabolic intermediates, represents established, "classic" inflammation (where leukocyte emigration is a hallmark of inflammation) as a primary pathophysiologic mechanism involved in endocrinopathic laminitis. Since in agreement with previous reports, very little to no evidence of leukocyte emigration into lamellar tissue was observed in response to this model. Rather, the lamellar inflammatory signaling may instead be an evidence of "cross talk between metabolic regulatory signaling pathways and inflammatory pathways" occurring in response to altered intracellular concentrations of energetic metabolites, which has been documented to occur in multiple cell types in vitro and in vivo[41]. Moreover, the lack of significant lamellar inflammatory cell infiltrates accompanying this model suggests that the source of pro-inflammatory mediators detected within lamellar tissue is likely to be the major cell type in this tissue, the lamellar keratinocyte.

Equine laminitis also involves epidermal cell proliferation, in this case in the epidermal basal cells of the digital lamellae. Hyperinsulinemia produces a more hyperplastic lesion where proliferation and distortion (stretching) of lamellar epidermal basal cells results in lamellar thinning and lengthening (Figure 9). The proliferative component of the response suggests a growth factor type role for insulin in the instigation of laminitis.

In insulin-dysregulated ponies, mean, post-prandial EGF concentrations were found almost three times higher than in healthy ponies, but the same authors in an investigation of the equine epidermal growth factor (EGF) system have found no evidence of an increased expression (neither gene nor protein) of the lamellar EGFR (Epithelial grow factor receptor) during experimentally-induced laminitis[42]. EGFR is unlikely to be a pathogenic factor in insulin-associated laminitis pathophysiology, but it might play a role, at least in part, in epidermal repair. Although the EGFR does not appear to play a major pathogenic role in hyperinsulinemic laminitis, the significance of increased EGF in insulin-dysregulated ponies deserves further investigation.

Very few insulin receptors (InsR) were identified in the lamellae[43], and more importantly they are not located on the epidermal basal cells[44]. Therefore the mechanism by which insulin causes cell proliferation is not known. However, in other species, insulin at high concentrations is known to directly stimulate receptors for insulin-like growth factor-1 (IGF-1), which is a powerful cell mitogen. Reorganisation of the cytoskeleton can occur in response to receptor activation, largely studied in the context of cancer cell motility, migration and epithelial to mesenchymal transition (EMT)[45].

Support limb laminitis. A work has established an experimental model to study preferential weight bearing and initial results have suggested that lamellar hypoxia may act in the pathogenesis of supporting limb laminitis[18].

Further experimental mechanistic research is warranted to explore any causal relationship(s) between hyperinsulinaemia and cellular changes[15]. Investigations should focus on: (1) the earliest changes, possible invisible histologically or ultra-structurally; (2) analyses of specific tissue compartments using NGS (Next Generation Sequencing) and/or proteomics, in particular of basal lamellar epithelial cells; and (3) investigation of possible links between insulin, IGF-1R, cellular tensegrity, inflammatory mediators, and BM composition/structure.

CONCLUSIONS

Laminitis, rather than a distinct morbid entity, must be better considered a clinical syndrome associated with (1) a systemic inflammatory response syndrome (SIRS), inflammatory laminitis, or with (2) a systemic pathology with endocrinopathy, endocrinopathic laminitis, or (3) associated, much less frequently, with protracted traumatism or biomechanical causes, load laminitis, support limb laminitis.

Studies in the Europe and USA agree that endocrine-associated laminitis (endocrinopathic laminitis) is the predominant form of 90% in horses presenting primarily with lameness [55].

The main endocrinopathic disorders that result in laminitis are the pituitary pars intermedia dysfunction (PPID) and/or other endocrine diseases (today collectively called equine metabolic syndrome - EMS -), the first characterized by obesity, insulin resistance and laminitis[35].

Instead of the pathogenetic definition of laminitis as a primary and serious pathology of the basement membrane [48 62], current

investigations on the predominant endocrinopathic laminitis propose as a key early pathophysiological event a variable subclinical phase centered on the stretching and elongation of the cells of the epidermal lamellae (lamellar epithelial cell stretching)[23] (Figures 9 and 11).

In endocrinopathic laminitis, hyperinsulinemia is expected to alter the metabolism of basal epidermal cells, not with a direct effect on cells, but through the activating binding of insulin with the IGF-1Rc receptor [insulin-like growth factor (IGF)-1(tyrosine kinase) receptor] also present on epidermal cells[56]. As has been observed in other cell types, the IGF-1Rc could interact dynamically also with integrins, intercellular adhesion molecules, and cytoskeleton. The alteration of the cytoskeleton of the cells of the secondary epidermal lamellae[62] would reduce their ability to support normal biochemical stress on the lamellae[57]. The cytoskeleton is in fact an arbiter of the shape and mechanical properties of the cell with microtubules and actin filaments that act respectively as compression-resistant elements and as traction components.

To complete the IGF-1Rc intervention there is also the regulation of two important signaling pathways: p44 / 42 MAPK (mitogen-activated protein kinase) and PI3K (phosphatidylinositol 3-kinase) / Akt, the latter also capable of regulate cell adhesion, by dissolving hemidesmosomes[58] (Figure 6), and altering the normal organization of the cytoskeleton. In turn, the activation by the two pathways of proteins, via phosphorylation of serine, threonine and tyrosine, can converge in the downstream activation of mTORC1 complex and RPS6 29, proteins both involved in producing epithelial cell dysplasia.

Cytomorphological alterations can result from damage to the cytoskeleton, such as loss of perpendicular orientation of the nuclei of the secondary epidermal lamellae with respect to their basement membrane, assumption of the ovoid shape of the nuclei that from apical become more central in the cytoplasm (Figure 11), while the nucleoli become prominent and with more random orientation. And the weakening and elongation (stretching) of the lamellar epithelial cells (which translate into alteration of their tensegrity, an architectural system in the structures that stabilizes them) may well induce some secondary alteration in the structure and / or elasticity of the basement membrane, and thus a further relaxation of the lamellae followed by loss of adhesion of the layer of basal epithelial lamellar cells with the underlying basement membrane[59].

The basal lamellar epithelial cells participate in the suspension of the distal phalanx on the stratum internum of the hoof capsule and have firmly maintained hooks with their basement membrane. Therefore the first motive that begins to compromise the suspension system of the distal phalanx in the hoof causing its descent is the alteration of the basal epithelial lamellar cells which determines their detachment from the basement membrane.

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* The term *equine metabolic syndrome* was first introduced in 2002 to propose that obesity, insulin resistance and laminitis were all components of a single clinical syndrome identified in horses and foals. Similarly to the human syndrome of the same name, equine metabolic syndrome is a condition of insulin resistance that derives from the hypersecretion of tissue cortisol (under the effect of 11-β-hydroxysteroid-dehydrogenase) present in adipose tissue, without any pituitary involvement (equine Cushing's syndrome). The associated clinical signs are a tendency to obesity and the notable prevalence of chronic laminitis in middle-aged animals. Diagnosis is based on clinical data and confirmation of persistent hyperinsulinemia and hyperglycaemia after intravenous administration of a glucose bolus.

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