

# LAMINITIS THEORY – SHOTS AROUND THE TARGET

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## Introduction

Laminitis is the most serious disease of the equine foot and causes pathological changes in anatomy that lead to devastating loss of function. The simplest definition of laminitis is: failure of the attachment between the distal phalanx and the inner hoof wall. A horse has laminitis when the lamellar architecture of the inner hoof wall, which normally suspends the distal phalanx from the inner surface of the hoof capsule, fails. Without the distal phalanx properly attached to the inside of the hoof, the weight of the horse and the forces of locomotion drive the bone down into the hoof capsule, shearing and damaging arteries and veins, crushing the corium of the sole and coronet, causing unrelenting pain and a characteristic lameness (Fig 1).



*Fig 1. Horse with severe laminitis in both front feet showing typical laminitis gait. The hind feet are placed as far forward as possible before the horse attempts painful shuffling steps.*

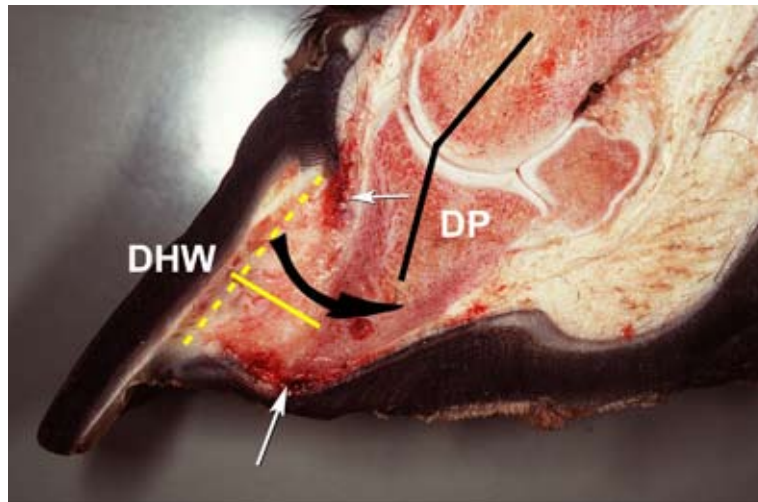
## The phases of laminitis

A *developmental phase*, during which lamellar separation is triggered, precedes the appearance of the foot pain (*the acute phase*) of laminitis. This is around 24 - 40 h in the case of excessive ingestion of high

starch grain (Garner *et al.* 1975; Obel 1948; Pollitt 1996) or fructan (van Eps and Pollitt 2006). During the developmental phase and prior to the clinical appearance of foot pain the horse or pony usually experiences a problem with one or more of the following organ systems: gastrointestinal, respiratory, reproductive, renal, endocrine, musculoskeletal, and immune. Multi-systemic aberrations in organs anatomically remote from the foot result in the lamellar tissues of the feet being exposed to factors which lead to separation and disorganisation of lamellar anatomy. The exact nature of the laminitis trigger factors, apparently reaching the lamellar tissues via the circulation, has yet to be elucidated. Sometimes no developmental phase can be recognized: the horse or pony is discovered in the acute phase with no apparent ill-health or inciting problem occurring beforehand. Obesity and related endocrinopathic problems have recently been incriminated in the pathogenesis of this insidious form of laminitis (Johnson 2002; Johnson *et al.* 2004). Grass founder can also appear without warning and this has now been linked to seasonal variations in the concentration of the soluble sugar fructan by temperate pasture species (Longland and Cairns 1998; Watts). Fructan can suddenly reach very high concentrations in the stems of grass and trigger a laminitis inducing gastrointestinal disturbance when consumed by horses and ponies. That laminitis can be induced by such sugars has been verified experimentally using oligofructose, a closely related compound (Pollitt *et al.* 2003; van Eps and Pollitt 2006). The parenteral injection of potent long acting corticosteroid preparations for the treatment of skin disease may precipitate iatrogenic acute laminitis (Eustace and Redden 1990).

The *developmental phase* merges into the *acute phase* of laminitis which lasts from the onset of clinical foot pain and lameness at the trot, to the time when there is clinical (usually radiological) evidence of displacement of the distal phalanx within the hoof capsule (Fig 2 and 3). After the acute phase, if the horse does not die from the disease process inciting the development of laminitis, it can make an apparent complete recovery or develop palmar/plantar displacement of the distal phalanx, the hallmark of chronic laminitis. The *chronic phase* can last indefinitely with clinical signs ranging from persistent, mild lameness, continued severe foot pain, further degeneration of lamellar attachments, recumbency, hoof wall deformation and even sloughing of the hooves (Hunt 1993). It is important to realize that the process initiating the destruction of the lamellar attachment apparatus begins to operate during the developmental phase before the first clinical sign of laminitis, foot pain, is apparent. During the developmental phase the specific problems of the horse often have to be attended to urgently (e.g. acute abdomen, grain overload acidosis, electrolyte imbalance, rhabdomyolysis, retained placenta) and unfortunately the feet are often left out of therapeutic equation until the first signs of foot pain (shifting weight from one foot to the other, lameness when trotted out, especially when turned) appear. By the time foot pain is apparent lamellar pathology is underway. In other words foot pain is the clinical sign that lamellar disintegration is occurring. To wait and see if foot pain is the

sequel to a metabolic crisis is to miss the opportunity to prevent or at least ameliorate lamellar pathology.



*Fig 2. Sagittal section of a foot with severe chronic laminitis and a large lamellar wedge. The attachment between the distal phalanx (DP) and the dorsal hoof wall (DHW) has failed and hoof and bone are now widely separated. The dashed yellow line shows the original position of the distal phalanx. The solid black line shows that the distal phalanx has rotated (in the direction of the curved black arrow) off the normally straight axis of the proximal and middle phalanges. The material now between the inner hoof wall and the bone is abnormal and consists of epidermal tissue proliferating to form a weak, disorganised mass called the lamellar wedge (yellow line). The descent of the unattached distal phalanx into the hoof capsule has distorted the growth of the proximal hoof wall tubules and has caused the sole to become convex instead of concave (dropped sole). Two dark haemorrhagic zones (white arrows) show the sites of greatest pressure and trauma.*



*Fig 3. Lateromedial radiograph of a digit with severe chronic laminitis. The distal phalanx (DP) has dropped deeply into the hoof capsule and is close to the ground surface which is shown by the horizontal radiopaque marker. The hoof distal phalanx distance (HDPD) is 42% of L the length of the palmar cortex (L) of the distal phalanx. The HDPD in the normal horse is approximately 25% of L. There is a linear radio lucency beneath the hoof wall (arrowheads) indicating that the epidermal lamellae of the inner hoof wall have separated from the dermal lamellae.*

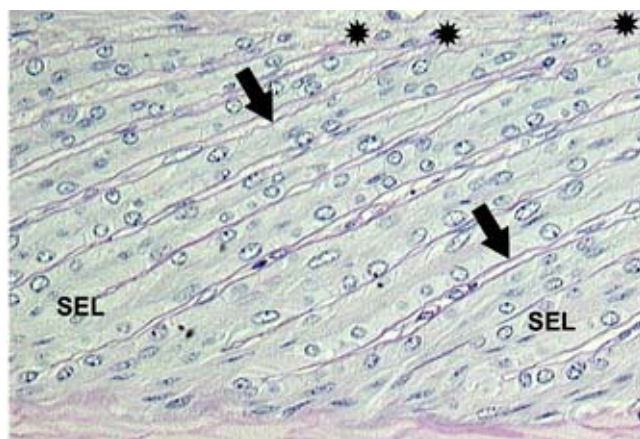
There is a good correlation between the severity of laminitis histopathology, as seen with the microscope, and the degree of lameness (Obel 1948) shown by the horse (Pollitt 1996). When a horse first starts to show laminitic pain, the anatomy of the hoof wall lamellae is being destroyed. The higher the lameness grade, the more severe the microscopic damage. Any activity that places stress on an already weakened lamellar attachment apparatus (such as forced exercise) causes further damage and is contraindicated. The use of nerve blocks to eliminate pain will also encourage locomotion and precipitate more damage.

### **The laminitis histological grading system**

As laminitis develops a sequence of microscopic (histopathological) changes occurs. Three grades of histological laminitis have been identified based on the degree of severity of the changes. Making the lamellar basement clearly visible is important and requires staining lamellar tissues with either periodic acid Schiff (PAS) or by immunohistochemical methods using basement membrane (BM) specific antibodies (Pollitt and Daradka 1998).

#### ***Grade 1 histological laminitis***

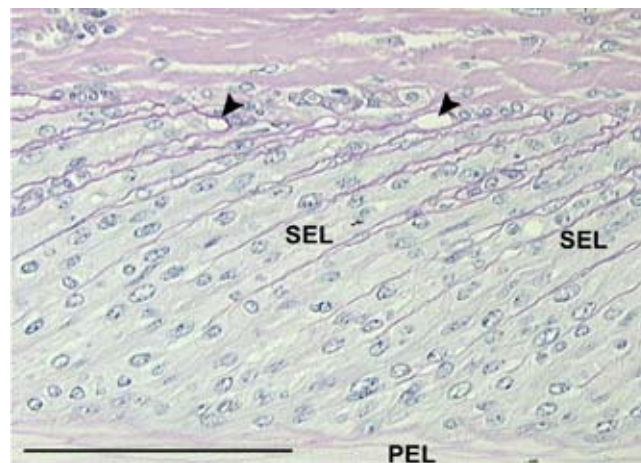
During the developmental phase lamellar basal and parabasal cells lose their normal shape, become elongated and appear to slide over one another and, as a consequence, the secondary epidermal lamellae become attenuated with tapering, instead of club shaped, tips (French and Pollitt 2004c; Pollitt 1996). While this is going on, the BM of the SEL loses its attachment to the basal cells. This is first noticeable at the tips of the SELs where small teat-shaped bubbles of loose BM form. (Fig 4).



*Fig 4. Grade 1 histological laminitis (PAS stain). Micrograph showing hoof lamellar tissues stained to highlight the basement membrane. The basement membrane (arrowed) is stained dark magenta. At the now tapered tips of the secondary epidermal lamellae (SELs) the basement membrane has lifted away (stars) from the underlying basal cells. Between the SEL bases the BM is in its normal position, close to the primary epidermal lamella (PEL). PAS stain. Bar = 10  $\mu$ m.*

### ***Grade 2 histological laminitis***

As dysadhesion between the lamellar BM and SEL basal cells continues the BM is drawn further away with each cycle of foot loading by the horse. The lamellar basement membrane is now absent between the bases of adjacent SELs (Hood 1978; Pollitt and Daradka 1998). The BM retracts from between the SELs and takes with it secondary dermal lamellar (SDL) connective tissue and SDL capillaries (Fig 5). The loss of these capillaries may explain why resistance to blood flow was increased 3.5 times (the bounding digital pulse) in horses during early laminitis (Allen *et al.* 1990) and why blood appears to bypass the lamellar capillary bed through dilated shunts in horses with acute laminitis (Hood 1978). Both of these phenomena are now placed after the triggering of MMP production and occur as a consequence of it. The epidermal basal cells that have lost their BM attachment do not appear to undergo necrosis, at least initially, and clump together to form amorphous, BM-free masses, on either side of the primary lamellar axis.

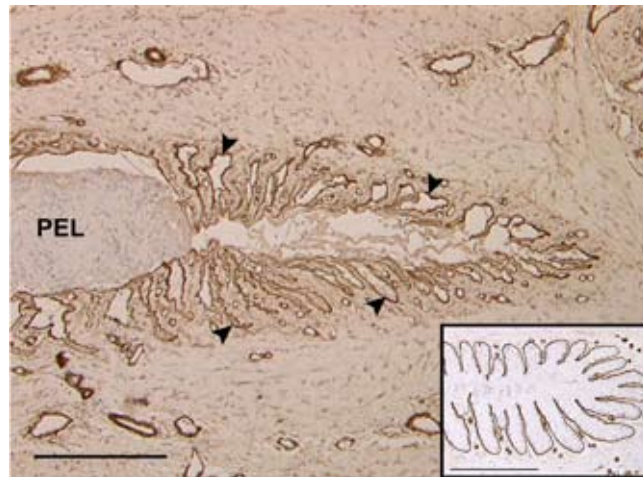


***Fig 5. Micrograph showing hoof lamellar tissues (PAS stain) with histological grade 2 laminitis. The basement membrane is stained dark magenta. At the tips of the now pointed secondary epidermal lamellae (SELs) the basement membrane (BM) has continued to lift from the underlying basal cells to form empty, teat-shaped, caps (arrowheads). The BM has disappeared from adjacent SEL bases and there is little connective tissue and few capillaries between. The lamellar BM is no longer close to the primary epidermal lamella (PEL). Bar = 10  $\mu$ m.***

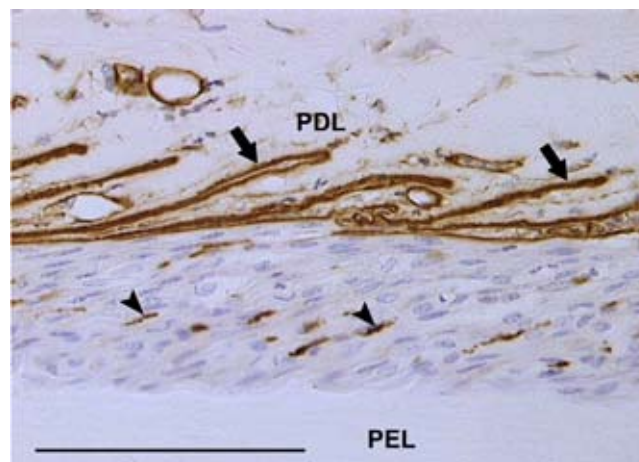
### ***Grade 3 histological laminitis***

In laminitis, the worse case scenario is a rapid and total BM separation from all the epidermal lamellae. Sheets of BM peel away to form aggregations of loose, isolated BM in the connective tissue adjoining the lamellae. The epidermal lamellar cells are left as isolated columns with no connection whatsoever with the dermal connective tissue. At first the lamellar tips slide away from the BM connective tissue attachments microscopically, but as the degree of separation increases the distance between hoof and distal phalanx

becomes measurable in millimetres (Figs 6, 7 and 8).



*Fig 6. Grade 3 histological laminitis (immunostain). The basement membrane of a lamellar tip is highlighted by type IV collagen immunostaining. The tip of the primary epidermal lamella (PEL) has completely detached from its basement membrane. The PEL basal cells are now an unattached, amorphous mass. Collapsed tubes of basement membrane, now empty of epidermal cells, are still attached to connective tissue (arrowheads). The PEL has already moved 0.03 mm from its dermal compartment and soon the distance will be measurable, in millimetres on a radiograph. The inset shows a normal lamellar tip, immunostained the same way. Type IV collagen immunostain. Bars = 10  $\mu$ m.*



*Fig 7. Grade 3 histological laminitis (immunostain). Only remnants (arrowheads) of the basement membrane (BM) remain between the now disorganised secondary epidermal lamellae. Most of the lamellar epidermal cells have coalesced into an amorphous mass no longer effectively attached to any connective tissue. The remainder of the lamellar BM lies free, in strands (arrowed), among the connective tissue of the primary epidermal lamella (PDL). Type IV collagen immunostain. Bar = 10  $\mu$ m.*

This is manifest clinically as the “sinker”. Since the BM is the key structure bridging the epidermis of the

hoof to the connective tissue of the distal phalanx, it follows that the wholesale loss and disorganization of the lamellar BM inexorably leads to the failure of hoof anatomy so characteristic of the chronic stage of laminitis.

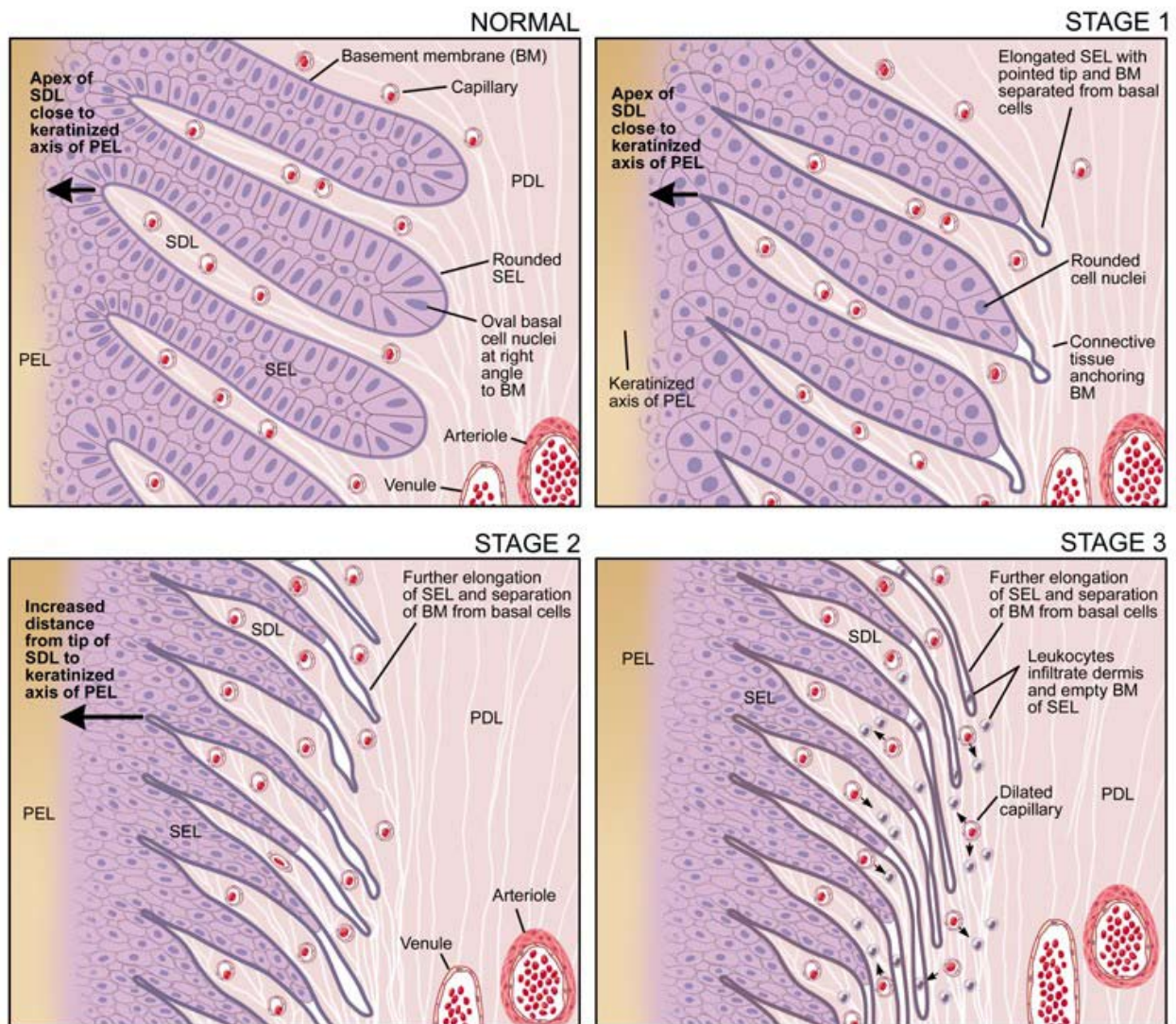


Fig 8. Diagrams showing normal lamellar histology and 3 grades of laminitis histopathology in order of increasing severity.

## The Pathophysiology of laminitis

The spectacular disintegration of the lamellar attachment apparatus, initiated during the development phase of laminitis, compromises a normally robust and trouble free hoof, distal phalanx attachment apparatus in a surprisingly short period of time. Logic suggests that it is a normally tightly controlled metabolic process that is thrown into disarray to cause the lamellar specific lesion of laminitis during its developmental phase (Pollitt *et al.* 2003).

The enzymatic (MMP) remodelling of the epidermal lamellae, assumed to be mandatory as the continually proliferating hoof wall (Daradka and Pollitt 2004) moves past the stationary distal phalanx, appears to be accidentally recruited in the pathogenesis of the laminitis disease process. MMPs are found in increased

quantities from lamellar tissues affected by laminitis (Pollitt *et al.* 1998). The epidermal cells of other species have been shown to readily increase their production of MMP when exposed to inflammatory signalling proteins called cytokines. Cultures of human oral mucosal cells respond to the addition of the cytokines by increasing production of MMPs (Pirilä 2003). Lamellar tissues affected by laminitis also increase transcription of MMP (Kyaw-Tanner and Pollitt 2004) and produce MMPs in their active forms (Pollitt *et al.* 1998) but whether in response to circulating cytokines or some other trigger factor is yet to be established. Evidence from our *in vitro* studies, using equine lamellar explants, suggests that lamellar MMPs are not activated by exposure to human cytokines (Mungall *et al.* 2001). Recently however we have shown very large increases in the lamellar transcription of proinflammatory cytokines at the time of lameness; notably interleukin-6 and 8 (IL-6 and IL-8) (Belknap *et al.* 2006). Increased cytokine expression may lead to downstream events resulting in lamellar failure, similar to organ failure in human sepsis.

The enzymatic theory of laminitis aetiology based on lamellar MMP activation challenges the alternative view that laminitis develops because of vascular pathology affecting the circulation of the foot. A current theory is that vasoconstriction and high hydrostatic interstitial fluid pressure (compartment syndrome) impede the flow of blood in the lamellar microcirculation to cause ischemic necrosis of epidermal lamellae (Allen *et al.* 1990). Current data do not support a role for global ischemia of the laminae in the development of laminitis induced with BWE. Lamellar genes expected to be upregulated if hypoxia/ischaemia were occurring are not (Cochran *et al.* 2006). These authors rightly question treatments aimed at ameliorating ischemia (vasodilators) in the early stages of laminitis.

Epidermal cell necrosis, intravascular coagulation and oedema are not identified in sections made from tissue in the early stages of laminitis in our laboratory (Pollitt 1996). The vessels in the primary dermal lamella, even the smallest, were generally dilated without evidence of microvascular thrombi (Weiss *et al.* 1994). Further, no abnormalities in the systemic coagulation and fibrinolytic cascades are found in horses with carbohydrate induced acute laminitis (Prasse *et al.* 1990). The gross anatomical appearance of freshly dissected laminitis tissue is one of dryness. Sometimes the lamellae peel apart. Tissues affected by a compartment syndrome exude fluid.

### **Laminitis trigger factors**

What are the laminitis trigger factors? Since the carbohydrate overload model of laminitis is characterized by endotoxin production it would seem a safe presumption that monocytes throughout the body would be subject to endotoxin stimulation just as they are during other acute gastrointestinal diseases (Barton *et al.* 1996). Monocytes express cytokines within minutes of exposure to endotoxin. The cytokine cascade originating from abdominal disease such as colic and colitis is responsible for most of the pathological effects

of endotoxemia. However laminitis has never been triggered by the experimental administration of endotoxin into the bloodstream (Hunt *et al.* 1990) or the peritoneal cavity and the actual trigger factors of laminitis remain unidentified. What appears certain in the light of recent research is that the lamellar disintegration of laminitis is mediated by the uncontrolled release of excess MMP (Kyaw-Tanner and Pollitt 2004).

### **Laminitis in vitro**

We have successfully developed a test tube or *in vitro* model (Mungall *et al.* 2001; Pollitt *et al.* 1998) for equine laminitis using small explants of tissue taken from the inner hoof wall of normal, freshly killed, abattoir horses. Each explant consists of hoof and its lamellar layer and the sub-lamellar connective tissue. After incubation for 48 h in tissue culture medium, plus the laminitis trigger factor under investigation, each explant is subjected to tension. The force required to separate epidermal from dermal lamellae is recorded. When dermal-epidermal lamellar separation occurs readily (as occurs in field cases of laminitis) we consider the tissue to have developed *in vitro* laminitis. Lamellar explants can be cultured for up to 7 days in normal medium and no lamellar separation occurs. It is virtually impossible to separate normal lamellar explants. One event that readily causes separation of lamellar explants is MMP activation. The addition to the culture medium of an MMP activator, readily induces explant lamellar separation. The presence or absence of MMP activation in the tissue culture fluid is detected visually using zymograms. Histological sections show a clear zone of complete separation between the basement membrane and the basal cells of the epidermal lamellae. This is a characteristic of *in vitro* laminitis and resembles the basement membrane lesion of natural *in vivo* laminitis.

We have used the *in vitro* laminitis explant model to investigate most of the proposed causes of equine laminitis. Equine lamellae have tested resistant to virtually all known cytokines, tissue factors and prostaglandins. Gram negative bacterial endotoxin and even anaerobic culture conditions fail to induce lamellar separation or significant MMP activation. However there is one notable exception. A factor present in the supernatant of cultures of *Streptococcus bovis* isolated from the equine caecum activates equine hoof MMP-2 and causes lamellar separation (Mungall *et al.* 2001). During grain overload *S. bovis* is the principal microorganism responsible for the rapid fermentation of carbohydrate to lactic acid in the equine hindgut. In the presence of virtually unlimited substrate its population explodes exponentially. We are currently investigating the role of the *S. bovis* MMP activator in natural cases of equine laminitis. If it crosses the mucosal barrier of the hindgut and enters the circulation it may be a “cause” of laminitis (at least in the carbohydrate overload model). In other words it may be an exogenous laminitis trigger factor (LTF). On the other hand fermentation by *Strep bovis* of large quantities of carbohydrate substrate severely damages the epithelium of the hindgut and creates a leaky mucosal barrier. Rapid epithelial remodelling of a repairing hindgut may be a source of

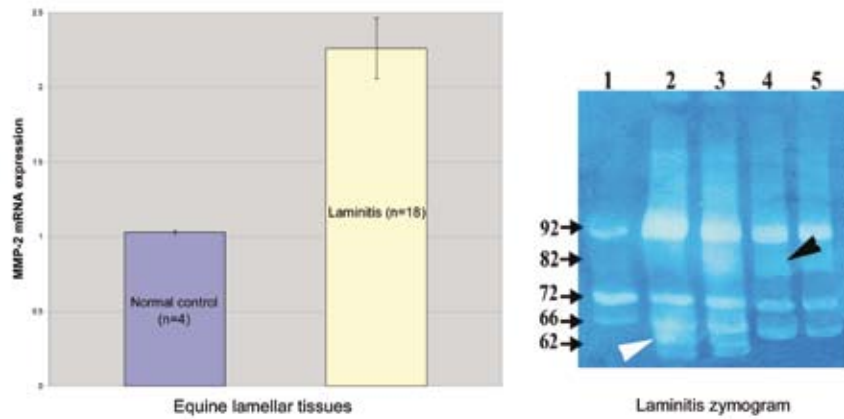
factors that ‘overflow’, via the circulation, and trigger ‘accidental’ remodelling of hoof lamellae. The low pH of the hindgut during carbohydrate overload results in ‘die-back’ of the *Strep bovis* population (Milinovich *et al.* 2006) and peptidoglycan exotoxin and toxic microbial nuclear degradation products released from dying microbes are also candidate LTFs or at least factors that may stimulate a cytokine cascade.

### **MMPs and laminitis**

We have recently cloned the genes responsible for MMP expression in lamellar hoof (Kyaw-Tanner and Pollitt 2004). Horses with acute laminitis show increased expression of the MMPs, 48 hours after alimentary carbohydrate overload (Fig 9). For MMP gene expression to have doubled by the time lameness is manifest implies that the factors signalling the increased expression have been present for some time. This places perturbation of MMP equilibrium early in the cascade of events leading to the foot pain of acute, clinical laminitis. Indeed biopsies of lamellar tissue taken as laminitis develops all show some of the histopathology of the laminitis grading systems (French and Pollitt 2004a; Pollitt 1996). At 24h, lamellae had intact basement membranes but SELs were attenuated with round basal cell nuclei. At 36h, SEL attenuation had progressed and SEL basal cells with rounded nuclei were disorganized; SEL tips were pointed instead of rounded. Only at 48h was the BM not attached to SEL basal cells suggesting that the dysadhesion process commenced somewhere between 36 and 48h. However the molecular and biochemical events contributing to BM disattachment, as evidenced by nuclear rounding and SEL attenuation, were in place by 24h. The basement membrane lesion of laminitis is insidious in nature and well under way by the time clinicians are aware of laminitis foot pain. Any preventive (van Eps and Pollitt 2004; van Eps *et al.* 2004) or treatment strategies must be in place before overt foot pain develops if horses are to survive the development phase of laminitis without significant lamellar damage.

### **MMP inhibitors**

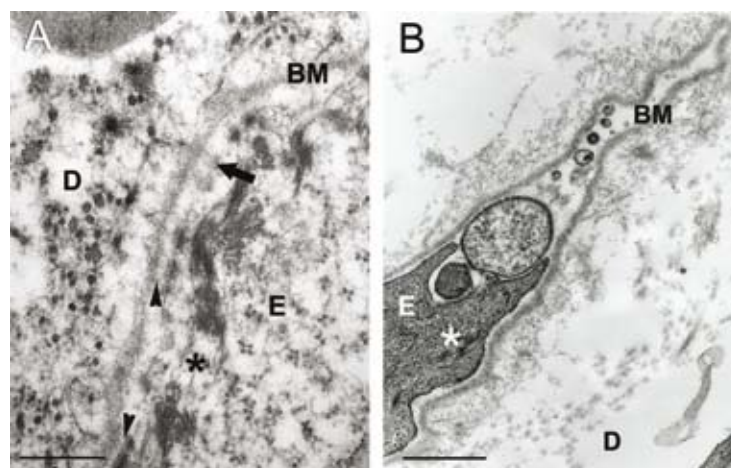
There is a wide range of chemical agents capable of inhibiting MMP activity both *in vitro* and *in vivo* (Roach *et al.* 2002). We have shown that one of these (Batimastat or BB-94, British Biotech, Oxford) blocks the activity of the laminitis MMPs *in vitro* and has the potential to be a useful tool in the prevention and management of acute laminitis (Pollitt *et al.* 1998). Trials to test whether MMP inhibitors can prevent or ameliorate field cases of laminitis are currently underway in the Australian Equine Laminitis Research Unit at The University of Queensland.



*Fig 9. Graph (left) showing the significantly different ( $p < 0.01$ ) mean values of MMP-2 expression between 4 normal hooves and 18 laminitis affected hooves. Polyacrylamide gel zymography (gel contains 0.1% gelatin) of lamellar explants from a horse with laminitis (right). Lane 1 = normal hoof explant supernatant. Lanes 2 & 3 = laminitis fore hoof explant supernatants. Lane 4 & 5 = laminitis hind hoof explant supernatants. Molecular weights are derived from standards (not shown). There is a significant increase in the amount of active MMP 9 (82 kDa - black arrowhead) and MMP2 (62kDa - white arrowhead).*

### The ultrastructure of laminitis

Laminitis studied by transmission electron microscopy (TEM) and immunofluorescence microscopy (IFM) has provided new insight into the mechanism of the disease. The hemidesmosome (HD) is the attachment plaque responsible for maintaining contact between the SEL basal cell and its underlying basement membrane. In lamellar SEL samples taken at the onset of acute laminitis many HDs are absent or disrupted (Fig 10). Loss and disruption of HDs is accompanied by BM separation, cytoskeleton damage and rounding of the basal cell nucleus.



*Fig 10. Transmission electron micrographs (TEMs) of lamellar SELs at the onset of acute laminitis. In A many hemidesmosomes (arrow) are absent or faded. Anchoring filaments (arrowhead) are present where hemidesmosomes are still relatively normal. Loss and disruption of hemidesmosomes is accompanied by the commencement of BM separation and damage of the cytoskeleton(\*). Bar = 500 nm. In B The BM has separated from the*

*attenuated, pointed SEL tip and formed a typical, empty BM enclosed bubble. There are few recognizable hemidesmosomes and only fragments of cytoskeleton (\*). Bar = 200 nm. D = dermis, E = epidermal basal cell.*

Hoof lamellae cultured in vitro without sufficient glucose separate under tension and the intracytoplasmic components of their HDs fade and collapse (French and Pollitt 2004b). Glucose starvation may be operating in vivo when toxemia and the various endocrinopathies associated with laminitis limit the supply of lamellar glucose. Activation of constituent lamellar MMPs also causes lamellar separation under tension but without affecting HD ultrastructure. Activated MMPs appear to cleave laminin5 anchoring filaments and set the BM adrift; also a process now shown to occur in vivo (French and Pollitt 2004a).

Ideas on laminitis pathophysiology abound (Hood 2004) and this review has focused on the MMP, enzymatic theory of laminitis pathogenesis, an hypothesis that depends on the generation of circulating toxins, or proinflammatory mediators (laminitis trigger factors) in the gastrointestinal (carbohydrate overload) or reproductive (septic metritis) tracts. A weakness of this hypothesis is how can laminitis trigger factors pass through the lung, kidney and liver without inducing significant pathology (Hood 1999)? Perhaps it all comes down to the unique anatomy of digitigrade equids. MMP activation and basement membrane dysadhesion may be ubiquitous to the epithelia of many organs but without the influence of weight bearing any resultant pathology is transient. However weight bearing BMs such as those within the lamellar dermal epidermal interface of horse's feet, separate under tension, a process that may escalate into a cascade of ever increasing severity. The validity of this proposition will be tested when veterinary researchers learn how to unload the feet of horses during the developmental phase of laminitis.

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