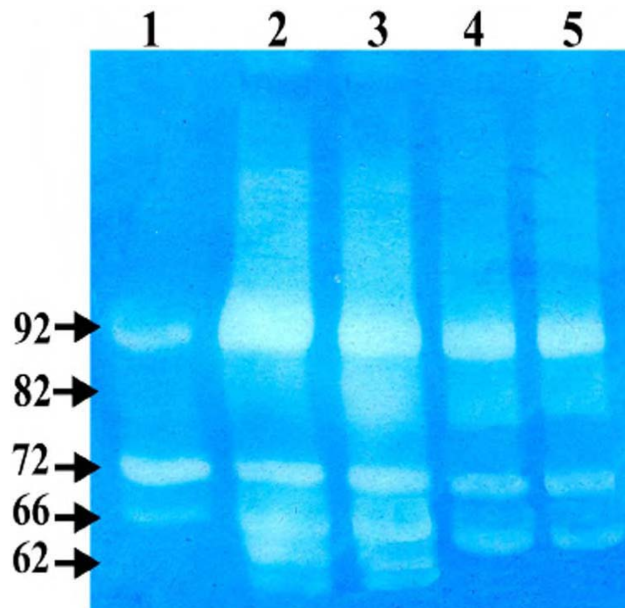


Fig 10. Polyacrylamide gel zymography (gel contains 0.1% gelatin) of lamellar explants from a horse with laminitis. Lane 1 = normal hoof explant supernatant. Lanes 2 & 3 = laminitis fore hoof explant supernatants. Lane 4 & 5 = laminitis hind hoof explant supernatants. Molecular weights are derived from standards (not shown). There is a significant increase is the amount of active MMP 9 (82 kDa) and MMP2 (62kDa).

While optimising the conditions for conducting the lamellar explant experiments we discovered that lamellar epidermal basal cells were quite sensitive to fluctuations in the concentration of glucose in the medium in which they were being incubated. Without glucose, or if their glucose metabolism was poisoned with 2-deoxygluose, explants underwent similar dermal/epidermal separation process as those with activated MMPs (Fig 11). Thus a metabolic pathway to laminitis was proposed. This fitted well with the idea that a form of laminitis developed when horses encountered problems with glucose delivery to peripheral tissues. Thus, a possible explanation for the development of laminitis caused by conditions such as acute inflammatory conditions, corticosteroid therapy, Equine Cushing's disease, obesity related insulin resistance and hyperlipidaemia was provided. It would be expected that these conditions would induce a major hormonally-mediated metabolic shift away from glucose consumption by many peripheral tissues. It was suggested, therefore, that if the metabolic change occurred faster than the hoof tissue could adapt to an alternative energy substrate, then hoof separation and laminitis would occur.

PASS, M.A., POLLITT, S. and POLLITT, C.C. (1998). Changes in glucose metabolism: a trigger for laminitis. Equine vet. J., Suppl.26: 133-138

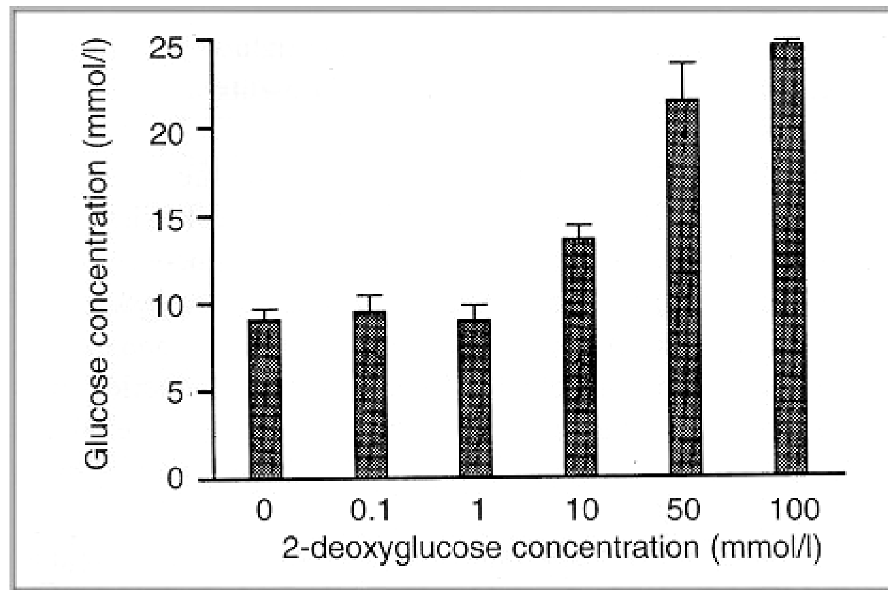


Fig 11. Effect of 2-deoxyglucose (2-DG) on glucose consumption by hoof explants cultured for 2 days in Dulbecco's modified Eagle medium (D-MEM). The results are the mean ± s.e. of 5 estimations of the glucose concentration in the medium at the end of the incubation period. Explants separated when glucose consumption was blocked.

To settle the question of whether laminitis induction was preceded by a period of ischaemia Craig Davies conducted a series of experiments inducing laminitis in a climate controlled laboratory.

POLLITT, C. C. and DAVIES, C. T. (1998). Equine laminitis: its development post alimentary carbohydrate overload coincides with increased sublamellar blood flow. Equine vet. J., Suppl. 26: 125-132

The temperature inside the laboratory was 10°C and small temperature probes, connected to data loggers, were inserted into the dorsal hoof wall of the left and right front feet. It was accepted that changes in hoof temperature reflected the state of the circulation in the sublamellar tissues. Thus, low temperatures indicated vasoconstriction

and high hoof temperatures indicated vasodilation. The ambient temperature of 10°C was chosen as a thermo-neutral temperature against which hoof temperature could be compared. The hypothesis for the experiment was that during the developmental stage of laminitis there would be a period of hoof cooling (and therefore ischaemia) of sufficient duration to cause ischaemic necrosis and/or reperfusion injury of the hoof lamellae and hence laminitis. Laminitis was induced using wheat starch alimentary overload and histopathology, as well as clinical signs, was used to determine if laminitis had occurred.

The results were the opposite of what we expected (Fig 12) and challenged the hitherto accepted premise that laminitis resulted from hypoxia of the lamellar region. Horses that maintained their feet in a vasoconstricted, cool state did not develop laminitis while those that remained hot and therefore vasodilated, did develop laminitis. This was despite all horses developing the clinical signs common to carbohydrate overload laminitis. The only parameter which significantly differentiated the laminitis positive from the laminitis negative horses, between 12 and 32 h after carbohydrate overload, was foot temperature, which was significantly higher in laminitis positive horses ($P<0.05$). Therefore, a period of sublamellar vasodilation, 12 to 40 h after alimentary carbohydrate overload preceded the onset of laminitis. If the digital circulation sustains vasoconstriction during this period then laminitis does not occur.

We proposed that the period of increased digital blood flow in laminitis positive horses, concomitant with the severe metabolic crisis brought on by the alimentary carbohydrate overload, exposed the lamellar tissues to a concentration of blood borne factors sufficient to trigger lamellar separation. This was the first time we emphasised the haematogenous laminitis trigger factor theory and the beginning of our abandonment of the ischaemia dogma. The experiments, conducted on horses with short summer hair coats and therefore not physiologically adapted to a low environmental temperature (10°C), appeared to have serendipitously revealed that digital vasoconstriction conferred protection during the developmental phase of carbohydrate induced laminitis. From this we deduced that deliberately making the distal limb cold (cryotherapy) may be a useful laminitis preventive strategy.

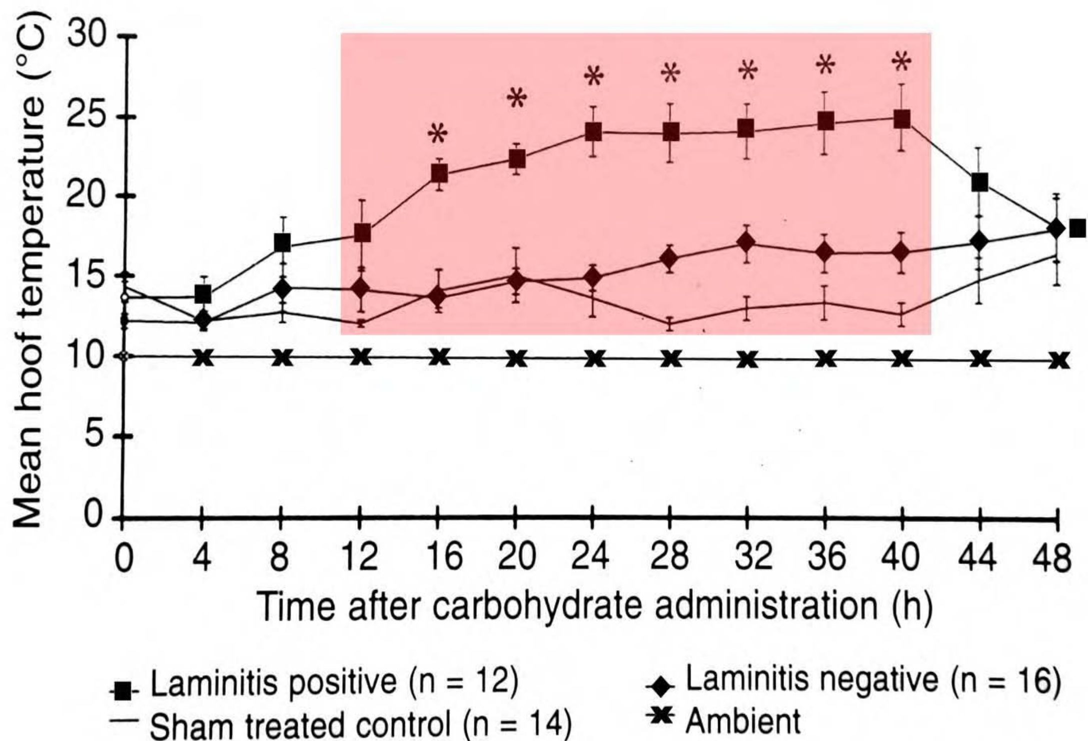


*Fig 12. Mean ± s.e. hoof temperatures of the sham treated control horse group (n = 14) compared to the mean ± s.e. hoof temperatures of the laminitis positive (n = 12) and the laminitis negative horse group (n = 16). The hoof temperature of the laminitis positive group was significantly higher than the laminitis negative group and the sham treated controls between 16 and 40 h after the first administration of carbohydrate (time 0). The indicators of statistical significance refer only to the differences between the laminitis positive and negative groups. *P<0.05.*

The search was now on to establish that laminitis trigger factors existed and could, at least theoretically, reach hoof lamellar tissues via the circulation. To qualify as a laminitis trigger factor the substance had to be able to activate lamellar MMPs and resemble factors known to utilize MMP activation as a pathogenic mechanism. Since by now we recognized that the alimentary carbohydrate overload model of laminitis induction was characterized by an overgrowth of Gram +ve hindgut Streptococci we concentrated on bacteria as a source of potential laminitis trigger factors. Central to our thinking was the observation that horses pre-dosed with the antibiotic Virginiamycin were protected from CHO induced laminitis because Streptococci were absent from the hindgut. If their absence prevented laminitis

surely their presence caused it. This was the key paper from Dr Bruce Mungall's PhD thesis.

MUNGALL, B.A., KYAW-TANNER, M. & POLLITT.C.C. (2001). In vitro evidence for a bacterial pathogenesis of equine laminitis. Vet. Microbiol. 2070: 1-15

Dr Mungall succeeded in calibrating the *in vitro* laminitis explant model by using a force transducer to measure the amount of force required to separate explants. This refinement made the use of hoof wall explants a potent technique for performing large numbers of trials without the need for live horse experimentation. Bacterial broth cultures and purified bacterial proteases activated MMPs and altered the structural integrity of cultured equine lamellar hoof explants. Four Gram-positive Streptococcus spp. and three Gram-negative bacteria all induced a dose-dependent activation of MMP-2 and MMP-9 and caused lamellar explants to separate. We had already shown that Batimastat prevented explant separation by inhibiting APMA induced MMP activation. Dr Mungall's experiments extended this observation to show that Batimastat also blocked bacterially induced MMP activity and also prevented lamellar separation. These results suggested the rapid increase in Streptococcal species in the caecum and colon, observed in parallel with carbohydrate induced equine laminitis, may directly cause laminitis via production of exotoxin(s) capable of activating resident MMPs at the hoof lamellar interface. Once activated, MMPs degraded key components of the basement membrane (BM) hemidesmosome complex, ultimately separating the BM from the epidermal basal cells resulting in the characteristic laminitis histopathology. This study provided a unifying aetiological mechanism for the development of carbohydrate induced equine laminitis.

When Dr Myat Kyaw-Tanner joined the AELRU team, as a post-doctoral fellow, she added the important technique of molecular biology to our investigative armamentarium. Since increased MMP2 production activation seemed to be a key player in the dysadhesion and destruction of lamellar basement membrane of laminitis her first task was to clone and sequence the cDNA encoding lamellar MMP-2. Rabbit anti equine MMP-2 and labelled MMP-2 riboprobe were also developed to analyse and quantitate MMP-2 expression.

KYAW-TANNER M. AND POLLITT C.C. (2004) Equine laminitis: increased transcription of matrix metalloproteinase-2 (MMP-2) occurs during the developmental phase. Equine vet. J. 36: 221-225

Total RNA was isolated, fragmented by RT-PCR, cloned into vector and sequenced. Rabbit anti-equine MMP-2 and labelled MMP-2 riboprobe were developed to analyse and quantitate MMP-2 expression. The results vindicated the effort; lamellar pathology of laminitis was associated with increased transcription of MMP-2. Western immunoblotting with anti-MMP-2 detected 72 kDa MMP-2 in hoof tissue homogenates and cross-reacted with human MMP-2. Immunohistochemistry and *in situ* hybridisation detected MMP-2 in the cytoplasm of basal and parabasal cells in close proximity to the lamellar basement membrane (Fig13). Northern analysis and quantitative real-time PCR showed MMP-2 expression significantly ($P<0.01$) elevated in laminitis affected tissues (Fig14). Real-time PCR analysis of lamellar MMP-2 accurately monitored laminitis development at the molecular level and gave us the potential to objectively test our preventive strategies. Thus we were able to add MMP Real-Time PCR to histopathology and clinical signs, to validate our claim that laminitis had occurred in our experimental tissues. This strengthened our claim as serious laminitis researchers.

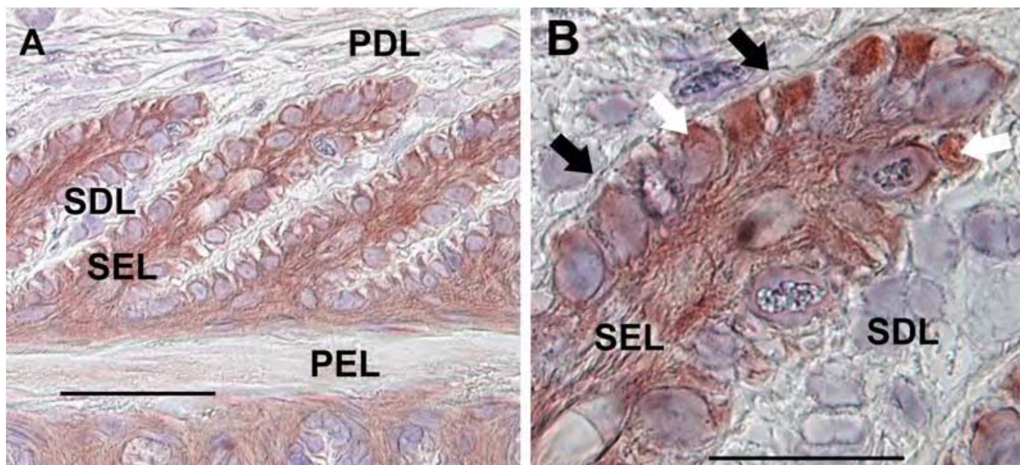


Fig 13. Immunostaining of normal hoof secondary epidermal lamella (SEL) with anti MMP-2. Dark brown, positive cytoplasmic staining was located mainly in lamellar basal and parabasal cells (A). Primary epidermal lamellae (PEL) and primary dermal lamellae (PDL) did not stain. Basal cell MMP-2 of SELs (B) was located in cytoplasm (solid white arrows) adjacent to the basement membrane (solid black arrows). Nuclei of epidermal cells and

fibroblasts stained blue by the haematoxylin counterstain. Bar in A = 50 µm. Bar in B = 20 µm.

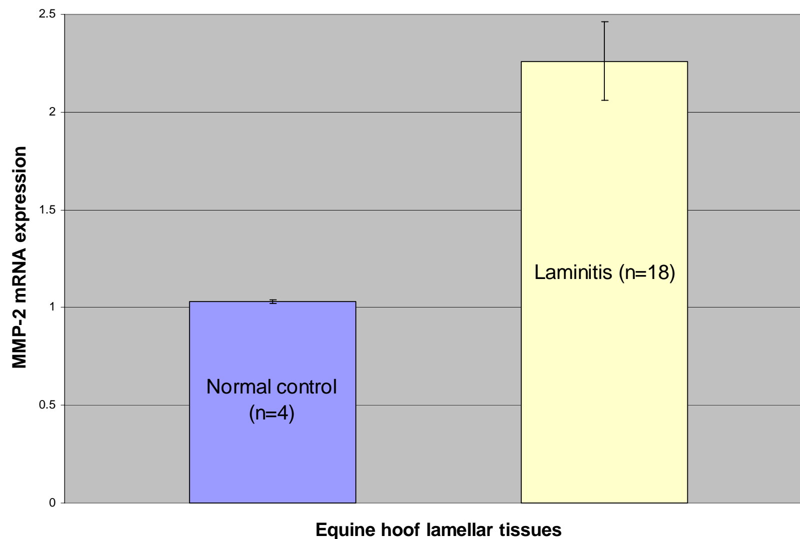


Fig 14. Graph showing the significantly different (P<0.01) mean values of MMP-2 expression between 4 normal hooves and 18 laminitis affected hooves.

Around 2000 it came to our attention that pasture grasses accumulate carbohydrate (CHO) in the form of fructan, sometimes at quite high concentrations (<50% DM). Fructans are polymers of fructose and, unlike the situation with starch, escape digestion by the mammalian small intestine as there is no enzyme with which to digest it. Instead, virtually all ingested fructan is fermented in the hindgut. There is a strong association between certain pasture species and laminitis especially at the times of the year when pasture fructan concentrations are high (spring and autumn). Andrew van Eps began his PhD project by dosing horses with commercially available fructan in the form of oligofructose (Raftilose P95®) giving us the opportunity to test the hypothesis that fructan, fermenting in the hindgut of horses, would induce laminitis. This seemed a logical, more natural progression over the starch alimentary overload model as, after dosing with grain starch, some horses died, required euthanasia or failed to develop the disease. In addition we sought a correlation between oligofructose (OF) dose, clinical and biochemical disturbances and laminitis severity.

VAN EPS, A & POLLITT, C.C. (2004). Equine laminitis: controlled induction by alimentary overload with oligofructose. Equine vet. J. (in press)

Three OF treatments (7.5, 10 & 12.5 g/kg bwt.) and 1 sham treatment were administered to clinically normal Standardbred horses. Clinical observations were made and data collected at 4 h intervals over a 48 h study period. Stained sections of the hoof wall lamellae, examined by light microscopy, were graded for laminitis severity. The effects of OF dosing on clinical signs and blood biochemistry were analysed for between treatment and treatment vs. time interactions. All OF dosed horses developed laminitis in at least one foot that was more severe in the high OF dose groups. Similarly, clinical and biochemical disturbances were more pronounced in horses receiving higher OF doses. All the horses survived without developing severe colic or serious illness. Plasma D-lactate concentrations peaked at around 24 h supplying strong proof of the rapid proliferation of D-lactate producing organisms in the hindgut (Fig 15). Mammals can only synthesize the L isomer of lactate. Interestingly although *S. bovis* and *S. equinus* are both lactic acid producing organisms neither can synthesise D-lactate.

Alimentary overload with OF proved to be a valid laminitis induction model. Laminitis of greater severity resulted when larger amounts of OF reached the hindgut suggesting a dose/response relationship. After this experiment, dosing with OF became our standard laminitis induction model. Since laminitis results from OF fermentation by hindgut microbes, reducing pasture fructan consumption and the numbers of fructan fermenting bacteria in the hindgut are feasible preventive objectives and bookmarked for further investigation.

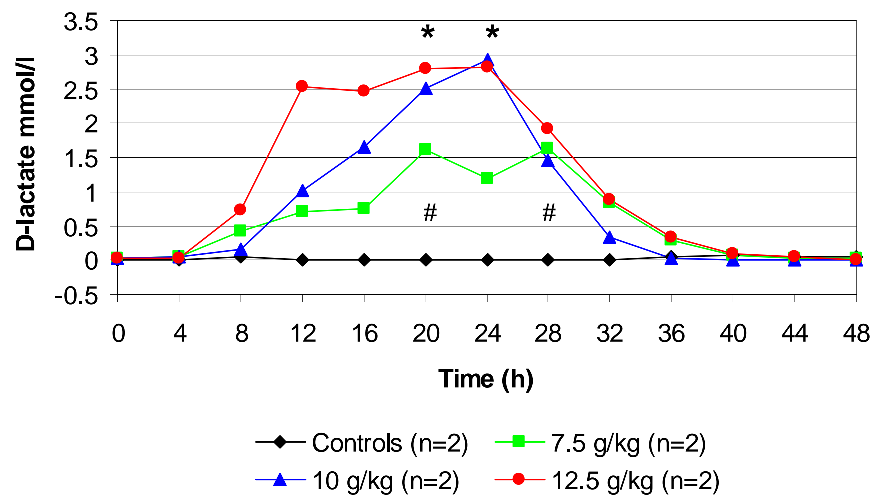


*Fig 15. Oligofructose dosing caused blood D-lactate concentrations to rise in all treatment groups. Blood D-lactate of horses dosed with 10 and 12.5 g/kg bwt. were significantly *($p<0.05$) above horses dosed with 7.5 g/kg bwt. at 20 and 24 h. D-lactate of OF dosed horses was significantly #($p<0.05$) above controls at 20 and 28 h.*

Armed with the more reliable OF induction model Andrew van Eps was able to test the hypothesis that efficient distal limb cryotherapy would prevent laminitis. The literature on cryotherapy for horses seemed to have been extrapolated from human medicine and was not grounded in reality. The recommendation that cold be not applied for more than 30-45 minutes contradicts the basic observation that horses survive severe cold weather in northern hemisphere winters, with no harmful effects, for weeks at a time. Nevertheless we first established that the prolonged application of an ice and water slurry to the distal limb of normal horses caused no harm.

POLLITT, C.C., VAN EPS A.W. (2004). Prolonged, continuous distal limb cryotherapy in the horse. Equine vet. J. 36: 216-220 .

A slurry of ice and water was applied to the right forelimb of 4 Standardbred horses for 48 h. The ice and water mixture was contained in a vinyl boot and was in direct contact with the limb. Hoof temperature, ambient temperature and ice boot temperature were logged continuously and clinical observations recorded every 2 h. Hoof temperature thermistors were housed in stainless steel probes attached to stainless steel brackets. The probes were inserted into holes, 7 x 2 mm, drilled into the sagittal midline of the dorsal hoof wall 20 mm distal to the coronet of both forelimbs and secured to the hoof wall with 2 screws. Lameness examinations were performed prior to cold application and 1 week, 6 months and 1 year after removal of the ice boot.

Continuous cryotherapy was well tolerated and resulted in marked cooling of the treated foot (Fig 16). No significant variation in clinical parameters occurred, and no lameness or gross pathology noted in the treated limbs at any examination period. The horses completely ignored the intense coldness of the treated limb. The only horse management problem was boredom, remedied by the supply of abundant feed. The continuous application of ice and water proved to be a safe and effective means of cooling the equine distal limb. The next experiment was to determine if the extremely low, constant temperature achieved was able to affect the outcome of induced laminitis.

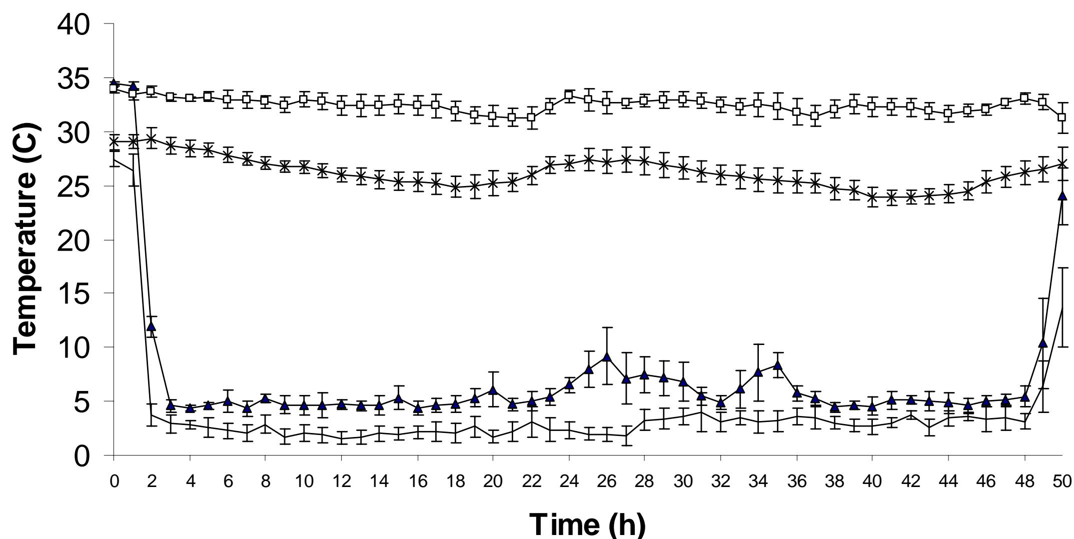


Fig 16. The hoof temperature (HT) of the untreated limbs (n = 4) displayed a general trend of variation similar to, though significantly greater than (P<0.05), that of the ambient temperature. Treated limb (n = 4) HT decreased rapidly after 0 h. After 2 h the HT of the treated limbs (mean 5.3 ± 0.3°C) remained significantly less than that of the untreated limbs for the remainder of the experimental period (P<0.05). At 50 h , 2 h after removal of the ice from the boot, the HT of the treated limbs (mean 24.0 ± 2.7°C) was still significantly less (P<0.05) than that of the untreated limbs (mean 31.5 ± 0.7°C).

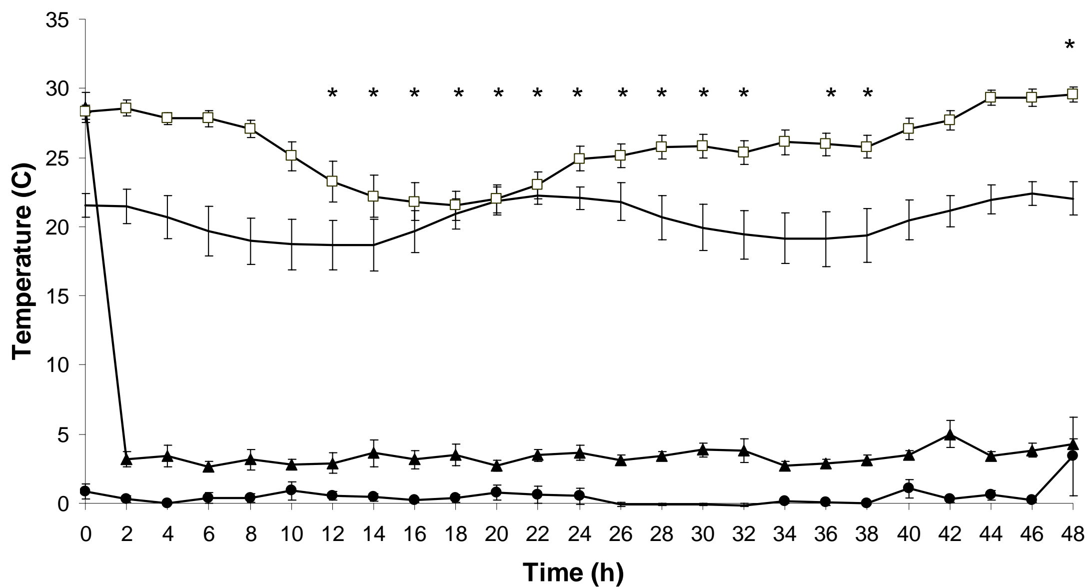
Andrew van Eps induced laminitis in 6 Standardbred horses that had one front limb continuously cooled in an ice/water mixture. Lameness evaluation, blinded lamellar histological grading and analysis for lamellar matrix metalloproteinase-2 (MMP-2) mRNA expression were used to evaluate the severity of laminitis. Again, as in the validation trial, cryotherapy was well tolerated and effective in cooling the feet (Fig 17). Interestingly, foot lifting, characteristic of the laminitis observed in the untreated limbs, was absent in the treated limbs. The apparent resilience of the equine distal limb to the potentially damaging effects of prolonged, extreme cold confirmed the results of the validation trial.

In each horse no lameness was observed in the treated limbs. Laminitis histology scores in the treated limbs were significantly less than those of the corresponding

untreated forelimbs ($P<0.05$). Expression of MMP-2 mRNA in the iced feet was significantly ($P<0.05$) less than that detected in the untreated feet.

Cryotherapy, when applied to one foot, markedly reduced the severity of acute laminitis. We proposed that vasoconstriction (preventing delivery of haematogenous trigger factors) and hypometabolism (reduction in lamellar MMP activity) were the primary therapeutic mechanisms. What was not determined here was whether laminitis could still have developed after cryotherapy ceased. It remains for clinicians in practice to establish if cryotherapy is an effective prophylactic strategy in horses at risk of developing acute laminitis in the clinical situation. Notably we avoided advocating cryotherapy in established acute or chronic laminitis cases as we had no data to support this one way or the other.

The reliability of our histopathology grading system, established in 1996, was rigorously validated in this trial by measuring the level of inter-evaluator agreement in assessing blinded laminitis histopathology. We applied the Kappa statistic and the inter-evaluator agreement in this study ranged from moderate to substantial, with the mean agreement (0.56) marginally below what is considered substantial. This compares favourably with the agreement between pathologists evaluating neoplasia of the colon/rectum and prostate in human patients, where Kappa statistics were 0.20-0.45 (slight to moderate). We recommended that any laminitis research, claiming clinical laminitis as an end-point, should be validated using several blinded evaluators.



A Treated limbs HT (n=6) Ambient Boot Untreated Limbs HT (n=18)

*Fig 17. Mean ± s.e. hoof temperature (HT) of the treated limbs compared to the mean ± s.e. HT of the untreated fore and hind limbs. The mean ± s.e. ambient and ice boot temperatures are also shown. The mean HT of the treated limbs was significantly less (P<0.05) than that of all the untreated limbs by 2h. The mean HT of all the untreated limbs at 12-32 h and 36-38 h after dosing was significantly less than that at 0 h (P<0.05, indicated by *).*

Background information was still needed regarding some basic functions of equine hoof wall. There were two theories explaining how the hoof wall ‘grows’ and moves past the stationary distal phalanx, both based on incomplete evidence. Continuous basal cell proliferation in the proximal region of the hoof wall lamellae occurs, causing distal movement of primary epidermal lamellae (PELs). Mitotic figures (MFs) among the basal cells of the proximal lamellar zone are frequent and a steady rate of distal PEL ‘growth’ is not contested. However convincing evidence that lamellar basal cells, in the remaining lamellae, proliferate to the same degree, is lacking. Movement in the lamellar region could occur by cell proliferation or by an enzyme-based remodelling process. Since laminitis pathogenesis appeared to involve increased transcription and activation of enzymes normally involved in tissue remodelling it seemed important to know precisely which process dominated the lamellar region of the hoof. Dr Mousa Daradka investigated

epidermal cell proliferation in the equine hoof wall and calculated a proliferative index (PI) for the coronet, lamellae and toe.

DARADKA, M. & POLLITT, C.C. (2004). Epidermal cell proliferation in the equine hoof wall. Equine vet. J. 36: 236-241.

We needed an objective technique for measuring cell proliferation that was quantitative and accounted for all cells undergoing mitosis during the study period. Fortunately this was available in the form of an analogue of thymidine, 5-bromo-2'-deoxyuridine (BRdU) that, doubles for thymidine during the synthesis stage of cell division and can later be tracked by immunohistochemistry. BRdU was infused intravenously into 5 ponies. Sixty minutes later lamellar tissue was harvested and BRdU (now incorporated into the DNA of all dividing cells) was detected immunohistochemically using mouse anti BRdU. Proliferative indexes (PIs) were calculated for the coronet and 10 levels of the dorsal hoof wall lamellae.

The highest proliferative indexes (mean ± SE) were in the coronet; 12.04% ± 1.59 and proximal lamellae (7.13% ± 1.92) and were thus growth zones of the proximal hoof wall. PIs of more distal lamellae ranged from 0.11% ± 0.04 to 0.97% ± 0.29; significantly lower ($p=0.05$) than the proximal lamellar growth zone (Fig 18).

A 20-fold PI decrease between proximal and more distal lamellae suggested that the majority of the normal lamellae are non-proliferative and their main function was to suspend the distal phalanx within the hoof capsule. Since the cells composing horse hoof lamellar tissue appear to remodel using enzymes capable of destroying key components of the very extracellular matrix involved in the apparatus suspending the distal phalanx from the inner hoof wall this may explain why, when lamellar MMPs are inadverdently activated *en masse*, horses are peculiarly susceptible to the destructive effects of laminitis. We have provided good evidence that that over expression of MMP-2 occurs in laminitis affected inner hoof wall tissue thus putting laminitis into the disease category of a normal process gone wrong. Understanding this process offers hope that laminitis may yet be preventable if not curable.

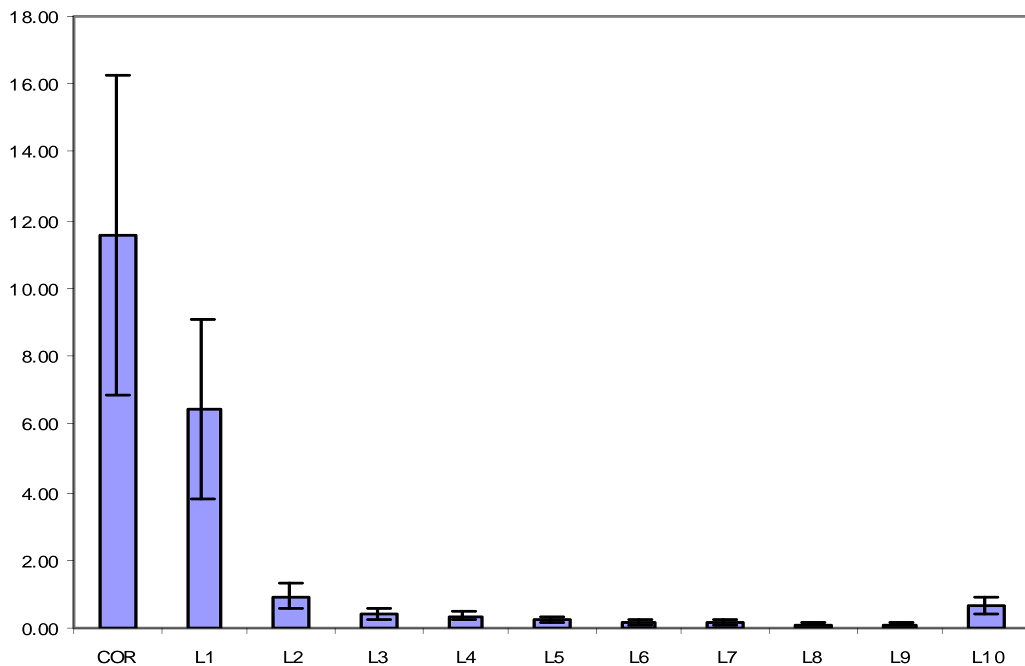


Fig 18. Graph of the means (± standard deviations) of natural logarithms of the percentages (Ln %) of proliferative indices for the coronet and lamellae of 5 normal ponies. COR and L1 were not significantly different. There was a significant drop in the mean Ln% from L1 to L2 and from L2 to L3. Thereafter the lamellar levels were not significantly different from each other until L10.

The next question was asked to shed light on another key function of the hoof wall – how does the hoof wall heal when it is injured? Dr Mousa Daradka investigated wall strip wound healing to better understand the biology of hoof lamellae and thus the pathophysiology of laminitis.

POLLITT, C.C. & DARADKA, M. (2004). Hoof wall wound repair. Equine vet. J. 36: 210-215.

It is well known that surgical stripping of the hoof wall results in a wound that heals remarkably well. However, exactly how lamellae of the hoof wall recover from wall stripping has never been reported. In contrast lamellae recovering from laminitis are often deformed and, notoriously, heal but poorly. We thought investigating lamellar wound healing in normal horses may aid understanding of laminitis. Dr Mousa Daradka documented temporal changes in the lamellar basement membrane (BM), dermis and

epidermis after wall stripping surgery. Wall strips were made in the dorsal hoof wall midline of 6 adult horses. Immunohistochemistry was used to document changes in the basement membrane (BM) and detect proliferation of epidermal cells in lamellar tissues harvested at intervals. A conforming metal plate was screwed to the hoof wall to maintain alignment of the wound edges.

. Wall stripping caused lamellar tips to snap and remain behind in the dermis along with the majority of the lamellar BM and some lamellar basal cells (Fig 19). The discovery that stripping the equine hoof wall causes the tips of the PELs to snap and remain embedded in the dermis had not been reported before. Three days later the BM was intact and new lamellae had been reconstructed by proliferation of surviving epidermal cells. By 5 days the surface of the stripped zone was covered with yellow epidermis that subsequently thickened and hardened. Eventually the hoof wall deficit was replaced by new wall growing down from the coronet. The conforming metal plate and postoperative analgesic ensured minimal lameness. In wall stripped lamellae, the BM survived virtually intact and was used as a template for cells proliferating from the snapped-off lamellar tips, to migrate and quickly achieve repair to near normality. Immunostaining with anti BRdU detected proliferation amongst migrating mid-lamellar basal cells. This contrasts with the results of BRdU staining of normal, mid-lamellar tissue that showed virtually no proliferation. Thus lamellar wounding triggers lamellar proliferation.

Uniquely, the act of tearing away the hoof wall, automatically invoked a mechanism that provides not only a source of cells but a BM template on which they coold rapidly migrate. This option is not available to laminitis affected tissue. In laminitis the pathology is reversed; in laminitis epidermal dysadhesion and lamellar BM destruction occurs and lack of a functional BM template may explain the prolonged and abnormal repair of affected lamellae. Furthermore the lamellar tips and the BM template are far removed from each other making anatomical reconstitution difficult. Perhaps this explains the irreversible nature of chronic laminitis and why cases of full recovery are so rare. The results emphasised the need, during laminitis development, to minimise physical separation of epidermal basal cells from their basement membrane. Future experiments attempting to achieve this are being planned.

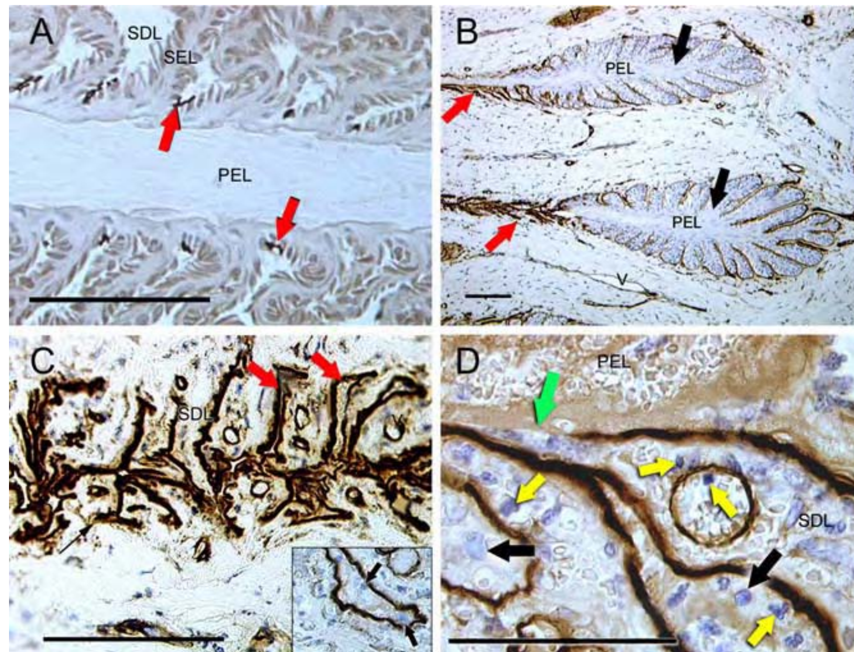


Fig 19. In stripped away hoof lamellae (A) there are remnants of brown, immunostained BM (red arrows), between SEL bases. One day after surgery (B) BM of stripped lamellae (red arrows) and the tips of 2 snapped-offf PELs (black arrows) are isolated in the dermis. Empty SELs (C) have a bi-layered appearance (red arrows) but contain basal cells (inset; black arrows). Gaps in the continuity of the lamellar BM (D) are mainly at SDL tips (green arrow). Capillaries, and all lamellar compartments contain PMNs (yellow arrows). Surviving basal cells have rounded nuclei and are not attached to the BM (black arrows). BM = basement membrane, PEL = primary epidermal lamella, SEL = secondary epidermal lamella, SDL = secondary dermal lamella, PMNs = polymorphonuclear cells, V = veins. Bars = 100 µm.

Acute laminitis is characterized by hoof lamellar dermal-epidermal separation at the basement membrane zone. Hoof lamellar explants cultured *in vitro* can also be made to separate at the basement membrane zone and investigating how this occurs may give insight into the poorly understood pathophysiology of laminitis. Dr Kathryn French used electron microscopy to investigate why glucose deprivation and metalloproteinase (MMP) activation in cultured lamellar explants led to dermo-epidermal separation.

FRENCH, K.R. & POLLITT, C.C. (2004). Equine laminitis: glucose deprivation and MMP activation induce dermo-epidermal separation in vitro. Equine vet. J. 36: 261-266

Explants, cultured without glucose or with the MMP activator *p*-amino-phenol-mercuric acetate (APMA), were subjected to tension and processed for transmission electron microscopy (TEM). Without glucose, or with APMA, explants under tension separated at the dermo-epidermal junction (Fig 20). This i*n vitro* separation occurred via two different ultrastructural processes. Lack of glucose reduced hemidesmosomes (HDs) numbers until they disappeared and the basal cell cytoskeleton collapsed. Anchoring filaments (AFs), connecting the basal cell plasmalemma to the basement membrane (BM), were unaffected although they failed under tension. APMA activation of constituent lamellar MMPs did not affect HDs but caused AFs to disappear, also leading to dermo-epidermal separation under tension. We suggested natural laminitis occurred in situations where glucose uptake by lamellar basal cells was compromised (eg. equine Cushing's disease, obesity, hyperlipaemia, ischaemia and septicaemia) or when lamellar MMPs were activated (alimentary carbohydrate overload). Thus therapies designed to facilitate peripheral glucose uptake and inhibit lamellar MMP activation may prevent or ameliorate laminitis.

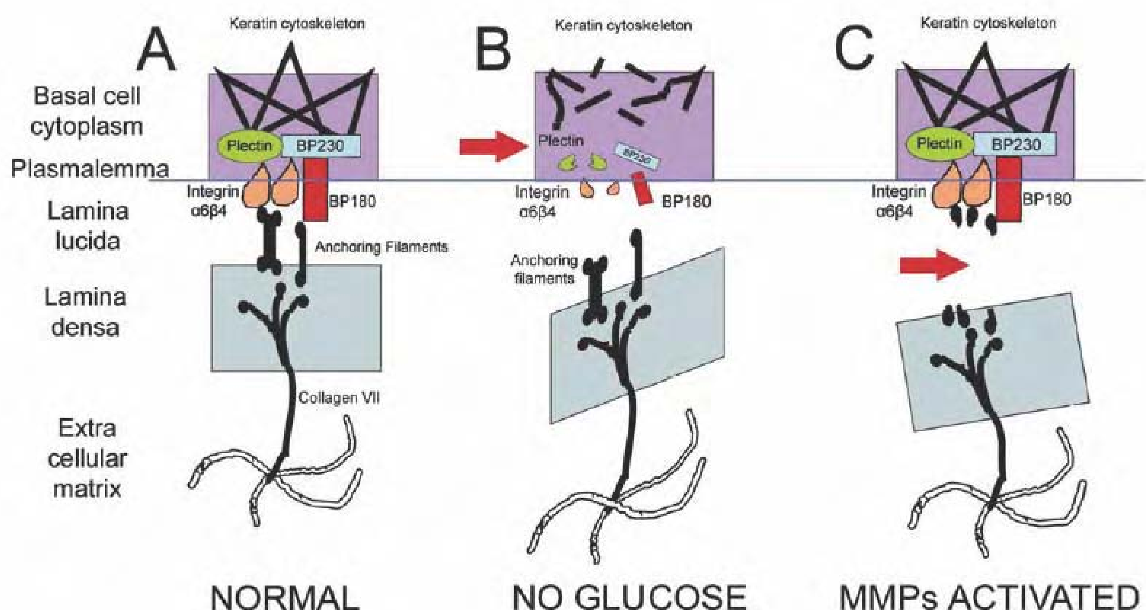


Fig 20. Diagram showing effects of no glucose and MMP activation on the hemidesmosomes of lamellar explants. The major components of the HD intracellular plaque (A) are plectin, integrin 64, BP180 and BP230. Intermediate filaments of the basal cell cytoskeleton insert into the HD thus connecting the cytoskeleton to the cell plasmalemma. Anchoring filaments connect the HD to the lamina densa of the basement membrane. Without glucose the HD dysassembles (B) causing failure of cytoskeleton insertion into the HD, dysadhesion of anchoring filaments and BM separation under tension. The target for activated MMPs (C) is anchoring filaments and their loss allows wholesale movement of the BM away from the epidermal basal cell plasmalemma leaving HDs unaffected.

Dr French sought more precise knowledge of the damage occurring in the lamellar basement membrane zone in laminitis affected tissue by using the newly developed OF laminitis induction model to examine the ultrastructure of acute laminitis as disease of greater severity was induced by increasing oligofructose (OF) dosage.

FRENCH, K.R. & POLLITT, C.C. (2004). Equine laminitis: loss of hemidesmosome ultrastructure correlates to dose in an oligofructose induction model. Equine vet. J. 36: 230-235

Three pairs of normal horses, dosed with OF at 7.5 g/kg, 10 g/kg and 12.5 g/kg bwt via nasogastric intubation, developed laminitis 48 h later. Their forefeet hoof wall lamellae were processed for transmission electron microscopy. Lamellar basal cell hemidesmosome (HD) numbers and the distance between the basal cell plasmalemma and the *lamina densa* of the basement membrane were estimated and compared to control tissue.

Increasing OF dosage caused greater HD loss and more severe laminitis (Fig 21). The characteristic separation of the basement membrane, cytoskeleton failure and rounded basal cell nuclei resulted from combined HD dysassembly and anchoring filament failure. Without properly assembled HDs, dysadhesion between the lamina densa of the BM and epidermal basal cells occured, emphasising the fundamental importance of HDs in maintaining attachment at the lamellar interface. Medical conditions that trigger lamellar MMP activation and/or compromise entry of glucose into lamellar basal cells appeared to promote loss and failure of HDs and thus laminitis development. A correlation between lameness severity and escalating loss of lamellar HDs now existed. Therapy aimed at protecting the lamellar

environment from haematogenous arrival of matrix metalloproteinase activators or from glucose deprivation may control laminitis development.

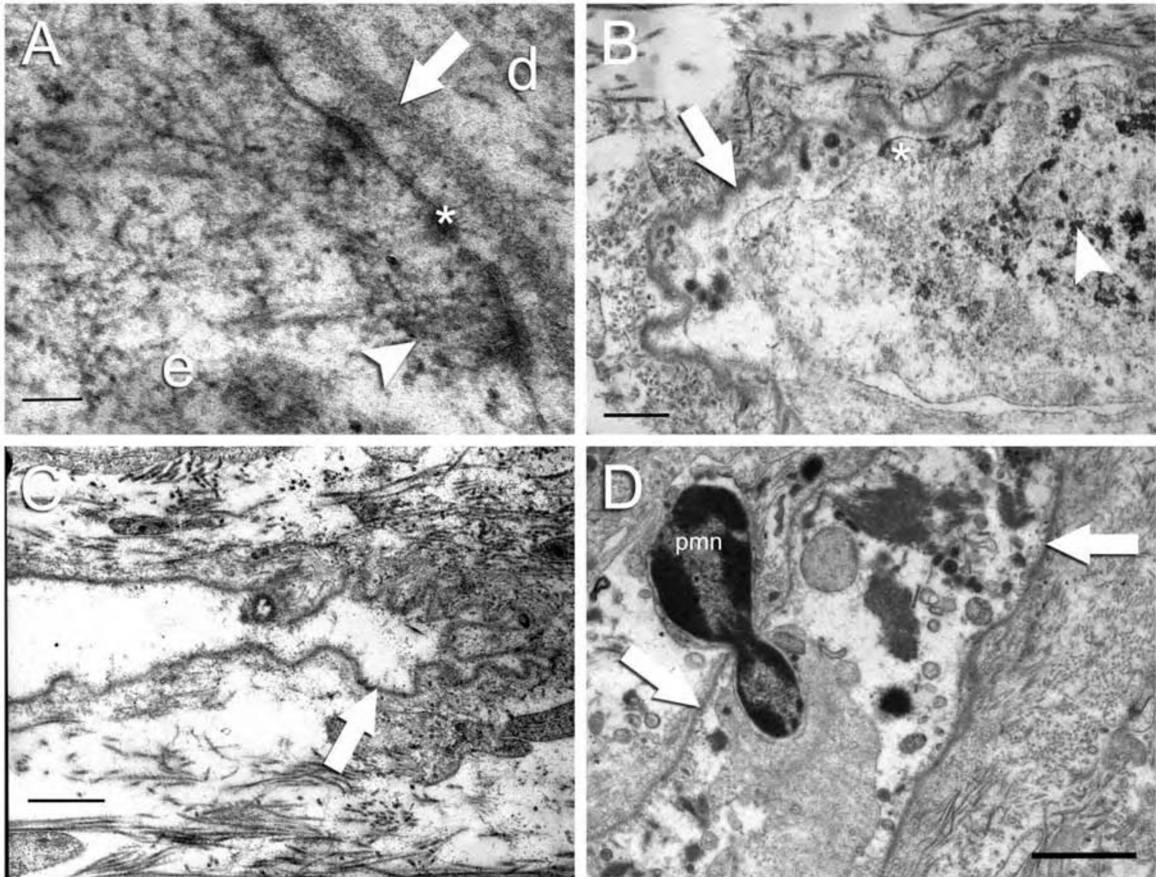


Fig 21. TEMs of lamellar BM zone of horses dosed with 12.5 g/kg. HDs (asterisk) were fewer in number (A), small and unevenly distributed in the plasmalemma of basal cells (e). HD intracytoplasmic dense plaques were pale and disorganised as if in the process of dysassembling (bar = 1 nm). At SEL tips (B) the lamina densa (arrowed) was detached from the basal cell plasmalemma which was often disrupted and feintly stained. Between the lamina densa and the occasional, surviving HD (asterisk) were a few anchoring filaments. Basal cell cytoplasm was pale and amorphous interspersed with clumps of cytoskeleton (arrowhead). Empty bubbles of lamina densa (C) that had completely separated from the basal cells of SEL tips were lined with the remnants of anchoring filaments. Lamina densa enclosed tubes (arrowed in D) contained debris of basal cell organelles, pale, diffuse cytoplasm and dense granules of cytoskeleton - like material. Polymorphonuclear leukocytes (PMNs) were often within damaged epidermal compartments and one is shown apparently

passing through a gap it is has created in the lamellar lamina densa (bars in B, C and D = 500 nm.

Neonatal laminitis is extremely rare and a call from an astute practitioner (Dr Graham Stabler) in Mackay, Queensland, describing clear laminitis clinical signs in a newly born Quarterhorse foal prompted an investigation. At the time of examination the foal was 45 days old and had been clinically lame from birth. Dr French utilised light, immunofluorescence (IFM) and transmission electron microscopy (TEM) to determine the nature of the hoof lamellar lesion.

FRENCH, K.R. & POLLITT, C.C. (2004) Equine laminitis: congenital, hemidesmosomal plectin deficiency in a Quarter Horse foal. Equine vet. J. 36: 299-303

The foal was examined clinically and showed the clinical signs and gross pathology of chronic laminitis. Characteristic laminitis histopathology was present only in the front feet. In all feet TEM showed the intracytoplasmic plaques of lamellar hemidesmosomes were small, misshapen and not associated with the cytoskeleton. In all feet IFM showed the hemidesmosomal, intracytoplasmic plaque protein, plectin, was absent.

The foal, a rare case of congenital epidermolysis bullosa simplex, had likely inherited failure to express plectin. Lacking plectin, the cytoskeleton and hemidesmosomes of the hoof lamellae were unstable, resulting in laminitis when the front feet first bore weight (Fig 22). This clinical investigation, aimed at determining the pathogenesis of a rare case of neonatal laminitis, revealed an unexpected hemidesmosome defect that precipitated the disease. The implications of these findings for understanding laminitis are far reaching. The foal demonstrated that laminitis can result from disruption or damage to a single molecule of the hemidesmosome adhesion complex of the inner hoof wall lamellae. Thus, it is immaterial whether the hemidesmosome defect results from an inherited gene “knockout” or a metabolic crisis in adulthood, dermo-epidermal separation within the hoof lamellae, the signature lesion of laminitis, still occurs. This case underpins the essential, functional importance of hemidesmosome integrity in hoof lamellar health. It also shows how closely the lesions of laminitis follow foot load distribution. The forefeet support the majority of the body weight. In the foal there was no laminitis histopathology in the hind feet despite the hind feet possessing the hemidesmosome defect. If clinicians could do more to lessen the impact

of weight bearing during the developmental stage, the destructiveness of laminitis pathology in adult horses could be diminished.

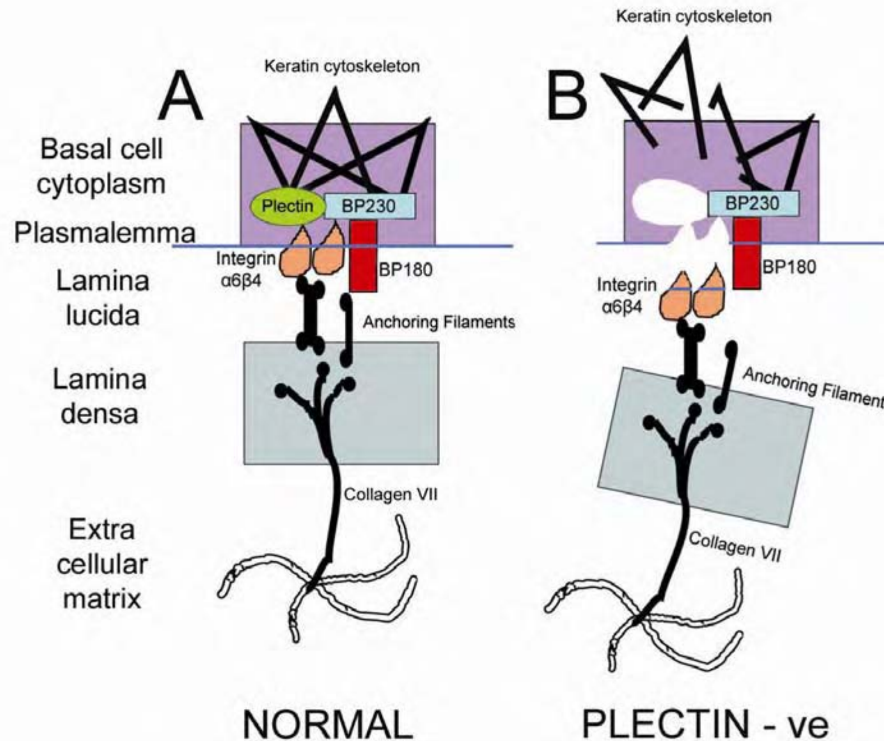


Fig 22. Diagram showing the effect of plectin deficiency on hemidesmosomes (HD). The major components of the HD intracellular plaque (A) are plectin, integrin a6b4, BP180 and BP230. keratin cytoskeleton filaments insert into the HD thus connecting the cytoskeleton to the cell plasmalemma. Loss of plectin (B) prevents correct hemidesmosome assembly and hinders insertion of the cytoskeleton into the plasmalemma, resulting in the collapse of the cytoskeleton and dysadhesion of the basement membrane.

The structural and adhesion proteins of the basement membrane zone must be altered if the key lesion of laminitis, separation at the hoof lamellar dermal-epidermal interface, is to occur. Ultrastructural studies of OF induced laminitis had demonstrated HD and cytoskeleton damage and BM dysadhesion. Which proteins and how damage to them leads to the lamellar separation of laminitis is unknown and was the next project for Dr Kathy French. She next investigated the pathological changes of OF induced laminitis using immunofluorescence microscopy (IFM).

FRENCH, K.R. & POLLITT, C.C. (2004). Equine laminitis: cleavage of key hemidesmosome proteins associated with basement membrane dysadhesion. Equine vet. J. 36: 242-247

Cryostat sections of lamellar tissues from 2 control and 6 Standardbred horses with oligofructose induced laminitis were studied using LM and IFM. Plectin, integrin α_6 and BP230 antibody was used to label hemidesmosome intracellular plaque proteins and anti-BP180 and anti-laminin 5 (L5) was used to label anchoring filament (AF) proteins. Cytoskeleton intermediate filaments were labelled using anti-cytokeratin 14. The primary antibodies of selected sections were double labelled to show protein co-localisation.

Laminitis caused reduction of transmembrane integrin $\alpha_{6,}$ the AF proteins BP180 and L5, and failure of co-localisation of BP180 and L5 (Fig 23). Proteins of the inner hemidesmosomal plaque, plectin and BP230, were unaffected.

Loss of co-localisation of L5 and BP180 suggested that, during the acute phase of laminitis, L5 was cleaved and thus, the AFs connecting the epidermis to the dermis, failed. Of particular interest was the fate of each protein after co-localisation was lost. BP180 remained localised to basal cells of the epidermal lamellae, concentrated in the basal cytoplasm and plasmalemma. Much of the L5 label remained with the BM as it separated from the basal cells and was visible as a thick fluorescent line far removed from the SEL tips. However, there was still L5 fluorescence associated with basal cells. This dual localisation of L5 in laminitis affected tissue, where the BM had separated from the epidermal cells, suggested that L5 was cleaved during the process of separation, leaving part of the molecule in the epidermal cell plasmalemma and part embedded in the detached BM.

We have well documented evidence that during laminitis there is up-regulation of transcription and increased enzymatic activity of MMP-2. L5 is a known substrate for MMP-2 activity and cleavage of L5 is a widespread trigger for migration in epidermal cells in contact with a BM. During laminitis development cleavage of L5 by MMP-2 would destroy the AFs that connect HDs to the BM and would contribute to movement of EBCs away from the BM by generating fragments of the L5α2 chain. Our studies showing localisation of MMP-2 in the basal and parabasal cells of the SEL, conveniently close to L5, provides further support for the role of MMP-2 in the development of laminitis.

Without a full complement of AFs separation at the lamellar dermo-epidermal junction occured. Suppressing or inhibiting metalloproteinase activity may prevent L5 cleavage and thus the lamellar dermo-epidermal separation of laminitis.

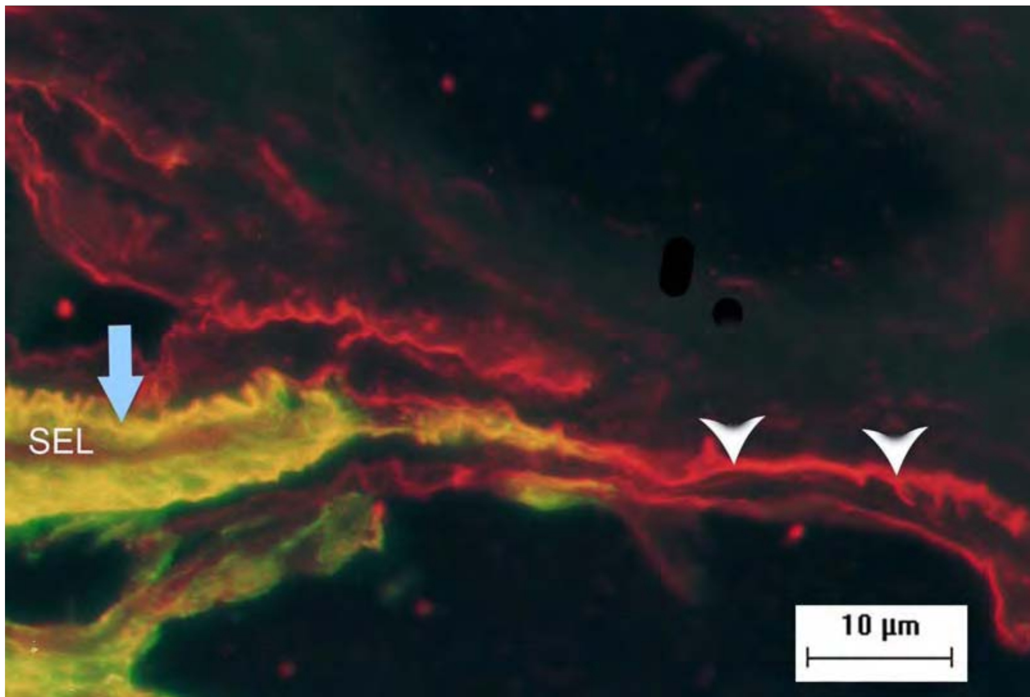


Fig 23. In laminitic tissue yellow co-localised BP180 and L5 was present in SEL basal cells (blue arrow). However only red L5 label was associated with BM that had separated (white arrowheads). Thus, during laminitis development, L5 was cleaved, leaving red in SEL basal cells (co-localised with BP180 to give yellow) as well as in the separated BM. Bar = 10 µm.

The Future... Where to now?

To achieve the body of work, now in the archives of AELRU, we have met each new investigative challenge by adopting new technology. Thus we have progressed from discovering that the right choice of histochemical stains could reveal the basement membrane lesion of acute laminitis to using MMP-2 real-time PCR to validate our claims that clinical laminitis was being prevented by cryotherapy. Over 26 scientists have contributed directly to this research effort and more are being encouraged whenever the opportunity arises.

We follow the evidence trail and build on the discoveries from each project by attempting to answer the new questions that arise. For example:

1. **Weight bearing.** We plan to develop ways of freeing horses of weight bearing during the developmental phase of laminitis. We believe that the period driving basement membrane dysadhesion is quite short and that, as our wall stripping experiments showed, the lamellar BM interface can repair remarkably quickly. The period of weight, relief required to prevent laminitis, at least with the OF induction model, may need to last no longer than 48 hours. We are investigating slings and flotation devices to achieve non weight bearing in horses.

2. **MMP inhibitors.** We have long known that MMP inhibitors deserve testing to determine if this class of drug can effectively block laminitis. Our *in vitro* trials have clearly established this. However the large amount required for horses, of these largely research-only substances, has prohibited our utilising their *in vivo* use experimentally. We are exploring new methods of local perfusion to the distal limb that will enable the use of much smaller amounts and are hopeful MMP inhibitor trials can commence soon.

3. **Cryotherapy**. Did cryotherapy, as it was applied to one limb, really prevent clinical laminitis in the long term? Was the onset of laminitis only delayed by cryotherapy? We couldn't ascertain this at the time as the horses were euthanased 48 hours after laminitis induction. Andrew van Eps is conducting further trials to establish, with certainty, the long term viability of cryotherapy. A large volume cold water bath has been constructed. Horses can stand in it, with all 4 distal limbs immersed in very cold water, for long periods of time (up to 3 days). The water is kept at a constant cold temperature by being circulated through an insulated reservoir attached to a refrigeration unit designed for the job. After laminitis induction and an appropriate period of cryotherapy the horses will be observed for a total of 7 days before their lamellar tissues are analysed for laminitis.

4. **Fructan epidemiology.** We have established that fructan in the hindgut of a horse is a potent antecedent of clinical laminitis. This is important information but it doesn't help horse owners struggling to decide if it is safe to let their horses out to graze today. We need to know which pasture species

produce and store the most fructan and at what time of the year or day. In which climates do they occur and on which landscapes? Are there safe pasture species that never accumulate significant fructan? If hay is made from grass rich in fructan at the time of cutting is the danger of fructan overload retained? After drought, when rain returns, is the new pasture growth dangerously high in fructan? Research, in both the epidemiological and agronomy areas, needs to be done to answer these questions so that horse owners have guidelines on which to base their management decisions.

5. **Laminitis trigger factors.** What are the laminitis trigger factors? We have some evidence that they may be of bacterial origin; exotoxins of *Strep. bovis* for instance. This may be true but it doesn't explain how laminitis develops after retained placenta/metritis, pleuritis and rhabdomyolyis where very different microbes are involved or none at all. Our Holy Grail is a unifying theory; a laminitis trigger factor common to all these conditions. Dr Kathy French's discovery that cleavage of the adhesion molecule laminin-5 occurred during laminitis development is an important clue and one which we are currently investigating. Fragments of L5 are potent triggers for epidermal cell dysadhesion and migration. It is certain that they are produced at the lamellar basement membrane interface and propagate the lesion but are they produced elsewhere as well? Are injured tissues in organs remote from the foot, a source of such fragments? If so they must affect epidermal cell basement membrane adhesion throughout the body. There is little evidence of this. Perhaps they only seriously damage the unfortunate soliped equids destined to bear weight on their hoof lamellar basement membranes forever.

6. **Hindgut microbiology.** Soluble carbohydrate in the horse's hindgut is rapidly fermented by a suite of anaerobic microbes some of which may be producing, yet to be discovered, exotoxins, vasoactive substances or proteases. Do these products of rapid hindgut fermentation of soluble carbohydrate cause laminitis directly or indirectly? They certainly cause serious and widespread damage to the caecal and colonic mucosa – in fact every case of carbohydrate overload is really a case of colitis. Idiopathic colitis or colitis-X is a notorious prelude to severe laminitis. Perhaps it is the

damaged mucosa, rapidly repairing its population of epidermal cells (a task requiring massive proliferation and migration of epithelial cells) that is the source of laminitis trigger factors. This returns us to the theme that laminitis is a normal process out of control. We have invested considerable resources into surveying the changing hindgut biota as horses develop fructan induced laminitis. Soon we will have better understanding of the type of microbes that explosively populate the hindgut in the presence of unlimited substrate. Perhaps when we know their names they will tell us what they produce that leads so inexorably to laminitis.

7. **Chronic laminitis.** We are just starting to investigate chronic laminitis and why the acute lesion leads to hoof deformation and distal phalanx displacement. Just as we documented the histopathology of acute laminitis in 1996 now we are preparing a manuscript describing the situation with chronic laminitis. We are using retrograde venography to track the changes that occur in the soft tissue surrounding the distal phalanx in horses with induced laminitis. We have developed rabbit antibody specific to equine fibrin so we can investigate the role of thrombosis in chronic laminitis.

8. **Multi centre laminitis research**. An exciting development in the world of laminitis research was the 2004 announcement from Dr Jim Belknap's team in the US* that there was "**NO** molecular evidence of laminar ischaemia in the developmental stage of the Black Walnut model of equine laminitis". Much expense and hope is placed on vasodilatory drugs based on the premise that ischaemia IS present and needs to be reversed. Dr Belknap's team reasoned that if ischaemia does occur it will be evidenced by the differential expression of hypoxia/ischaemia sensitive genes. They selected an appropriate array of genes and used real time qPCR to quantitate expression. Laminitis was induced with black walnut extract (BWE) and lamellar tissue was obtained from anaesthetized horses, at the time point where a decrease in laminar/digital blood flow has been reported to occur. There was no molecular evidence of decreased blood flow in the

* Huggins H, Black S, Cochran A & Belknap J (2004). There is No Molecular Evidence of Laminar Ischemia in the Developmental Stage of the Black Walnut Model of Equine Laminitis. Proceedings of the AAEP Laminitis Focus Group meeting, Louisville, Kentucky, July 2004.

developmental stage of BWE equine laminitis and they concluded that drugs purported to address causes of laminar ischaemia are not indicated in the developmental stage of laminitis. Clearly this technology must be applied urgently to the carbohydrate laminitis induction model. All the laminitis pathophysiology evidence that AELRU has generated over the year has always indicated that ischaemia had nothing to do with lesion development. AELRU has entered into collaboration with molecular biologists at the University of Ohio (Dr Belknap's team) and the University of Pennsylvania (Dr James Orsini's team) to answer the crucial question: "is there molecular evidence of ischaemia/reperfusion in the developmental stage of carbohydrate induced laminitis".

Prof Chris Pollitt
(written in Norway Feb 2005)

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