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NARRATIVE REVIEW

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A review of cellular and molecular mechanisms in endocrinopathic, sepsis-related and supporting limb equine laminitis

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Abstract

Equine laminitis has both fascinated and frustrated veterinary researchers and clinicians for many years. The recognition that many ponies suffering from pasture-associated laminitis have an insulin-dysregulated phenotype (endocrinopathic laminitis, EL) and that prolonged insulin and glucose infusions can experimentally induce laminar pathology and functional failure are seminal discoveries in this field. Researchers have studied the molecular basis for disease pathogenesis in models of EL, sepsis-related laminitis and

Abbreviations: ADAM, a disintegrin and metalloprotease domain (family of proteins); ADAMTS, a disintegrin and metalloprotease domain (family of proteins) with thrombospondin repeats; aGLP-1, active-glucagon-like peptide-1; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AMPK activator); Akt, a serine/threonine protein kinase also known as protein kinase B; AMB, ambient temperature; AMPA, aminophenylmercuric acetate; AMP-kinase-α, AMP-activated protein kinase alpha; APC, adenomatous polyposis coli gene product (component of the β-catenin degradation complex; negative regulator of canonical Wnt signalling pathway); BCS, body condition score; BM, basement membrane; BPH, benign prostatic hypertrophy; BW, bodyweight; BWE, black walnut extract (model of laminitis); CDH, continuous digital hypothermia; CH, contralateral hindlimb; CHO, carbohydrate; CIS, Cytokine inducible SH2-continuing protein; CK1α, casein kinase 1α (negative regulator of canonical Wnt signalling pathway); COX-1 or 2, cyclo-oxygenase-1 or 2; CV, coefficient of variation; CXCL-(n), chemokine (C-X-C motif) ligand-(n) [chemokines of the CXC family, chemotractant]; DE, digestible energy; dsh, dishevelled (positive regulator of the canonical Wnt signalling pathway); DTP, developmental time point; EBC, epidermal basal cell; EGF, epidermal growth factor; EHC, euglycaemic hyperinsulinaemic clamp (model); EL, endocrinopathic laminitis; ELISA, enzyme-linked immunosorbent assay; EM, electron microscopy; EMS, equine metabolic syndrome; ER, endoplasmic reticulum; ERK1/2, extracellular signal-regulated kinases 1 and 2 (these are also members of the mitogen-activated protein kinase family; phospho-ERK1/2 = p44/42 MAPK); EMT, epithelial to mesenchymal transformation; FITC dextran, fluorescein isothiocyanate dextran; F/L, forelimb; FSIVGTT, frequently sampled intravenous glucose tolerance test; FZD4, frizzled 4 (receptor for Wnt positive regulator of canonical Wnt signalling pathway); GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GIT, gastrointestinal tract; GLUT-1, glucose transporter 1; Grp78/BiP, glucose response protein 78/binding immunoglobulin protein (BiP) (also known as heat shock 70 kDa protein 5); Grp94, glucose response protein 94 (also known as heat shock protein 90 kDa beta member 1); GSK3β, glycogen synthase kinase 3β; HD, hemidesmosome; H&E, haematoxylin and eosin (histological stain); HIF-1α, hypoxia-inducible factor 1-alpha; H/L, hindlimb; HMW, high molecular weight; ICAM-1, intercellular adhesion molecule-1; ICE-1, Interleukin-1 converting enzyme also known as caspase-1; ID, insulin dysregulated; IFN-γ, interferon gamma; IGF-1, insulin-like growth factor 1; IGF-1R, insulin-like growth factor 1 receptor; IGFBP-1 or 3, insulin-like growth factor binding protein 1 or 3; IH, ipsilateral hindlimb; IHC, immunohistochemistry; IL-(n), interleukin-(n) [cytokines of the interleukin family]; ImmunoF, immunofluorescence; iNOS, inducible nitric oxide synthase (also known as NOS-2); InsR, insulin receptor; IPC, ischaemic preconditioning; IR, insulin resistant; IRF, interferon regulatory factor; IS, insulin sensitive; JNK, Jun amino-terminal kinases (also member of the MAP kinase family); KA, keratinised axis; KA-SDL, Keratinised axis − secondary dermal lamellar (distance); L:G, lactate to glucose ratio; LOD, limit of detection; L:P, lactate to pyruvate ratio; LRP, low-density lipoprotein receptor-related protein (co-receptor for Wnt, positive regulator of β-catenin signalling pathway); MAPK, mitogen-activated protein kinase (see also ERK1/2 and MEK); MCP-1, monocyte chemoattractant protein-1 (also known as chemokine (C-C motif) ligand 2 [CCL-2]; MCP-2, monocyte chemoattractant protein-2 (also known as chemokine (C-C motif) ligand 8 [CCL-8]; MEK, mitogen-activated protein kinase kinase (a mitogen-activated protein kinase also known as extracellular signal-regulated kinase—see MAPK and ERK); MMP, matrix metalloproteinase; MPO, myeloperoxidase; MT1-MMP, membrane-type 1 matrix metalloproteinase (also known as MMP-14); mTORC1, mammalian target of rapamycin complex 1 or mechanistic target of rapamycin complex 1; MW, molecular weight; NFκB, nuclear factor kappa B; NL, nonlaminitic (no history of laminitis); NOS-2, nitric oxide synthase-2 (also known as iNOS); NS, not significant; NSC, nonstructural carbohydrate; OF, oligofructose; OG-1, Obel grade 1 lameness; OG-2, Obel grade 2 lameness; OG-3, Obel grade 3 lameness; OST, oral sugar test; P70S6K, p70S6 kinase also known as ribosomal protein S6 kinase beta-1; P90RSK, p90 ribosomal S6 kinase; PACE-4, proprotein convertase enzyme (family member); PAI-1, plasminogen activator inhibitor-1; PAS, periodic acid-Schiff (histological stain); PDL, primary dermal lamella; PEL, primary epidermal lamella; PEPCK, phosphoenolpyruvate carboxykinase; PGC1α, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (master regulator of mitochondrial biogenesis); PGK-1, phosphoglycerate kinase-1; PL, previously laminitic; PMN, polymorphonuclear leucocyte; PP1, protein phosphatase 1; PPARγ, peroxisome proliferator-activated receptor gamma; PPID, pituitary pars intermedia dysfunction; PWB, preferential weight bearing (limb or model); RBP4, Retinol binding protein 4; RIA, radioimmunoassay; RPS6, ribosomal protein S6; rs, correlation coefficient (r squared); RT-(q)PCR, real-time (quantitative) polymerase chain reaction; SAA, serum amyloid A; SAPK, stress-activated protein kinases (member of MAP kinase family); SDL, secondary dermal lamella; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SEL, secondary epidermal lamella; Ser, serine; siRNA, small interfering ribonucleic acid; SLL, supporting limb laminitis; SOCS, suppressor of cytokine signalling; SRL, sepsis-related laminitis; STAT1, signal transducer and activator of transcription 1; STAT3, signal transducer and activator of transcription 3; TCF4, T cell factor 4 (member of T cell transcriptional factor family); Thr, threonine; TIMP, tissue inhibitor of matrix metalloprotease; TLR-4, toll-like receptor 4; TNF-α, tumour necrosis factor-alpha; TXP2, TPX2 is a nuclear protein (repp86/p100) associated with cell proliferation that is specific for S, G2, and M phases of the cell cycle; Tyr, tyrosine; WB, western blotting; Wnt4, Wingless in Drosophila and integration 1 (positive regulator of β-catenin); XBP-1, X-box binding protein 1 (marker of ER stress).

For affiliations refer to page 23

supporting limb laminitis and generated much data over the last 15 years. This review attempts to synthesise those data, drawing comparisons between models and naturally occurring laminitis. A hypothesis is proposed that the basal epithelial cell stress is a central event in each category of laminitis. Furthermore, in naturally occurring pasture-associated laminitis, pathways that predominate in each type of laminitis contribute to laminar lamellar pathology to varying extents. Based on the molecular mechanisms determined in experimental models, interactions between these pathways are identified.

KEYWORDS
basal epithelial cell stress, horse, insulin dysregulation, laminitis pathogenesis, signalling pathways

1 | INTRODUCTION

Equine laminitis has fascinated and frustrated clinical veterinarians and researchers for many years. Progress has been made through the study of models where laminitis is experimentally induced and the timeline of pathology development studied to give clues to the pathogenesis. New models have been developed over the last 15–20 years with the application of new technologies to study these models in detail. The numbers of publications on the three main categories of laminitis (endocrinopathic (EL), sepsis-related (SRL) and supporting limb (SLL) laminitis) is impressive and provides much to contemplate. Publications studying the pathogenesis of naturally occurring diseases and animals, which are predisposed to repeated bouts of laminitis are, understandably, less common.

Laminitis is a difficult clinical disease to study the pathogenesis of because much has occurred prior to its clinical presentation. It occurs sporadically in at risk animals but not in a predictable fashion to facilitate intense study of these animals at times of high incidence. Yet relating data from experimental models to naturally occurring laminitis is key to establish their relevance and ensure the models can be used to provide insights into the pathogenesis.

The recognition that animals with insulin dysregulation (ID) are predisposed to pasture-associated (now called, by some, 'endocrinopathic') laminitis stimulated researchers to develop a new model, the euglycaemic hyperinsulinaemic clamp (EHC) model which opened up a whole new field of research into laminitis.[1] The epidemic of obesity-related insulin resistance in human medicine progressing to type 2 diabetes, with its attendant cardiovascular health risks has inevitably led to obesity-related ID in the horse being coined 'equine metabolic syndrome'.[2] Because of the human health impact of ID and type 2 diabetes there is now a wealth of experimental animal research into the detrimental effects of hyperinsulinaemia (direct or indirect) and the mechanisms by which these predispose to poor health outcomes. Understanding which of these mechanisms is of relevance to structure and function of the lamellae is key to future progress in laminitis research.[3]

The focus on ID as the underlying predisposing factor for equine laminitis recognises the fact that EL occurs most often clinically and reflects the paradigm shift in laminitis research this discovery has caused. Furthermore, laminitis can be induced by infusion of insulin and glucose or glucose alone (sub-clinical laminitis in this case), the latter probably resulting from the hyperinsulinaemia generated. Whether the pathogenesis involves a direct or indirect effect of insulin on lamellar tissue is an important question that remains to be resolved. Here we explore the hypothesis that the effects of ID on basal epithelial cell biology, predisposing to EL, are also ultimately activated in SRL and SLL, such that all three pathophysiological mechanisms are at least additive contributing (to varying degrees) to naturally occurring pasture-associated laminitis. Hence, understanding why ID perturbs the physiology of the lamellae such that they fail could help us to devise measures to protect against all forms of laminitis, as shared final common molecular pathways are likely involved.

This review attempts to (i) relate pathological and pathogenesis findings in experimentally-induced laminitis models to those reported in naturally occurring clinical cases and (ii) to use the pathological and molecular findings to identify signalling pathways that intersect between the different forms of laminitis (EL, SRL and SLL).

2 | HISTOPATHOLOGY

2.1 | Naturally occurring laminitis

Whilst much has been published about the pathology affecting the lamellae in the different models of laminitis, relatively few publications document lamellar pathology when the disease has occurred naturally. Table 1 summarises the findings from four papers in the literature where naturally occurring laminitis was studied and reported in enough detail to identify the major features.[4–7] Figure 1 aims to help the reader understand the anatomical terminology used in Table 1 and throughout this review.[8] The selection of cases for these studies is biased towards cases with documented endocrinopathic risk factors, which may be appropriate because EL has been shown to be the most common category of laminitis encountered in the field.[9] Nevertheless, the exclusion of other diseases leading to inflammation and sepsis, that could contribute to the pathophysiology in field cases means these data present a spectrum of

TABLE 1 Pathological descriptions of naturally occurring laminitis cases.

Reference	Clinical details	Structural pathology	Epidermal cell response	Basement membrane changes	Inflammatory cell infiltrate
Karikoski et al., 2015[4]	Cases ($n = 14$) with classical signs of laminitis but no history of inflammatory disease and documented basal plasma insulin of >20 miU/ml. Cases with classical signs of PPID were excluded. Divergent growth rings noted in all but one laminitis case Comparisons made to age and breed matched nonlaminitic animals	Lengthening of PELs which were either standard or curving Increased numbers of tapering PELs Increased keratinisation of PELs (abaxially[a]) Increased proportion of tapering SELs (axially[a]) High proportion of fused, separated and keratinised SELs abaxially Interlamellar epidermal bridging occurred (variably) Entrapped rounded islands of dermal tissue Acute lamellar separation seen in 1/3 of cases; sometimes PEL torn from SEL (little inflammation present)	Apoptotic cells in SELs where no separation had occurred Mitotic figures in SEL (variable but most numerous where separation of SEL from PEL had occurred)	No evidence presented of basement membrane disruption	Little inflammatory infiltrate documented
Cassimeris et al., 2019[5]	Selected 12 cases of EL from a repository. All animals had chronic active laminitis of the F/L (veterinary diagnosis) and were confirmed as EL by appropriate phenotypic tests. Six of the 12 were PPID cases and 6 were obese with regional adiposity. All lacked other predisposing factors for laminitis. Gross pathology score of all F/L hooves included was 3 or 4 out of 4. A comparator group of tissues from 8 age, breed and sex-matched controls were obtained from the same repository subjected to euthanasia due to lameness (nonlaminitic cause $n = 7$) or infertility ($n = 1$). Gross pathology score of all the controls was 1 of 4 (normal). In	Dimensions of the PEL and SEL were rigorously analysed (Supporting Information). Structural changes include: PEL length of laminitic fore feet were 73.5% longer than controls KA-SDL distance was increased (by 3.75- to 6-fold) at axial, mid and abaxial points and the KA was displaced by 1.5 mm compared with controls. None of these parameters differed significantly from controls in the hind feet SEL generally were longer in laminitic F/L than controls although highly variable. Axial SELs were 117% longer (laminitics vs. controls) SELs were commonly merged within or between PELs and had an abnormal	Qualitative scoring of epidermal cells from fore feet of laminitis showed epithelial hyperplasia and metaplasia were usually regional or global whereas in controls these features were absent, focal or multifocal in distribution. Cellular necrosis was much more often present in the laminitic fore feet compared with controls. Islands of epidermal cells were much more common (laminitic fore feet vs. control).	Qualitative histo-pathology scores indicated that the laminitic front feet had thickened and fragmented BM with SDL retraction. These lesions were found regionally or globally whereas they were absent, focal or multifocal in controls	Qualitative scores for mononuclear and plasma cell, PMN and haemosiderocyte infiltration were higher in the laminitic fore feet (vs. control) Perivascular inflammation was regional or global in the laminitic fore feet and absent, or focal in the controls. Endothelial cell activation was seen more commonly (laminitic fore feet vs. controls).

(Continues)

TABLE 1 (Continued)

Reference	Clinical details	Structural pathology	Epidermal cell response	Basement membrane changes	Inflammatory cell infiltrate
	addition, the hind feet of the cases were included as a separate group (none of the cases had H/L laminitis clinically; gross pathology score 1 out of 4)	shape in laminitic fore feet (vs. controls)			
Karikoski et al., 2016[6]	Cases ($n = 16$) diagnosed with PPID, 6 of which had veterinary-diagnosed laminitis (basal insulins >40 miU/ml; immulite assay) and 10 of which did not have laminitis (basal insulin <20 miU/ml). Rigorous criteria for diagnosis of PPID (including pituitary histopathology). Ten animals without either laminitis or PPID were used as controls.	PPID animals without clinical evidence of laminitis had normal lamellae morphology (measurements of PEL length and width did not differ from age and breed-matched controls). PPID cases with laminitis compared with the controls and PPID cases without laminitis had: • longer and wider PELs • more tapered PELs axially • more tapering, fusion, dermo-epidermal separation—mid-region • more fusion, dermo-epidermal separation, keratinisation abaxially	Increased apoptotic cells in SEL (4 of 6 laminitics and 3 of 10 PPID cases without laminitis) Mitotic figures in SEL (4 of 6 laminitics)	Acute separation through the BM in the abaxial and mid regions (2 of 6 laminitics) with necrosis of both compartments	Very little leucocyte infiltration
Wattle 2000[7]	Includes tissues from 3 horses subjected to euthanasia within 30 hours of first showing clinical signs of acute laminitis. Horse 1 was a mare with retained placenta. Horses 2 and 3 developed signs shortly after being turned out to grass (horse 2 following a minor injury to its H/L).	Measurements not systematically made and examples are given in the publication. All hooves showed varying degrees of pathology (horse 1 mild; horse 2 the most severe case) which included elongation of PEL and SEL.	Cell proliferation in the suprabasal layers axially and abaxially. Numerous mitoses were seen. Rate of proliferation is highest in the mid-region Rounding of nuclei and cellular oedema is described	Horse 2 had complete dissociation of the epidermal and dermal lamellae in left F/L. (Destruction of the BM is not described, however).	No mention is made of an inflammatory infiltrate in any of the cases.

Abbreviations: BM, basement membrane; EL, endocrinopathic laminitis; F/L, forelimb; H/L, hindlimb; KA, keratinised axis; PEL, primary epidermal lamella; PMN, polymorphonuclear leucocyte; PPID, pituitary pars intermedia dysfunction; SDL, secondary dermal lamella; SEL, secondary epidermal lamella.
[a]For definitions of axially and abaxial, please see Figure 1.

disease that may not be completely representative of the field cases of laminitis as a whole. Earlier descriptions of the pathology of laminitis in naturally occurring cases do exist in the literature, but either the clinical characterisation of the cases is not detailed enough or the definition of pathological lesions not precise enough for these to be helpful[10] or they describe a single case.[11]

As would be expected, most of the cases presented in Table 1 have chronic active disease. Usually, this is for >7 days and in many instances,

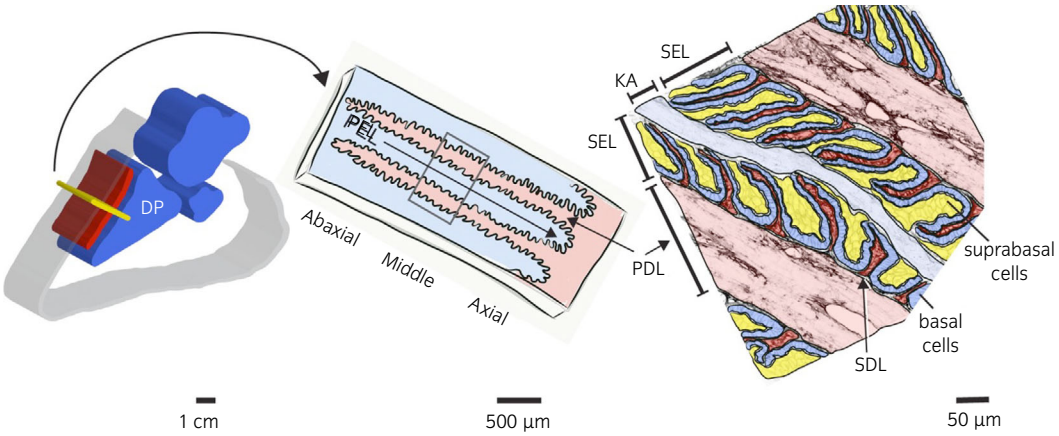

FIGURE 1 Schematic overview of equine hoof lamellar tissue anatomy. *Left panel*: cutaway view of the midsagittal section of an equine foot outlining the position of lamellar tissue (red) relative to hoof wall (grey) and distal phalanx (DP), middle phalanx and distal sesamoid bones (blue). The transverse section plane used for tissue collection is shown in yellow. *Centre panel*: a portion of the transverse section plane diagrammed schematically. The interdigitating primary epidermal lamellae (PEL) and primary dermal lamellae (PDL) are highlighted. Positions relative to the skeletal axis are denoted as axial (closest to DP), middle and abaxial (farthest from DP and closest to the hoof wall). *Right panel*: higher magnification illustration of lamellar tissue organisation. Colours are overlaid on a tissue section stained to highlight cell membranes and extracellular matrix (rhodamine-tagged wheat germ agglutinin, see Methods). Darker blue overlays mark basal epidermal cells, yellow overlays mark suprabasal epidermal cells, light blue overlay highlights the keratinised axis (KA) of each PEL. The PEL consists of combined secondary epidermal lamellae (SEL) and KA. Dermal tissues are shown in red overlays, with light red highlighting PDL and darker red marking the secondary dermal lamellae (SDL). DP, distal phalanx; KA, keratinised axis; PDL, primary dermal lamella; PEL, primary epidermal lamella; SDL, secondary epidermal lamella; SEL, secondary epidermal lamella. Reproduced from Cassimeris et al.[8]

recurrent laminitis has been recognised in these horses and ponies for years prior to euthanasia and tissue harvest. Active disease at the time of euthanasia was an inclusion criterion in these studies. The absence of clear descriptions of the pathology associated with either SRL or SLL is surprising as examples of such cases would be expected to present to university referral hospitals for treatment. Much work has been done characterising the pathology of SRL models of laminitis (see below), yet the comparison with field cases seems to be lacking within the literature.

The molecular pathology of naturally occurring cases of SLL has been studied[8] but the histopathological lesions were not reported in detail. However, they have been summarised in a review publication.[12] Ischaemia is thought to be the pathogenic factor involved in SLL leading to pathology in the epidermal cells furthest away from the hoof vasculature. Unlike other types of laminitis (naturally occurring or models), necrotic cell death (indicated by cell shrinkage and hypereosinophilia with nuclear pyknosis and karyorrhexis), the end result of ischaemic cell death,[13] is a feature of naturally occurring SLL accompanied by parabasal acanthosis. The cells most affected are those adjacent to the primary epidermal lamellae's keratinisation axes (KA). Importantly, lesions are not confined to the supporting limb and can be identified in multiple limbs.[8,12] Some pathological lesions similar to those found in other forms of laminitis are noted, namely SEL elongation and thinning. In the mid-PEL region, there is displacement of the KA with clefts opening and becoming filled with fibrin. It is likely that the histopathology of naturally occurring SLL will be replicated in the model that has recently been developed to represent this form of laminitis although, in the naturally occurring disease, concomitant sepsis and pain-induced insulin resistance[14] might also contribute to the pathogenesis and therefore influence the pathology seen. A full description of the lamellar pathology seen in naturally occurring SLL cases would therefore be of value.

2.2 | Models of laminitis

Much work has been published over the last 25 years characterising experimentally induced models of laminitis in the hope that this would give rise to novel ways of managing this disease. Tissues from models have been shared which has reduced the number of animals involved. A pathological timeline for some models has been characterised as tissues have been sampled at different time points related to the onset of clinical signs of lameness, which is usually where, for humane reasons, the models are stopped and the horses are subjected to euthanasia to prevent the suffering that would otherwise ensue. In many of the publications, particular molecular pathways have been studied to determine the onset of expression of mRNA and proteins associated with that pathway in relation to development of pathological lesions, which lead to the onset of clinical signs (see below). This section focuses on the timeline of histopathological changes that are seen in each model in studies where these have been clearly described. Table 2 provides a summary of the histopathological descriptions of EL and SRL (mostly the Oligofructose model)[15–21] and Figure 2 illustrates lamellar tissue histology in normal horses and in horses subjected to the EL and SRL (oligofructose) models of laminitis. Table 3 presents more detailed studies of the associated basement membrane (BM) changes in these models.[22–28] The histopathology of the black walnut extract (BWE) model of SRL is not well described. The paper first characterises this model[29] presents some description of acute changes in three horses seen after 12 h (onset of lameness) but the descriptions are brief. They describe mitotic figures in the epidermal cells which were absent from the normal control horse tissue. They also describe vacuolation of the SDL and loss of cellular definition of the tips of the PELs. As the BWE model appears to differ

TABLE 2 Pathology of laminitis models.

Reference	Model employed, time of sampling number of animals	Structural pathology	Epidermal cell response	Basement membrane changes	Inflammatory cell infiltrate
Pollitt 1996[15]	SRL; CHO overload; 48 h ($n = 8$ horses with three horses used as controls; age, breed and sex not stated in the paper)	PELs appeared unchanged until grade 3 when became tapered and shrunken SELs were longer and thinner (tips more pointed) compared with controls in laminitis Obel grade 1, becoming more tapered in grade 2. In grade 3 SELs mostly not recognisable (amorphous mass); the few that were recognisable were devoid of connective tissue between them and were extremely elongated and thin	Nuclei of epidermal basal cells rounded and more central in grade 1; basal cell nuclei became pyknotic with cellular vacuolation near SEL tips in grade 2. Mitoses noted at the tips of PEL in grade 2. In grade 3—these changes were more generalised.	With PAS staining, BM at the base of SEL was lost (SELs adhered to each other) in grade 1. Line of BM often wavy and sometimes broken. Separation of the BM from the tips of SEL in grade 2 giving a teat-shaped bubble. In grade 3 the bulk of the BM appeared to have been stripped away from epidermal basal cells and was lying free besides the PDL connective tissue	Neutrophils present around PEL tips, particularly from Obel Grade 2 onwards. In grade 3, blood vessels close to the PEL tips were surrounded by PMNs but blood vessels in the dermis appeared normal
Morgan et al., 2003[16]	SRL; CHO overload; 10 horses treated and biopsy samples obtained at onset of lameness or at 72 h if not lame. Pathology compared with 10 control horses similarly biopsied. Seven treated horses became lame.	Little detailed description of the PEL and SEL structure. Where the dissolution of the epidermal basal cells was evident, tearing of the lamellar interface occurred. No fusion or blunting of SELs was detected.	Epidermal basal cells exhibited vacuolation, pyknosis and dissolution of varying severity (<10% to >70% of lamellar interface). Cellular pathology more severe abaxially than axially.	The BM was intact in all horses, even those with severe dissolution of basal cells	Perivascular inflammation (mainly mononuclear cells) involving 10 to 50% of lamellar interface. Hypertrophy of the capillary endothelium
De Laat et al., 2011a[17]	SRL (OF at 48 h)—$n = 4$; comparisons made to 4 control horses (Standardbred horses used)	Marked attenuation of SELs along the length of PELs. Length of SELs was increased and width was decreased compared with control both axially and abaxially.	Increased mitoses in EBCs Increased apoptosis in EBCs Rounding of nuclei	Widespread BM damage noted (more than in the EHC model)	Leucocyte infiltration was heavier in the OF model compared with the EHC model in the dermis around the tips of the PEL.
Asplin et al., 2010[18]	EHC until Grade 2 laminitis or for 72 h; $n = 5$ ponies subjected to the model and 4 age-matched controls	Variable changes associated with the model—PEL undulated with loss of distinct PEL-SEL interface. PEL keratin increased. SEL axes contained increased disorganised keratin. At the tips of the PELs, SELs were swollen and club-shaped. Abaxially and mid regions, SEL were elongated and thin (confirmed by measurements)	Cells were swollen at the PEL tips, with cytoplasmic vacuolation and enlarged nuclei exhibiting dyskeratosis Mitoses were numerous in the SEL at the PEL tips. Highly variable number of apoptotic cells at the SEL-PEL interfaces and basal layers	Separation of SEL from BM at PEL tips but no separation abaxially or in the mid-PEL region.	Areas of BM separation from SEL infiltrated with degenerate neutrophils. Leucoyte infiltration noted in lamellar dermis of laminitics but not control animals. Perivascular lymphatic expansion and some dermal venous fibrin thrombi noted

TABLE 2 (Continued)

Reference	Model employed, time of sampling number of animals	Structural pathology	Epidermal cell response	Basement membrane changes	Inflammatory cell infiltrate
De Laat et al., 2011a[17]	EHC; 48 h ($n = 4$) in Standardbred horses—comparisons made to 4 control horses	PEL dyskeratotic to varying degrees Widespread disorganisation of SELs—most marked axially where basal cells lost but SEL still outlined by BM Loss of symmetrical angulation of SELs SELs wider at base and narrower at tip. Length of SELs was increased and width was decreased compared with control both axially and abaxially.	EBCs prominent nucleoli, rounded nuclei More frequent mitoses and apoptotic EBCs when compared with controls	BM dysadhesion from basal cells Patchy loss of BM staining (PAS) BM pathology highly variable	Leucocytes present perivascularly in dermis around tips of PELs Leucocyte infiltrated SELs where EBCs lost Endothelial cells of capillaries swollen
De Laat et al., 2013a[19]	EHC (6, 12, 24 h)—$n = 4$ per time point of Standardbred horses. Archived samples from similar horses ($n = 4$) subjected to the same model that had been harvested at the time of onset of lameness (48 h) and age and breed-matched controls were also analysed for histopathology.	Compared with controls, tapering and elongation of SELs appeared to be occurring at 6 h of EHC model and became more pronounced at 12 and 24 h. These subjective assessments were confirmed by objective measurements of SEL in forefeet where the length increased (of both axial and abaxial SELs) at each time point from 6 h onwards and width reduced (differences between groups were generally not significant attributed to small group sizes and variability between individuals).	EBC nuclei became parallel instead of perpendicular to the BM at 6 h and remained so. TXP2 was used to detect dividing cells which increased at 24 h compared with control. Large variation in numbers of apoptotic cells—seemed to peak at 6 h and decreased thereafter.	Slight blebbing of the BM or more significant separation of the basal cells from the BM was seen at the SEL tips of a few PEL axial tips at the 24 h time point (not a diffuse or severe change).	Calprotectin staining showed that leucocyte infiltration did not occur to any significant extent until 48 h.
Karikoski et al., 2014[20]	EHC (until onset of Obel grade 2 lameness); $n = 4$ ponies (treated) and 4 age-matched control ponies (infused with *n*-saline for 72 h prior to euthanasia)	PEL length was 15% greater in treated compared with control ponies (F/L and H/L). PEL width was not affected by treatment. Treatment led to elongation (33 to 142% increase) and narrowing of the SEL (axial, mid and abaxial regions) with the angle to the PEL keratinised axis becoming more acute. In general, these changes were similar in magnitude between F/L and H/L but were more marked in the axial and mid-regions compared with the abaxial region.	Basal epidermal cells undergoing mitosis were identified by TXP2 staining and apoptosis by cell (nuclear) morphology. In treated ponies, there were more mitoses (axially) and apoptotic cells (abaxially and mid-region). The number of cells per SEL increased (12%) in the treated group but normalising for the SEL length showed there were 75% fewer cells per μm of SEL with treatment.	No assessment of BM membrane structure was made in this study	No leucocyte infiltration was mentioned in this article.

(Continues)

TABLE 2 (Continued)

Reference	Model employed, time of sampling number of animals	Structural pathology	Epidermal cell response	Basement membrane changes	Inflammatory cell infiltrate
De Laat et al., 2012[21]	Male Standardbred retired horses ($n = 4$ treated and 4 controls) were subjected to a glucose infusion over 48 h (50% dextrose; 0.68 mL/kg/h—treated horses). This is a variation of the EHC model of EL. No horse became lame but all developed histological lesions in their lamellae. Serum insulins rose to 208 ± 26.1 μIU/mL on average over the second 24 h period compared with 10.6 ± 1.36 μIU/mL in the control group.	At least one foot affected in all treated horses. Lesions included lengthening and tapering of SELs with SELs being narrower at both axial and abaxial ends of the PEL. Confluence of SEL basal cells near the PEL axis led to obliteration of SDL.	EBCs had rounded nuclei, prominent nucleoli and evidence of mitoses and apoptosis (absent from controls). Architecture of regular epidermal basal cell layer disrupted	No separation of BM from tips of SELs but its appearance in treated horse is described as irregular	Three of the four treated horses had increased extravasated PMNs in the lamellar dermis adjacent to the tips of the PELs. None of the controls exhibited this.

Abbreviations: BM, basement membrane; CHO, carbohydrate; EBCs, epithelial basal cells; EHC, euglycaemic hyperinsulinaemic clamp; OF, oligofructose; PAS, periodic acid-Schiff (histological stain); F/L, forelimb; H/L, hindlimb; PDL, primary dermal lamella; PEL, primary epidermal lamella; SDL, secondary dermal lamella; SEL, secondary epidermal lamella; PMN, polymorphonuclear leucocyte; SRL, sepsis-related laminitis; TXP2, a nuclear protein (repp86/p100) associated with cell proliferation that is specific for S, G2 and M phases of the cell cycle.

considerably from the others in terms of pathogenesis, with infiltration of inflammatory cells occurring very early in the developmental phase,[30] this review focuses on the other three models and naturally occurring laminitis for the reasons discussed in the following paragraphs.

2.3 | Role of leucocytes in laminitis

Additional studies have focused on the presence of leucocytes within the lamellar tissue and related this to the onset of clinical and histopathological signs of laminitis. From the descriptions of the models presented in Table 2, it is clear that leucocyte infiltration is present at certain time points in both SRL and EL models. By comparison to Table 1, three of the four descriptions of naturally occurring laminitis do not describe a leucocytic infiltrate which is probably because most of the cases examined were aggravated chronic cases rather than peracute occurrences (which the models best mimic).

Based on the time-course studies that have been undertaken, for the EHC model of EL, epithelial cell stress (apoptosis evident from 6 h onwards) precedes any leucocyte infiltration or activation (mainly evident at 48 h), suggesting any inflammatory infiltrate occurs secondary to the events initiating lamellar pathology rather than inflammation initiating that pathology.[19] Detailed immunohistochemistry of markers of different leucocytes has not been undertaken in the EHC model to date, although the work of Watts et al.[31] also supports this sequence of events. However, in both carbohydrate models of SRL (oligofructose model and starch overload model—see Table 2), the question of what cell types infiltrate when has been extensively studied (Table 4). The conclusions drawn by the authors of these three studies are equivocal and do not definitively answer the question as to whether inflammatory cell infiltration drives the lamellar pathology or is a response to it. Whilst the use of serial biopsies of lamellar tissue certainly reduces animal use in these sorts of experiments, the regional variability in pathology is striking and may affect the power of such studies and the effect of palmar digital nerve blocks on pathological events in these models remains to be determined.

Based on the findings in the EHC model (where the time course of events clearly indicates leucocyte infiltration is in response to lamellar pathology; see Table 2,[19] most likely driven by stress to the epidermal cells) and the time course for signalling events in the lamellar epithelial cells (see below), the changes seen in SRL (which the oligofructose model and corn starch overload models most closely mimic), may be consistent with leucocyte infiltration being a response to, rather than the cause of, lamellar damage.

Unlike the EHC and two carbohydrate models of SRL, the BWE model of SRL clearly has an early inflammatory component. Black et al.[35] used an anti-equine monoclonal antibody to CD13 as a marker for neutrophils and monocytes/macrophages in this model. Tissues from two time points, a development time point (DTP; onset of leucopenia) and the acute onset of clinical signs of laminitis (lameness) were examined. No marginal pool of CD13-positive

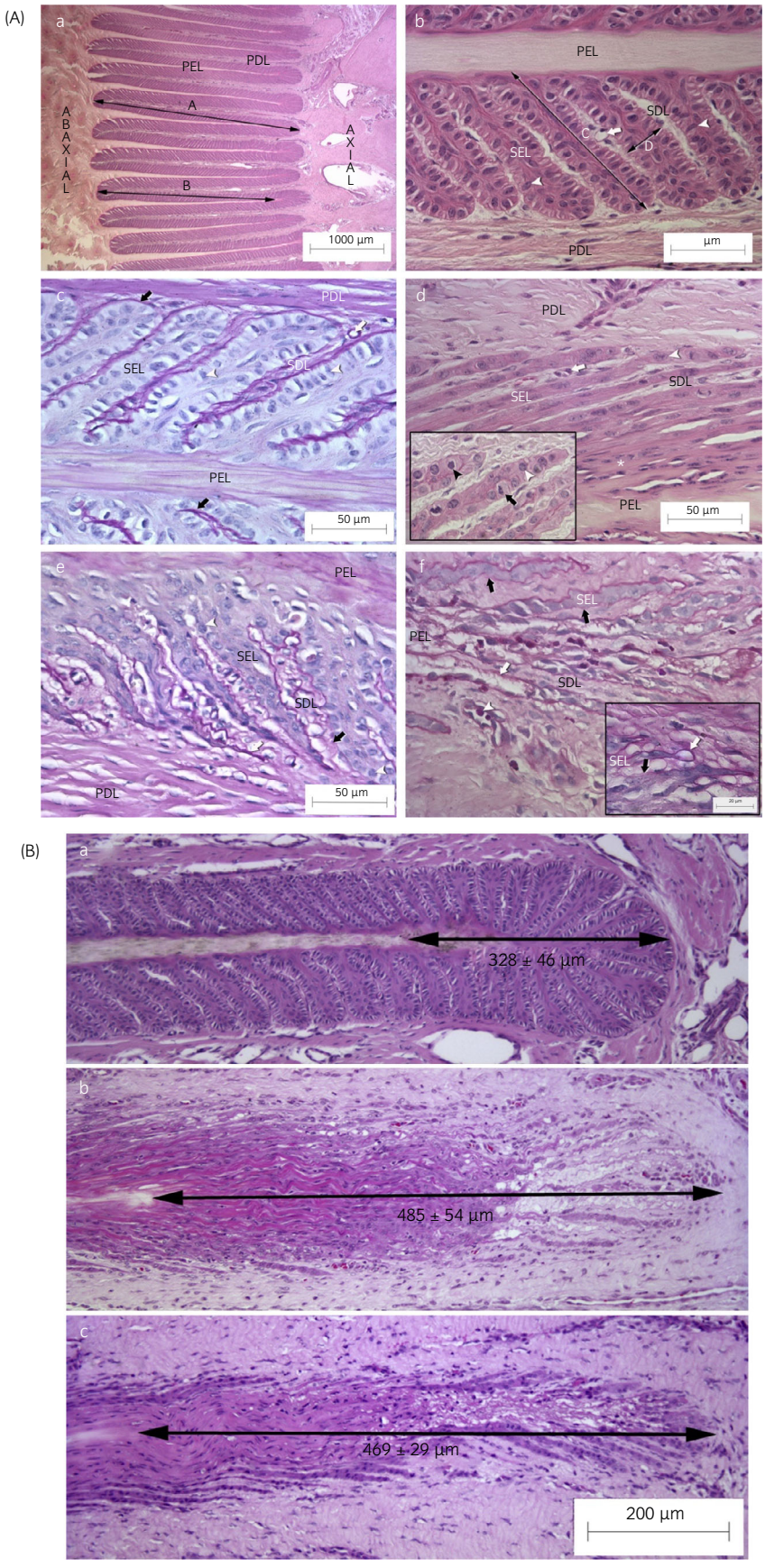

FIGURE 2 Photomicrographs of lamellar histology from experimentally-induced laminitis. (A) Photomicrographs of hoof lamellar histology from control (a,b,c) and insulin-treated (d,e,f) Standardbred horses in transverse section. Lamellar measurements (a, b) included the total (A) and keratinised (B) length of the PELs and the length (C) and width (D) of the SELs. In control horses, the PEL were straight and uniform in length, with the keratinised axis finishing before the PEL axis (a). The SEL was uniform in length and clearly separated by each SDL, which extended almost to the PEL axis (b, c). EBC nuclei (white arrowheads in b) were ovoid and located apically in the cell and the PAS-stained BM was tightly adherent to the rounded tips of each SEL and continued intact to within one or two EBCs of the PEL axis (black arrows in c). The microvasculature surrounding the SEL appeared normal (white arrows in b and c). In insulin-treated horses, the SEL appeared lengthened with sharp, pointed tips (d, e) and the EBC nuclei were variable in size and rounded with prominent, and often multiple, nucleoli (white arrowheads in d and e). Clear demarcation of individual SEL at the PEL axis was lost (asterisk in d). Pyknotic (black arrowhead in d inset) and mitotic (black arrow in d inset) figures were present. Loss of PAS-positive BM staining was apparent (black arrows in e and f) with empty profiles of BM (white arrows in f) and a lack of visible BM at SEL bases (e). The microvasculature appeared more prominent (white arrow in d and white arrowhead in f) when compared with control sections (white arrows in b and c). H&E (a, b, d) and PAS (c, e, f). (B) Photomicrographs of hoof lamellar histology at the axial end of PELs from (a) control, (b) oligofructose and (c) insulin-treated standardbred horses in transverse section. The average length from the end of the keratinised axis of the PEL to the axial tip of the PEL was shorter ($p < .05$) in control horses (328 +/− 46 mm) when compared with horses with oligofructose (485 +/− 54 mm) and insulin-induced (469 +/− 29 mm) laminitis. H&E. BM, basement membrane; EBC, epidermal basal cells; H&E, haematoxylin and eosin (histological stain); PAS, periodic acid-Schiff (histological stain); PEL, primary epidermal lamella; SDL, secondary dermal lamella; SEL, secondary epidermal lamella. Reproduced with permission from Morgan et al.[17]

TABLE 3 Detailed studies of the basement membrane of the lamellae in models of equine laminitis.

Reference	Model employed, time of sampling number of animals	Structural pathology
De Laat & Pollitt 2019[22]	EHC; at onset of Obel grade 2 laminitis (46 h) in three Standardbred horses; 3 age and breed matched normal horses for controls	Basement membrane (BM) zone appeared widened, disorganised and amorphous (patchy—some areas appeared normal). Plasma membrane of the EBC—often indistinct. BM was often devoid of hemidesmosomes (HD). HDs present were no longer orientated parallel to the lamina densa of the BM. The lamina lucida of the BM was abnormally wide and irregular or absent. The lamina densa also lacked uniformity varying in width (normal to wide). Intracellular cytoskeleton of the EBCs was disorganised and often parallel rather than perpendicular to the plasma membrane. Measurements showed the BM to be nearly double the width in laminitic horses compared with normal and the density of HDs reduced by 50% in laminitic horses compared with normal.
Nourian et al., 2009[23]	EHC model in ponies ($n = 4$ infused for 72 h and killed 5–10 h after infusion stopped; $n = 1$ given the same infusion and kept alive for 48 h after the infusion stopped). Compared with four control ponies (age and breed matched). All EHC model ponies given phenylbutazone	Measured the number of HDs per µm of BM—this was significantly lower in the laminitic ponies compared with controls (29% lower). The width of the BM was not significantly different between the laminitic ponies and controls. Numerous mitotic figures were noted in the epithelial basal cells (EBCs) of the laminitic ponies. Some EBC nuclei were abnormally close to the plasma membrane and the lamina lucida was absent. In most areas, the BM was intact, with no breaks. Occasionally it was detached from the plasmalemma forming short empty loops. PMNs were frequent in the dermis and occasionally penetrated the BM adjacent to a damaged EBC.
French & Pollitt 2004a[24]	SRL (OF model—7.5, 10 and 12.5 g/kg; $n = 2$ Standardbred horses per dose; subjected to euthanasia at 48 h and subjected to transmission EM. Two control horses (used for teaching) were examined for comparison. Four front feet per dose of OF were evaluated	Dose-dependent effects of the OF model on lamellar basal cell ultrastructure were noted. The SELs became progressively more pointed. EBCs had rounder nuclei, centrally or basally positioned. The BM zone became progressively more crenellated and the lamina densa less distinct. Progressive thinning of tonofilaments in cytoplasm was noted and these no longer attached to the HDs. Lamina lucida of the BM progressively lost its anchoring filaments and there was separation of the lamina densa from the plasmalemma where hemidesmosomes were absent. The number of HDs enumerated per µm of plasmalemma was 22%, 53.6% and 62.3% lower in horses treated with 7.5, 10 and 12.5 g/kg OF, respectively, when compared with controls. The measured distance from the HD to the middle of the lamina densa increased progressively with dose also (25% increase at the highest dose).
Nourian et al., 2007[25]	SRL (OF model—10 g/kg; $n = 4$ Standardbred horses with age and breed matched controls); samples collected at first sign of foot discomfort (24–30 h) and subjected to transmission EM	HDs were enumerated per 1 µm of BM in laminitics and control—the laminitics had 57% fewer. In laminitics, the BM was broken in many places and parts of the lamina densa were separated from the plasmalemma of the epithelial basal cells. The mean distance between the plasmalemma and the middle of the lamina densa of the BM was larger (11.5% larger)
Pollitt & Daradka 1998[26]	SRL (Alimentary CHO model at 48 h; $n = 2$ horses with eight control horses for comparison). Light microscopy study but used immunohistochemistry to stain laminin, collagen type IV and collagen type VII. The latter is specific for lamellar basement membrane.	Laminitis histopathology was graded as grade 3 according to Pollitt (1996).[12] Demonstrated that type VII collagen antibody stained the lamellar BM but not that of blood vessels. Showed that two processes occurred to differing extents in different areas of the lamellae, namely loss of contact (separation) of EBCs with the BM (which remained intact and anchored to the dermis) and disintegration of the BM. Detachment of EBCs occurred without obvious necrosis of the cells. Loss of immunostaining of three proteins that constitute the BM suggests genuine disintegration of this structure. When the BM loses contact with the basal cells it slides to form dilated empty SEL tips (tubes of BM) which sometimes break away completely to form vessel-like structures—but their positive staining for type VII collagen demonstrates their BM origin. Numerous PMN were present within the dermis surrounding the tube-like structures, some being located between the layers of BM.

TABLE 3 (Continued)

Reference	Model employed, time of sampling number of animals	Structural pathology
French & Pollitt 2004b[27]	SRL (OF model 10 g/kg; $n = 6$ Standard-bred horses killed 48 h after dosing). Control tissue was from 2 age-matched normal horses. Undertook immunohistochemistry for three HDs proteins BP230 (human monoclonal 5 E-HY-4B), plectin (mouse monoclonal 417D1), and integrin α_6 (rat monoclonal GoH3), two anchoring filament proteins, BP180 (mouse monoclonal 283) and laminin 5 (rabbit polyclonal J18) and cytoskeleton intermediate filaments cytokeratin 14 (mouse monoclonal LL-0023).	The classical pathology of the lamellae was demonstrated on histopathology—elongated thinner SELs with sharp rather than rounded tips. PAS staining demonstrated strands of BM no longer attached to SEL present in the adjacent dermis. Plectin staining was unaffected by OF treatment—located in the basal cells—basal cytoplasm and plasma membrane. BP230 was localised at the bases of SEL at the dermo-epidermal junction penetrating between bases of adjacent SEL. OF treatment caused intermittent BP230 loss around SEL perimeter. That remaining stayed associated with basal cell plasma membrane despite BM separation from EBCs. Integrin α6 staining outlined EBCs in control tissue—brighter at the basal plasma membrane adjacent to BM. Tissue from treated horses had weaker staining but same localisation even when BM separated from EBCs BP180 staining was localised to SEL basal cells, concentrated adjacent to the BM. Reduced staining in OF-treated horses. Where the BM separation from SEL occurred, staining stayed with epidermal cells Laminin 5 staining was concentrated at the rounded SEL tips in control horse tissue, at the dermo-epidermal junction. In tissue from treated horses, Laminin 5 staining was diminished at SEL tips and associated with strands of BM present in the dermis, detached from SEL. In tissue from normal horses, BP180 and Laminin 5 were shown to co-localise. Where BM had separated from EBCs and formed strands in the dermis, no BP180 was found with Laminin 5 showing these two proteins had also separated as the EBCs and BM separated. These data suggest that Laminin 5 is cleaved to give rise to BM separation from SELs. Cytokeratin 14 staining was in the cytoplasm of EBC cells leading to outlining of the oval nucleus in tissue from normal horses. The intensity of the staining was unchanged in tissue from OF-treated horses but the shape and orientation of the nuclear shadow had changed, becoming rounded and with its long axis paralleling the BM.
Visser & Pollitt 2011a[28]	OF model of SRL; 5 Standardbred horses given 10 g/kg of OF and lamellar tissue biopsied (prior to and at 12, 18, 24, 30 and 36 h following dosing). Biopsies were taken from dorsal hoof wall of F/L (three positions per front foot [medial, lateral and central]—alternating between feet) with the horses sedated, standing having received a palmer digital nerve block. Horses were subjected to euthanasia at 48 h and lamellar tissue harvested. Control horses ($n = 3$) were sham treated with water and biopsied in the same way.	In this study, lamellar BM was stained with a panel of different antibodies directed against Laminin 332 subunits, polyclonal antibody ab14509 which is against all three subunits of the protein, the monoclonal antibody D4B5 and the polyclonal Pab26 which are directed against the human γ2 subunit and antibodies K140 and BM165 which are directed against the human α3 and β3 subunits, respectively. Antibody CIV22 against collagen IV α2 and α3 chains were also used. Tissue from normal horses showed staining with all Laminin 332 antibodies except D4B5. In samples from horses subjected to euthanasia after 48 h, these antibodies demonstrated long gaps in the BM. Moreover, the remnants of the BM now stained with D4B5 after induction of laminitis. A semi-quantitative scoring scheme for Laminin 332 and collagen IV staining (1 for 100% stained through to 4 for <25% stained) showed time-dependent loss of staining with most antibodies for laminin 332 and collagen IV from 12 or 18 h with a concomitant increase in staining with D4B5 antibody over the same time-frame. Regions where epidermal basal cells had separated from the BM to form a bubble, the BM remained intact (positive staining with most antibodies)—representing a different pathology where Laminin 332 separates from its HD.

Abbreviations: BM, basement membrane; CHO, carbohydrate; EBC, epidermal basal cell; EHC, euglycaemic hyperinsulinaemic clamp; EM, electron microscopy; F/L, forelimb; HD, hemidesmosomes; OF, oligofructose; PAS, periodic acid-Schiff (histological stain); PMN, polymorphonuclear leucocyte; SEL, secondary epidermal lamella; SRL, sepsis-related laminitis.

leucocytes was detected in the perivascular dermal region of the lamellae of control horses. Three of the 5 horses taken to the DTP had CD13-positive cells perivascularly in the dermal lamellae (lamellar venules) as did all of the horses at the point of onset of lameness. Nuclear morphology of the CD13-positive cells suggested they were mainly neutrophils. Infiltration of the skin with CD13-positive leucocytes was also demonstrated in samples taken from the same horses. Superficial dermal vessels demonstrated perivascular accumulation of CD13-positive cells in the developmental and acute clinical phases. This was not seen in the deep dermal skin vessels, only the superficial ones whereas the deeper lamellar dermal vessels also demonstrated perivascular accumulation of CD13-positive cells (although the data demonstrating this were not shown in the paper). The authors of this paper suggested that cytokines derived from these emigrating CD13-positive cells drove the lamellar inflammatory response seen in this model, which was responsible for the lamellar damage and pathology. Similar findings were reported by Faleiros et al.[36] who used calprotectin as a marker of activated leucocytes (neutrophils and monocytes) and of stress in keratinocytes and by Faleiros et al.[30] who showed modest extravasation of CD163-positive (a monocyte/macrophage marker) cells in the SDL at the early (1.5 h) and DTP (3–4 h). All these findings support the concept that leucocyte infiltration of the lamellae drives a stressful and damaging inflammatory process in the BWE model.

Thus, it appears that the BWE model of laminitis is quite different from the other SRL models (alimentary carbohydrate overload and oral oligofructose) and the EHC model, when epidermal stress precedes evidence of inflammation. Although relatively little has been published to date, the SLL model seems also likely to result from stress to the epidermal cells, possibly caused by hypoxia.[37] On the basis that elements of carbohydrate overload (SRL), SLL and EL might all occur to varying degrees in naturally occurring pasture-associated laminitis, the remainder of this review will focus on research findings in these models.

3 | BASEMENT MEMBRANE DEGRADATION: POTENTIAL MECHANISMS

A major advance in understanding equine lamellar structure and function is underpinned by the detailed work of Pollitt and others showing that in normal horses, the EBCs are anchored to the BM via hemidesmosomes (adhesion plaques; HDs). HDs are found on either side of the cell's plasma membrane and attach to the cells' cytoskeletal elements from the inner surface and to the BM from the outer surface. Fibrils of type VII collagen attach the BM (lamina densa) to the lamellar dermal connective tissue. Fine anchoring filaments bridge the gap between the lamina densa of the BM and the HDs, crossing the lamina lucida of the BM (Figure 3A,B). This microanatomy gives the dermal-epidermal connections the tensile strength to withstand the changing forces associated with locomotion, maintaining the structural organisation of the secondary epidermal lamellae, which is the platform for this interface.[38]

The maintenance of this structural organisation is disrupted in laminitis, leading to stretching of the SELs and/or their separation from the BM with or without dissolution of the BM structure itself. The initiating factors in these processes are the subject of intense debate in laminitis research. This microanatomy is not easy to study, requiring electron microscopy and careful immunohistochemistry by light microscopy to determine its state and the presence of constituent proteins at different time points of the development of laminitis. The published studies that are of high quality that describe these events in the EHC and carbohydrate overload SRL models are presented in Table 3. These elegant studies, together seem to suggest that two separate processes occur to differing extents. First, separation of the SEL from the BM through loss of HDs (BM stays intact and attached to the dermal connective tissue) and second, disintegration/dissolution of the BM itself. In the EHC model, loss of HDs seems to predominate[22,23] whereas both processes seem to occur in a dose and time-dependent way in the oligofructose model of SRL.[24,28]

The activation of enzymes responsible for digesting the various elements of connective tissue within the BM and/or anchoring the HDs to the BM or to the EBC has been proposed as the principal mechanism triggering this pathology. Whether this is initiated by stress to the epithelial cells or infiltrating leucocytes has also been the subject of investigation. Table S1 summarises the key publications to date involving tissue from models of laminitis and naturally occurring disease. Initially, the focus was on matrix metalloproteinases (MMPs). In vitro studies demonstrated that MMP-2 and MMP-9 could be produced by lamellar explants cultured in the presence of the MMP activator aminophenylmercuric acetate (APMA).[39] APMA treatment of lamellar explants led to separation of lamellar tissue from the hoof wall at the dermal-epidermal junction when tension was applied, an effect that was prevented by the MMP inhibitor batimastat.[39] Early studies of naturally occurring laminitis focused on MMP-2 and MMP-9 and demonstrated increased expression of these proteins but did not determine the relative proportions of active and inactive forms of these proteases within the tissue.[40] Later studies suggested that these enzymes were not free of inhibition[41] and that MMP-9 expression was associated with leucocyte infiltration. Leucocyte-derived MMP-9 is not likely to be driving the initial lamellar damage associated with HD and BM pathology, which seems to occur prior to leucocyte infiltration (Tables 3 and 4). No evidence of activation of MMP-2 has been demonstrated in either model of SRL.[41,42] All published studies to date have extracted proteins and RNA from whole tissue and only in some cases was immunohistochemistry used to localise proteins shown to be upregulated by western blotting. This means that localised upregulation of enzyme activity may not be detected and could still play an important role in the pathophysiological events leading to the underlying pathology. Laser dissection microscopy and single-cell sequencing technologies seem appropriate tools to apply to this question in the future.

The EHC model has not been extensively studied for activation of extracellular matrix metabolising enzymes although ultrastructural studies (Table 3) suggest detachment of HDs from their anchoring filaments does occur, leading to their detachment from the apical surface

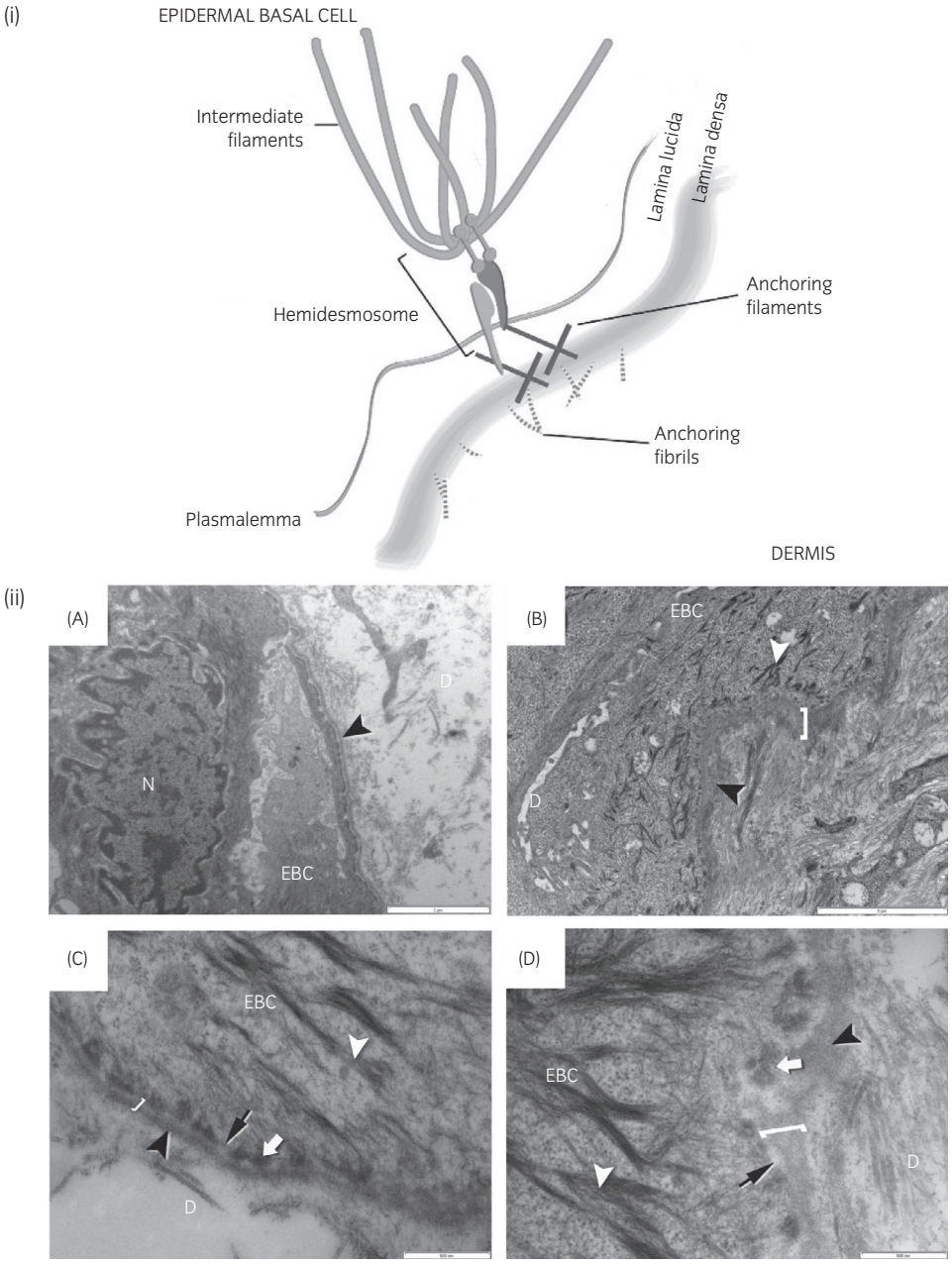

FIGURE 3 (i) Schematic diagram of the lamellar basement membrane zone of the equine hoof. The epidermal basal cell has a plasmalemma that contains hemidesmosomes. Anchoring filaments cross the lamina lucida and engage with the lamina densa. (ii) Transmission electron micrographs of the lamellar basement membrane (BM) zone of healthy horses (A and C), and horses with insulin-induced laminitis (B and D). The epidermal basal cells (EBC) are separated from the lamellar dermis (D) by the BM (black arrowhead). The cytoplasm of the EBC contains the nucleus (N). (A) Low magnification ultrastructural image of a healthy horse lamellar BM zone. (C) At higher magnification the cytoskeleton intermediate filaments (white arrowhead) of the EBC connect to hemidesmosomes (white arrow) of the plasmalemma (black arrow) of the BM zone (black arrowhead pointing to the lamina densa). The thickness of the BM zone is indicated by the white bracket. (B) In the same zone in treated horses, the BM was extensively disorganised. The width of the BM zone (white bracket) was greater in treated horses, and the lamina densa (black arrowhead) was often absent. (D) At higher magnification, fewer hemidesmosomes (white arrow) were apparent on the plasmalemma (black arrow) and the lamina densa (black arrowhead) of the BM zone (white bracket) of treated horses appear thickened (also visible in B). Cytoskeleton intermediate filaments (white arrowhead) appears disassociated from the hemidesmosomes. Magnification = A; 8000×, B; 15 000×, C; 50 000×, D; 50 000×. Bar = A; 2 μm, B; 5 μm, C and D; 500 nm. BM, basement membrane; EBC, epithelial basal cell. Reproduced with permission from de Laat and Pollitt.[22]

of the EBCs. One study focussing primarily on MMP-2 and MMP-9 and their regulators and ADAMTS-4 failed to demonstrate enzyme activation that occurred concomitantly with the appearance of BM pathology.[43] Given the very localised BM-related pathology of the EHC at the dermal–epidermal junction, looking at the whole tissue level for evidence of enzyme activation is possibly a very blunt tool.

TABLE 4 Leucocyte infiltration in carbohydrate overload models of sepsis-related laminitis.

Reference	Model employed, time of sampling number of animals	Summary of findings
Faleiros et al., 2011b[32]	CHO overload (SRL; corn starch and wood flour mix (80:20) 17.6 g/kg by nasogastric tube). Three groups of eight normal adult horses were used. Two groups received the corn-starch and one group received 6 L of deionised water in its place. Tissues were harvested at the onset of fever (Group 1), at the onset of lameness (OG-1; Group 2) and after 24 h (control horses).	Groups 1 and 2 had six responders and two nonresponders (no fever or signs of lameness detected) each. The responders reached the end-point between 10 and 20 h (group 1) and between 24 and 48 h (group 2) post-carbohydrate administration. Evidence of epidermal cell stress (nuclear rounding, SEL elongation) was objectively scored and did not differ between control and group 1 (onset of fever—developmental time point); Group 2 (onset of lameness) differed from both control and group 1. Calprotectin staining in epidermal cells was also objectively scored as an alternative measure of epithelial cell stress and gave the same results (although large variation was noted between sections from same horse). BM pathology is not described in detail in this paper but is reported not to be present in horses except those in group 2 where it was evident in mild form in three of the six horses. Calprotectin-positive leucocytes were more numerous in Group 1 compared with control in both the dermis (13.75-fold higher) and lamellar regions (7.75-fold higher). Further increases in calprotectin-positive cells were seen in Group 2 (108.5- and 181.2-fold higher numbers in the lamellar and dermal regions respectively relative to the control group). CD163 positive cells (marker of type 2/anti-inflammatory macrophages and possibly other activated macrophages in the horse) were common in control tissues and Group 1 did not differ from control, whereas Group 2 horses had approximately 2-fold more CD163 positive cells in the deep dermis and the lamellar regions compared with both the control and Group 1. Conclude that infiltration of leucocytes is less marked and later than in the BWE model of SRL but precedes basement membrane pathology and epidermal cell stress so could contribute to these processes.
Visser & Pollitt 2011b[33]	OF model of SRL; 5 Standardbred horses given 10 g/kg of OF and lamellar tissue biopsied (prior to and at 12, 18, 24, 30 and 36 h following dosing). Biopsies were taken from dorsal hoof wall of F/L (3 positions per front foot [medial, lateral & central] – alternating between feet) with the horses sedated, standing having received a palmer digital nerve block. Horses were subjected to euthanasia at 48 h and lamellar tissue harvested. Control horses (n = 3) were sham treated with water and biopsied in the same way.	Leucocytes staining positively for calprotectin were first detected at 18 h in two horses and were detected in all treated horses by 30 h. Very few positive cells were detected prior to dosing or in the control biopsies (minimal leucocytes seen after 36 h surrounding biopsy sites). Positively staining leucocytes (both monocytes and neutrophils) were first seen around blood vessels in the primary dermal lamellae and then the sub-lamellar dermal tissue and larger dermal blood vessels. From 24 h onwards, calprotectin staining was detected in epidermal basal cells (primary lamellae) possibly suggesting leucocyte infiltration was responsible for stress and damage to these cells. However, data from the same group of horses (see Visser and Pollitt 2011a[25]; Table 3) suggests that BM damage precedes leucocyte infiltration, which could be a response to the ensuing structural damage. This study also evaluated IL-6 expression in lamellar tissue and showed upregulation of IL-6 gene expression—in four of the five horses, this preceded the increase in calprotectin staining by 6–12 h possibly suggesting IL-6 secretion was involved in leucocyte recruitment although the cell type expressing IL-6 was not identified.
Godman et al., 2016[34]	SRL (OF model); 14 horses were administered 10 g/kg of OF by nasogastric tube, one front leg was placed in an ice boot and seven animals were subjected to euthanasia at 20 h (developmental time point; DTP) and the remaining seven were subjected to euthanasia at the onset of OG-2 lameness (20–28 h). There was no control group included in this study.	Immunohistochemistry was used to define and enumerate MAC-387 (M1 macrophage phenotype, neutrophils and activated epithelial cells) and CD163 (M1 and M2 macrophage phenotype) positive cells. There were more CD163 positive cells than MAC-387 positive cells at both time points in all tissue samples. The vast majority of cells (staining positive for both markers) were located in the PDL and SDL (perivascular) with very few in the epidermal lamellae. CD163+ cells increased in number between the developmental and onset of lameness time points but there was no effect of continuous hypothermia on the number of CD163-positive leucocytes at either time point. MAC-387 positive cells were also higher in number at the lameness time point. Hypothermia had no effect on the number of MAC-387 positive cells at the developmental time point but reduced the number seen at the lameness time point. However, because of the low number of these cells the reliability and biological significance of this reduction is questionable. The lack of effect of hypothermia on the increase in CD163-positive cells was surprising and suggests that these inflammatory cells are not the drivers of pathology, as hypothermia is very effective at reducing clinical signs and pathology in this model of laminitis (van Eps et al., 2012).[53]

Abbreviations: BM, basement membrane; CHO, carbohydrate; DTP, developmental time-point; F/L, forelimb; IL-6, interleukin-6; OF, oligofructose; OG, Obel grade; PDL, primary dermal lamella; SDL, secondary dermal lamella; SRL, sepsis-related laminitis.

Only one in vivo study has been published which attempted to demonstrate the effect of selective inhibition of connective tissue degrading enzymes on the pathological changes in the lamellae resulting from oligofructose administration.[44] The inhibitor used was marimastat (a relatively broad-spectrum metalloprotease inhibitor with Ki values against a large range of MMPs enzymes in the lower micromolar range and IC_{50} values against ADAMTS-4 and 5 in the high micromolar range[45]), delivered by regional limb perfusion every 6 h to maintain the lamellar ultrafiltrate drug concentration above the concentration giving 90% inhibition of both enzymes throughout the inter-dosing interval. No effect of this intervention was seen when used as a controlled intervention in the oligofructose model of SRL although definitive evidence that the target enzymes were inhibited in vivo by the dosing regimen used is lacking. This is an important proof-of-concept study which needs to be extended to other drugs with differing matrix metabolising enzyme inhibitory profiles. For such interventions to be translated to the clinic, the safety of such inhibitors for use in clinical cases would depend on the route of administration and the spectrum of enzymes inhibited. Nevertheless, as experiments which test the hypothesis that extracellular matrix-degrading enzymes are the principal initiators of lamellar pathology in laminitis, further such intervention studies are warranted.

4 | DISCOVERY OF PATHOPHYSIOLOGICAL PATHWAYS THROUGH TRANSCRIPTOMICS AND PROTEOMICS

Following initiatives for researchers to share tissue collected from models of laminitis, whole lamellar tissues from different models have been examined at different time-points for differential gene and protein expression when compared with control tissues. A targeted rather than an unbiased approach has been used in most published studies, targeted to confirm or refute processes hypothesised to be important in laminitis pathogenesis. Table S2 summarises 14 studies undertaken over the last 15 years using tissue from models of SRL and EL. In general, the studies are limited by low numbers of animals, the variability of their response to the model (particularly the corn-starch overload model of SRL and the diet challenge model of EL) and the study of time points which make it difficult to tell whether the genes and proteins differentially expressed are a response to or causally related to lamellar pathology. In addition, most papers do not identify the cells expressing the genes and products of the genes (i.e. they work on extracts of whole lamellar tissue) although in some cases immunostaining does suggest the cellular location of the protein expression.

Nevertheless, by examining the information generated by these studies in the context of what is known about the time course for histopathological and ultrastructural changes seen in these models (Tables 2–4), some general conclusions can be drawn.

The first is that epithelial cell stress likely precedes any inflammatory process in laminitis, both in SRL and EL. This fits with the observations relating to leucocyte infiltration, which occurs once BM damage has been initiated in EL and probably SRL. The certainty of this conclusion is lower for SRL than it is for EL because most of the studies assessing protein and gene expression have used the corn-starch overload model. This model is inherently variable in terms of the timeline of events and there is overlap between the onset of fever and the onset of Obel Grade-1 (OG-1) lameness. Nevertheless, the evidence from the detailed studies of Visser and Pollitt suggests changes to proteins at the epidermal-dermal junction occur between 12 and 18 h in the OF model of SRL (which is more consistent in its onset and progression) and precedes the onset of fever (20–28 h), the DTP most frequently used in these gene and protein expression studies.[28] Furthermore, where studies have localised the cytokine and chemokine mediators expressed in the SRL models, they have been localised to the EBCs (particularly ERK 1/2, STAT-3, RPS6 phosphoproteins) and often the cytokines/chemokines have not been significantly upregulated until the OG-1 time point (see Table S2 for references).

The second conclusion is that although the time scale and the severity of lamellar damage is different between SRL (carbohydrate-induced) and EL, the initiating signalling pathways activated in the EBCs appear to be similar. These seem to involve growth factor signalling pathways, possibly initiated via energy sensing systems within the cell (mTORC1 pathway) which intersect downstream with pro-inflammatory pathways. Upregulation of phospho-RPS6 is particularly marked in both models and proven to be localised to epithelial cells at time points which suggests this is an early event indicating epithelial cell stress. The stressor in the case of EL is proposed to be insulin acting through the IGF-1R on the epithelial cell,[46] the gene for which becomes downregulated in the EHC model.[47]

Insulin's growth effects on lamellar EBCs through the IGF-1 receptor have been demonstrated in vitro[48,49] and the infusion of a monoclonal antibody to selectively block the IGF-1 receptor partially prevented acute laminitis in the EHC model.[50] Horses treated with mAb11 showed less sinking of the distal phalanx and milder histological changes, with markedly less elongation at the tips of the secondary epidermal lamellae. At the dose used, the mAb11 did not prevent signs of laminitis and the histological and radiographic effects, although statistically significant, were relatively small. Nevertheless, there were some features of this study which are particularly noteworthy. In this trial, insulin levels were lower than in previous EHC models (up to 500 μIU/mL) and in the insulin-infused positive control group, many EBCs had nuclei that appeared rounded, compressed and tilted; many had lost their apical position in the EBC cytoplasm. Mitotic figures and apoptotic EBC were frequently observed in the SEL located at the tips of the PEL, where the SEL were separated. However, in the antibody-treated group, the SELs located at the axial end of each PEL were less elongated, and the BM was still attached to the SEL. Furthermore, few samples showed any evidence of mitotic figures, apoptosis, or inflammatory cells. These findings, as well as implicating the IGF-1R in the EL model, seem to suggest again that both apoptosis and increased mitotic figures may be observed in this model, which is unusual considering that these pathways usually tend to be mutually exclusive. However, ER stress may be one common

factor that can lead epithelial cells either towards apoptosis or (depending on the presence of other signals from the microenvironment) towards cytoskeletal disruption and loss of HD attachments, which is part of the so-called epithelial-to-mesenchymal transition (EMT)[51] (Figure 4).

The stressor in the case of SRL remains to be determined although a number of possibilities have been proposed, which are linked to events in the hindgut resulting from carbohydrate overload. An indirect effect via circulating platelets is a possibility given the strong evidence of platelet involvement and the early timescale for their activation (within 8 h) in the oligofructose model.[52] Weiss and others also published evidence of platelet activation in the prodromal stages of corn-starch-induced laminitis in ponies and demonstrated the presence of platelets in venous thrombi in the dermal lamellar venules at the onset of lameness in this model.[53,54] It should, however, be acknowledged that venous thrombi are not reported by others in the small number of studies where detailed histopathological analysis of this model has been undertaken (Table 2). The same researchers went on to show that an experimental antiplatelet drug, a peptide inhibitor of fibrinogen receptors, prevented laminitis in ponies undergoing the corn-starch model of laminitis.[55] Unfortunately, the work of Weiss and co-workers focusing on platelet function and the platelet as a target in the prodromal stages of SRL has not been replicated by others, although we are not aware of negative results from such attempts being published. A candidate mediator released by platelets that could stimulate the signalling pathways discussed above is the platelet-derived growth factor given that its receptor is from the same family of receptors (tyrosine kinase) as the insulin-like

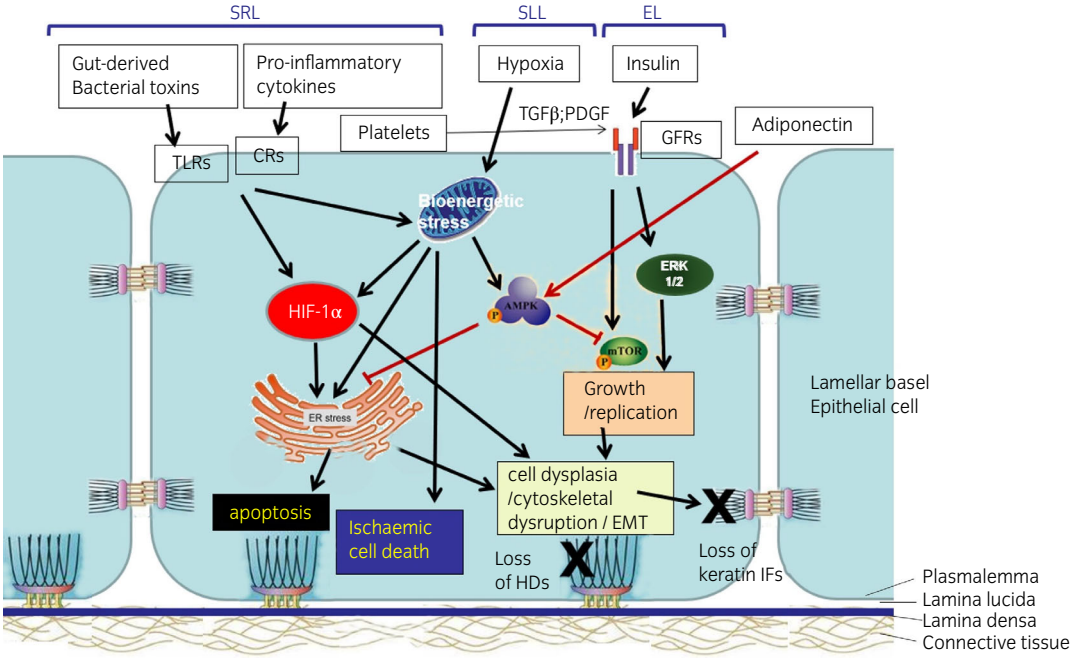

FIGURE 4 Interacting cellular pathways involved in the three experimental models of laminitis. The stabilisation and upregulation of the transcription factor, HIF-1α, is a key common element linking the inflammatory pathways that may be initiated by gut-derived bacterial toxins (such as lipopolysaccharide acting through toll-like receptor 4) or pro-inflammatory cytokines in sepsis-related laminitis (SRL); with the bioenergetic stress caused by hypoxia (supporting limb laminitis; SLL). HIF-1α and bioenergetic stress are important initiators of endoplasmic reticulum (ER) stress; which in turn can lead either to apoptosis or to cytoskeletal disruption and loss of hemidesmosomal attachments characteristic of epithelial to mesenchymal transition (EMT). Growth factors, such as insulin acting through the IGF-1 receptor (in the endocrinopathic laminitis model; EL) or potentially other growth factors such as TGFβ or PDGF from activated platelets, stimulate cell division and protein synthesis via the mitogen-activated protein kinase (ERK 1/2) and mTOR pathways. The mTOR (and Akt) pathway is also known to play a role in the induction in EMT in epithelial cells. Therefore, there is considerable overlap between the pathways. Systemic inflammation associated with SRL, and hypoxia associated with the SLL model, may tend to promote cell death. However, any inflammatory or energetic cell stress may influence the lamellar epithelial basal cell's response to growth factors and push it towards EMT and loss of hemidesmosomal attachments (and therefore laminitis). Adiponectin may suppress the activation of many of these pathways (and low adiponectin levels may lower the threshold for their activation). AMPK is activated when AMP/ATP ratio increases in a cell, such as hypoxia leading to bioenergetic stress. AMPK is also involved in the regulation of cell growth, apoptosis (via ER stress) and autophagy. It is counter-balanced by the mammalian target of rapamycin (mTOR) signalling pathway, and its activation may inhibit this pathway. Adiponectin, by supporting the activation of AMPK, may inhibit both ER stress and mTOR activation. AMPK, AMP-activated protein kinase; EMT, epithelial to mesenchymal transition; ER, endoplasmic reticulum; ERK 1/2, extracellular signal-regulated kinases 1 and 2 (these are also members of the mitogen-activated protein kinase family; HIF-1α, Hypoxia-inducible factor 1-alpha; IGF-1, insulin-like growth factor 1; mTOR, mammalian target of rapamycin complex or mechanistic target of rapamycin complex; PDGF, platelet-derived growth factor; SLL, supporting limb laminitis; SRL, sepsis-related laminitis; TGFβ, transforming growth factor beta.

growth factor-1 receptor.[56] Another mediator for which platelets are a major source is transforming growth factor β (TGF-β),[57] which has been specifically identified as a stimulus for EMT.[58]

The third conclusion from all these exploratory studies is that the classical NFkβ-mediated activation of the TNF-α pathway does not seem to be involved in the downstream inflammation that occurs in both SRL and EL models, albeit this is more marked in the SRL models. Multiple studies summarised in Table S2 have looked for upregulation of TNF-α and, to the surprise of the investigators, failed to demonstrate this at the gene level. In addition, in the studies that have examined signalling pathways activated by TNF-α, such as p38-MAP-kinase, no activation has been found. This suggests that the classical systemic inflammatory response syndrome is not operating at the level of the lamellar tissue to initiate laminitis.

All of the studies summarised in Table S2 are observational and do not include targeted interventions which inhibit what is thought to be a key pathway to determine the effect on the pathology and clinical signs in the model. Continuous digital hypothermia (CDH), which has been shown to prevent the pathology if started at the time of model initiation, has been used to determine if a particular pathway is inhibited by this intervention.[59] Many pathways are inhibited by CDH but for those that are not inhibited, despite CDH protecting against laminitis in the model, the investigators have concluded that these pathways are unlikely to be involved in the pathogenesis of laminitis (for example phospho-ERK1/2 is upregulated early in the time-course of oligofructose-induced laminitis but is not inhibited by CDH). However, in some cases, CDH itself appeared to have effects leading to upregulation of gene expression (e.g. SAPK/JNK and IL-10) which is contrary to the concept that CDH causes cellular quiescence totally. Positive results (identifying pathways upregulated and showing they are inhibited by CDH) merely provide the proof-of-concept to justify more definitive experimental studies prior to translation to clinical cases. Nevertheless, CDH is a preventative therapeutic modality that has been successfully used in veterinary practice in the early stages of acute laminitis.[60]

A less biased approach to identifying important pathways potentially involved in the pathogenesis of laminitis is to use unbiased transcriptomics and proteomics. To date, four papers have used an untargeted microarray approach to identify genes that are differentially upregulated or downregulated in the developmental phase of models of laminitis. One paper focused on the BWE model which, for reasons explained above, this review does not cover.[61] The second paper examined tissues for the oligo-fructose model of SRL prior to the onset of clinical signs (24–30 h).[62] Six horses were involved in this study (three treated and three control) and the microarray used was a bovine microarray with over 15 000 transcripts and GAPDH as a house-keeping gene. By today's standards, this is a relatively small number of transcripts for unbiased transcriptomic studies. No genes were downregulated and only 14 genes were more than 2-fold upregulated relative to the controls with just three being upregulated more than 5-fold. The top upregulated gene was ADAMTS-4 (aggrecanase 1). This and the other two upregulated genes were subsequently validated by RTqPCR. The authors of the paper acknowledge the many limitations to their cross-species approach to transcriptomics.

The third paper employed laser dissection microscopy and RNAseq methodology to identify genes up and downregulated in EBCs of secondary lamellae.[63] This study used the corn-starch model (alimentary carbohydrate overload) of SRL. Eight healthy Standardbred horses were involved and lamellar biopsies were collected at three times points (control – before dosing (left fore); DTP – onset of fever or 24 h if no fever had developed (left hind) and OG-1 time point – onset of OG-1 lameness ($n = 5$) or at 48 h ($n = 3$; right fore) whichever occurred first). One horse developed OG-1 lameness at 24 h without demonstrating fever so did not contribute a DTP biopsy sample. Epidermal lamellae were laser dissected, RNA extracted, sequenced and pairwise comparisons made (control vs. DTP; control vs. OG-1; DTP vs. OG-1) to determine differentially expressed genes. Ingenuity pathway analysis was used to determine common signalling pathways in which genes differentially expressed by more than 2-fold were involved. This study convincingly shows that epidermal cells are responding at the DTP to some form of stressor(s) leading to upregulation of inflammatory/immune-regulatory pathways (18% of genes differentially regulated at the DTP vs. CON) and pathways regulating the degradation of extracellular matrix (10% of the differentially regulated genes). Whether this is a direct result of factors absorbed from, the gut in response to carbohydrate overload or indirect effects of those factors activating white blood cells and platelets remains to be determined. Understanding the nature of the stressor(s) triggered by events in the gut is clearly of importance.

Studies such as Leise et al.,[63] are hypothesis generating. The choice of model used for this study is somewhat perplexing, however, since the alimentary carbohydrate overload model seems to generate much more individual variability in responses (both timing and extent) when compared with the oligofructose model, perhaps making the results less easy to interpret with the limited number of horses involved. The collection of biopsy samples from different feet adds to the potential variability. Furthermore, the relatively low annotation of equine-specific sequences at the time this study was undertaken may mean important epithelial cell pathways were missed.

One pathway upregulated in the OG-1 phase was that related to pattern recognition receptors for bacteria and viruses (specifically TLR-2 and TLR-4), possibly supporting the concept that epidermal cells are responding to bacterial products released from the damaged gut in this model. Interestingly, this unbiased study identified a small number of genes related to 5-HT function that were differentially regulated at the DTP so there is evidence of indirect effects as well. 5-HT release from circulating platelets, activated in the early stages of the oligofructose model, has been demonstrated which may be of relevance to this finding.[52] Many of the genes differentially regulated feature in whole tissue targeted transcriptomic studies of models of SRL, supporting EBCs as contributing to the whole tissue transcript levels measured (Table S2).

The fourth paper applied RNAseq with the highest quality samples (4 pooled from each phenotype) undergoing long read sequencing to look for differentially expressed genes in lamellar tissues (36 samples)

from 13 horses suffering from SLL (hospital-acquired) compared with seven control horses (subjected to euthanasia for lameness due to non-laminitic orthopaedic disease).[64] Each animal and limb from which the lamellar tissue was derived was scored from clinical, gross and histopathological findings as having no laminitis, developmental laminitis (no clinical signs but some histopathological lesions) or acute laminitis. Forty-three genes were reported to demonstrate differential expression that correlated with progression from developmental to acute stages of laminitis. The function of the products of most of these genes could be associated with four processes linked to laminitis pathogenesis: (i) loss of epithelial integrity; (ii) apoptosis/necrosis; (iii) changes in keratinocyte phenotype and (iv) leucocyte chemotaxis. This study lacked the ability to identify the cells differentially expressing these genes and focused on a type of laminitis (SLL) where hypoxia is thought to play a major role in stimulating epithelial cell stress (see below). In addition, because these were samples from clinical cases, all laminitics were at the stage of demonstrating clinical signs warranting euthanasia. Nevertheless, tissues from less affected limbs from the same horse were used when pathological lesions were present but no lameness was detected. These were classified as DTPs.

The authors of this article highlighted the *Erzin* gene, which was downregulated in both developmental and acute laminitis phases. The product is involved in regulating epithelial integrity and EMT, processes which warrant further study. Erzin also plays a key role in leucocyte adhesion and subsequent migration through the endothelium. The *NR1D1* gene was also downregulated in both developmental and acute phases of SLL. This gene encodes for a nuclear receptor that is important in circadian rhythm in growth (e.g., hair growth cycle) and in regulating the innate immune system. The transcription factor *FOXJ2* was upregulated in the developmental phase, which might inhibit differentiation of progenitor endothelial cells and inhibit EMT, the former impairing epithelial integrity.

Pathway analysis included in this article identified 15 canonical pathways influenced by differentially regulated genes in the developmental phase and 25 in the acute phase. From this analysis, the authors highlighted a common upstream regulator protein deacetylase *sirtuin-1* (SIRT-1) as a promising target with potential overlap to hyperinsulinaemia. Notably lacking from the pathway analysis in this study are hypoxia-responsive genes. The metabolism of lamellae in SLL models indicates they are hypoxic with increased lactate formation[12] and HIF-1α protein expression increases in response to increased weight bearing, a model of SLL.[65] Regulation of HIF-1α is at the post-translational level in cultured keratinocytes.[66] EBCs are hypoxic in their physiological state compared with many other cells, similar to epidermal cells elsewhere in the body but perhaps more so because the hoof wall prevents their access to oxygen in the air more than the keratinised epidermal tissue of skin. As a result, their expression of HIF-1α protein is high under normal conditions.[65] How these cells respond to hypoxia and how this response is manifest in terms of lamellar EBC stress is an important question in laminitis research and highly pertinent to SLL in particular.

The induction of hypoxia or the upregulation of HIF-1α by the hypoxia-mimetic, cobalt chloride, has been shown to upregulate the expression of MMP-1 and MMP-9 by cultured equine lamellar keratinocytes.[67] Furthermore, inflammatory cell signalling pathways are known to show synergism with HIF-1α. In equine digital vein endothelial cells, the combination of bacterial lipopolysaccharide (known to be increased in the OF model of laminitis)[52] plus hypoxia (5% O_2) showed a synergistic effect on HIF-1α stabilisation, leading to synergistic effects on neutrophil adhesion and monolayer permeability to FITC-dextran.[68] Therefore, these stimuli in combination are likely to have a powerful effect on leucocyte recruitment to the lamellar tissues. Furthermore, the hypoxia-mimetic cobalt chloride can also induce the expression of MMP-2 by equine digital vein endothelial cells (Poulet and Bailey, personal observation), which could also affect vascular permeability and tissue access by leucocytes.

The recent studies involving the SLL model showed that ischaemia can be a primary cause of lamellar tissue failure, with HIF-1α likely being a key mediator. The synergism between inflammation and ischaemia in various cell types, involving this factor, suggests that energy dysregulation at the cellular level may be one pathway in common between SLL and SRL (Figure 4).

In conclusion, much effort has been expended in identifying the key signalling pathways and mediators that initiate the lamellar pathology that ultimately progresses to lamellar failure and the structural damage that characterises clinical laminitis. There appear to be common pathways in the EBCs that are active in the early stages of the disease, possibly triggered by different mechanisms in SRL and EL. Understanding energy production and utilisation in this key cell and what predisposing factors do to the control of these processes seem to be important questions that remain to be answered in laminitis research.

5 | EFFECT OF EXPERIMENTALLY INDUCED LAMINITIS ON METABOLISM WITHIN THE LAMELLAE

Metabolic stress on the lamellar epithelial cells has long been suggested as a mechanism that triggers or contributes to the development of laminitis. The inaccessibility of the tissue in the live animal and the large number of arteriovenous shunts in the circulation (adaptation for thermoregulation) make this difficult to study over time in the different models of laminitis. A microdialysis method has been developed and validated to study this question.[69] Further refinement (to minimise hoof wall resection) and validation studies were undertaken to compare microdialysis probe placement (lamellar dermis vs. sublamellar dermis) and to compare glucose utilisation between skin/sublamellar and lamellar tissue.[70] The results suggest lower perfusion (based on urea clearance) and increased glucose utilisation (with adaptation to efficiently utilise generated lactate) in the lamellar dermis compared with the sublamellar dermis and skin. The results of the lamella lactate to pyruvate ratio (a measure of tissue redox state, dependent on mitochondrial function and oxygenation) do not suggest lamella tissue is relatively hypoxic and so are at odds with the observations of Pawlek et al.[66] based on HIF-1α protein expression. However, it is important to recognise that there are also oxygen-

TABLE 5 Results of microdialysis studies to assess metabolism in experimental models of laminitis.

Reference	Model	Stage	Summary of findings
Medina-Torres et al., 2016[73]	SRL (OF) with microdialysis probes inserted in F/L sub-lamellar dermis and skin dermis (tail head). Probe infusion rate 1 μL/min	Measurements made every 2 h in six horses treated with OF and six controls for 24 h with horses confined to stocks. Phenylbutazone was administered at onset of OG-1 lameness (18–22 h)	Histopathology at 48 h demonstrated laminitis was successfully induced in the OF-treated horses. The probe was in the sub-lamellar dermis in four and five horses and between the tips of PEL in two and one horse(s) in the OF-treated and control animals respectively. Urea concentration in lamellar dialysate fluid decreased by 33% (15–10 mmoL/L) in the OF-treated horses between 4 and 12 h, returned to baseline by 24 h and was significantly lower than OF-treated skin (4–16 h) and control lamellar (6–18 h) dialysates. Plasma urea decreased more slowly (by 2.3 mmoL/L at 16 h). Glucose dialysate concentration decreased from baseline in OF-treated lamellae and skin (no concomitant changes in plasma glucose were seen in these horses). Control lamellar dialysate glucose did not change from baseline. Lactate concentration in the dialysate fluid did not change from baseline in any tissue. Pyruvate concentration changed in OF-treated lamellar dialysate to be lower than baseline at 14 and 22 h. No change from baseline was seen in OF-treated skin or control lamellae. L:G ratio increased from baseline in OF-treated lamellar and skin dialysate from 6 h onwards (primarily due to the decrease in glucose) whereas there was no change in control lamellar dialysate L:G from baseline. L:P ratio tended to be higher than baseline from 10 h onwards in OF-treated lamellae and from 16 h in OF-treated skin. Control lamellar L:P did not change from baseline significantly although there was a general increase over time to 16 h and then a reduction. Authors speculate on the cause of the increased glucose utilisation in the lamellar tissue in the OF model. The reduced urea concentration suggests increased perfusion. They suggest increased utilisation by resident macrophages as a result of sepsis but the timing of onset seems to precede the rise in plasma LPS recorded in this model which is detected at 8 h (Bailey et al., 2009).[52] They conclude there was no evidence of bioenergetic failure in the OF model—despite increasing L:P in OF-treated lamellar dialysate, arguing that this never became significantly different from control lamellar dialysate despite increasing relative to baseline (whereas as control was never significantly higher than baseline). The number of animals in the study and variation in the placement of the probe make strong conclusions difficult to make with certainty. They also accept that L:P may not be a sensitive indicator of bioenergetic failure in the lamellar tissue as even with total ischaemia the increase is mild (and similar to that seen here).
Stokes et al., 2020b[74]	EL (EHC model) with microdialysis probes implanted 24 h before EHC established (to give baseline) and continuing for 48 h of the clamp. Probes were positioned in dermal lamellae (between PELs) and dialysate flow rate was 0.5 μL/min. Sampled every 4 h (control) and 6 h	Standardbred horses (n = 8 undergoing EHC and n = 6 controls) Retrospective controls were used from horses from the same source following the same protocol. All EHC-treated horses developed OG-1 lameness after 28–34 h of insulin infusion	This study did not demonstrate marked changes in metabolism or perfusion in the EHC-treated animals relative to the control animals. No evidence of reduced perfusion was found – urea clearance tended to increase relative to the control during the Clamp period which is not consistent with hypoperfusion and suggests an increase in perfusion. Glucose concentrations in the dialysate fluid did change in the Clamp period but there was no difference between EHC-treated horses and control. Lactate concentrations increased more rapidly in the EHC horses relative to control during the Clamp period suggesting faster glucose utilisation by the tissue. Pyruvate concentrations did not change significantly over time in the Clamp period in either group but there was an increase in pyruvate concentrations in EHC-treated horses relative to control.

(Continues)

TABLE 5 (Continued)

Reference	Model	Stage	Summary of findings
	(EHC). Confined to stocks for full 72 h		The authors suggest these results support two key conclusions – the EHC model of laminitis is not associated with reduced perfusion of the lamellae – if anything perfusion is increased. The lack of change in L:P ratio during the EHC clamp also does not support the presence of bioenergetic stress in lamellar tissue in this model. The limitations are the prolonged period of standing of the horses in this model, the use of historic controls and the differences in frequency in sampling times between the controls and EHC-treated animals.
Van Eps et al., 2021[37]	SLL (model of preferential weight bearing—PWB) Two dialysis probes inserted in one F/L hoof—one in the lamellar dermis and the other in the sub-lamellar dermis—fluid sampled every 4 h (perfusion rate 0.5 μL/min) for 96 h	Standardbred horses ($n = 13$), six subjected to PWB model and seven controls Four hours after dialysis probe implantation—the opposite F/L was destabilised to cause PWB on the limb with the probes fitted.	Rectal temperature increased in PWB horses between 12 and 48 h (>38.5°C) peaking at 24 h. Load on the instrumented limb was higher in the PWB group after 4 h relative to controls and did not change over time in either group (38.7% of BW vs. 27.3% BW in controls). Offloading frequency increased over time in controls but not in PWB animals. Lamellar glucose decreased suddenly after 40 h in both groups—decrease was faster in PWB horses. This change was not seen in sub-lamellar dialysate. Lamellar lactate concentration did not change over time in either PWB or control dialysates whereas sub-lamellar dialysate lactate decreased over time in control but not PWB groups. Lamellar dialysate pyruvate decreased in both groups but to a greater extent in PWB than in control such that the lowest pyruvate concentration of PWB was 50% of control. Sub-lamellar pyruvate concentration did not change over time in either group. Lamellar L:P ratio significantly increased over time in PWB but not in control such that the peak L:P ratio was >4-fold higher in PWB group than control. Sub-lamellar L:P ratio did not change over time in either group and did not differ between groups. Lamellar urea clearance decreased over time in PWB but not controls such that these clearances differed between the two groups. No change in sub-lamellar clearance was found in either group and no difference between groups was seen. For PWB, lamellar urea clearance was associated with L:P ($r = -0.76$) and limb load ($r = -0.3$) but not offload frequency whereas in controls lamellar urea clearance was associated with offload frequency ($r = 0.33$). Looking at patterns in individual PWB horses—abrupt increases in lamellar lactate and decreases in pyruvate and glucose occurred concomitantly—but variation in timing of these in each individual meant composite plots do not reveal this so clearly. Conclusion is that prolonged PWB gives rise to lamellar but not sub-lamellar ischaemia as indicated by the normal to high lactate, reduced pyruvate (consistent with ischaemia) and decreased urea clearance (consistent with hypoperfusion). The magnitude of the L:P ratio (>100) fits with observations from other ischaemic tissues. The authors speculate on the haemodynamic mechanism and accept that the metabolically active lamellae are relatively hypoxic (have higher L:P ratios than most tissue) and so highly sensitive to further reductions in perfusion.

Abbreviations: BW, bodyweight; EHC, euglycaemic hyperinsulinaemic clamp; EL, endocrinopathic laminitis; F/L, forelimb; L:G, lactate to glucose ratio; L:P, lactate to pyruvate ratio; OF, oligofructose; OG, Obel grade; PEL, primary epidermal lamella; PWB, preferential weight bearing; SLL, supporting limb laminitis; SRL, sepsis-related laminitis.

independent pathways that may activate this signalling molecule.[71] These oxygen-independent pathways may include PI3K but importantly (in the context of laminitis) also may involve growth factor effects, utilising the mTOR/RPS6 pathway. Finally, this model has been tested to determine the response to interventions which stop (tourniquet) or reduce perfusion (noradrenaline inclusion in the microdialysate) and demonstrated the expected results,[72] although total ischaemia was necessary to stimulate a reduction in urea clearance, suggesting this is a relatively insensitive index of tissue perfusion (possibly due to the relatively high dialysate perfusion rate used).

This method obviously is invasive and samples fluid from a restricted area of the lamellar interstitial fluid and the presence of the dialysis probe may influence microcirculatory haemodynamics, as does the requirement for the horses to be restrained in stocks during the perfusion and collection procedure. Accepting those limitations, this method has been used to study lamellar metabolism in three different models of laminitis (Table 5).

These studies, although not without limitations, certainly seem to demonstrate dramatic changes in lamellar tissue utilisation of glucose and perfusion early in the developmental stages of SRL, supporting the concept of epidermal cell stress being an early factor in the pathogenesis of this disease.[73] Considering measurements in this study were made from a probe in the sub-lamellar tissue primarily, these effects may not be accurately represented in lamellar tissue fluid.[37]

Targeted metabolomics on the microdialysates from the SRL study examining the concentrations of 44 intermediates of central carbon metabolism has been published showing that combining metabolomics with microdialysis is possible and could be useful in the future, if an unbiased approach can be taken.[75] Epidermal cell stress seems to be generated in a different way when hyperinsulinaemia is produced by the EHC model of EL. Although the rate of lactate generation increases relative to control in this model, indicating some changes in metabolism, there is no evidence of bioenergetic stress.[74] However, in SLL there is clear evidence of hypoperfusion and bioenergetic stress when lamellar tissue fluid dialysate was sampled.[37] Such evidence was not found in sampling sub-lamellar fluid. In a further study, where dialysis probes were placed in the lamellar tissue and microdialysis used to study lamellar metabolism during OF-laminitis and EHC-laminitis induction under ambient temperature and continuous digital hypothermia, some evidence of nonischaemic oxidative energy failure was identified between 24 and 36 h of induction as evidenced by a marked increase in the lactate to pyruvate ratio in both models.[76] The late occurrence of this change in the lamellar microdialysates suggests energy failure is unlikely to be a primary pathophysiological event in either EHC or OF model of laminitis but could be a complication of the primary pathology which is benefited by continuous digital hypothermia.

6 | NATURALLY OCCURRING LAMINITIS AND STUDY OF MOLECULAR MECHANISMS

Gene and protein expression have not been measured commonly in tissues from horses with naturally occurring laminitis. Expression of enzymes degrading the extracellular matrix proteins have been measured (Table S1[41,40]). Faleiros et al.[77] measured caspase 3 enzyme immunostaining and TUNEL staining in lamellar tissue collected from 4 acute laminitis cases (duration <1 week so probably better described as sub-acute) and 8 chronic laminitis cases (>8 weeks duration; full clinical details are found in Johnson et al.[78]) and compared the results to normal control horses and tissue obtained from model studies (CHO overload model; 10–18 h which is a DTP). The acute naturally occurring laminitis cases all had significant evidence of apoptosis occurring in the epidermal lamellar cells, particularly in the basal layer (17-fold increase relative to the control). In addition, TUNEL staining without accompanying caspase 3 staining was greatly increased (1025-fold) in keratinocytes of tissue from acute laminitis cases suggesting cell death is occurring with DNA fragmentation in these cells. These were not features of the early DTP of the CHO overload model (a finding confirmed by TUNEL staining only in Catunda et al.[79]) or of the chronic laminitis cases.

Cassimeris et al. examined tissue from naturally occurring cases of EL (6 cases of confirmed PPID and 6 cases with obesity (BCS >7) and/or regional adiposity) and demonstrated clear evidence of endoplasmic reticulum (ER) stress in the lamellar epithelial cells of the more severely affected forelimbs.[5] Data are not presented in the paper or supplementary files as to how many of the naturally occurring cases had been tested to confirm they had insulin dysregulation (ID). Markers of ER stress were measured in lamellar tissues including (a) 50 kDa XBP1s, the product of spliced mRNA for XBP1, (b) 78 kDa Glucose regulated protein also known as binding immunoglobulin protein (Grp78/BiP), an ER chaperone and (c) 94kDa Glucose regulated protein (Grp94), another ER chaperone. XBP1 and Grp78/BiP proteins were minimally expressed in lamellar tissue from control animals and in the much less affected hindlimbs from the clinical cases when compared with the affected lamellae of the forelimbs. All three markers were upregulated by approximately 2-fold on average and were significantly different when laminitics were compared with controls. Comparing F/L and H/L for Grp94 did not reach statistical significance although demonstrated the same pattern as the other markers. Immunofluorescence staining for Grp78/BiP qualitatively confirmed the western blotting results and demonstrated the protein was localised to suprabasal epithelial cells adjacent to the keratinised axis and was absent from the SELs and axial tips of PELs. The pattern of staining (punctate in the cytoplasm) suggested its localisation within the ER.[5] Prolonged ER stress can lead to apoptotic cell death. Whether ER stress and the resulting unfolded protein response is an early (primary) or late (secondary) event in laminitis development remains to be determined but is of interest given the underlying predisposing factors of insulin dysregulation in naturally occurring pasture-induced laminitis (see below).

In conclusion, based on the pathology and transcriptomics/proteomics data published from the different models of laminitis, it is possible that naturally occurring pasture-induced laminitis could have elements of all three experimental models underlying its pathogenesis (Figure 4). Persistent ID and high peaks of insulin following intake of pasture containing elevated concentrations of nonstructural

carbohydrates seem to be common predisposing factors to naturally occurring laminitis (see below). Some increase in colonic fermentation will occur with the excess carbohydrate (albeit to a much lesser degree than the extreme SRL models) and mild preferential weight bearing due to chronic changes in foot anatomy as a result of repeated episodes of sub-acute laminitis all could contribute. Since EBCs are thought to be the key initiating cell in all forms of laminitis, factors which predispose these cells to stress responses as a result of disturbed metabolism, perfusion or mechanical stretch should be considered. Looking at the risk factors for naturally occurring laminitis might provide some clues to this.

7 | HOW DOES CHRONIC INSULIN DYSREGULATION PREDISPOSE TO PASTURE-ASSOCIATED LAMINITIS?

The association between ID and predisposition to laminitis was first recognised in the 1980s by Coffman and Colles[80] and Jeffcott et al.[81] and proposed to be important in the pathogenesis of pasture-associated laminitis by Field and Jeffcott.[82] The first large cohort study to examine this association between ID and a history of laminitis was published in 2006 and involved 160 horses and ponies in the USA.[83] This study also proposed and tested a predictive algorithm to identify ponies that would develop laminitis in the next season which correctly predicted 11 of the 13 ponies that went on to suffer from laminitis. Further studies in an observational cohort of 80 ponies in the UK confirmed the association between high basal serum insulin (in the summer), elevated serum triglycerides and uric acid and relative hypertension and a history of recurrent laminitis.[84] These two studies recognised the similarities of the phenotype of laminitis-prone ponies with human metabolic syndrome. Further development and evaluation of the predictive algorithm were undertaken in the same closed herd showing that basal serum insulin and leptin concentrations, and scores for generalised (BCS) and localised (cresty neck score) obesity could be combined to predict future laminitis episodes.[85]

These early studies were undertaken on populations of ponies with a known predisposition to laminitis and animals kept under similar or identical conditions with no history of laminitis. This approach was criticised in a systematic review of research into the risk factors for laminitis which appraised the quality of the published literature assessing laminitis risk factors.[86] Since the publication of that systematic review, large prospective studies have followed ponies of unknown laminitis history and determined risk factors for naturally occurring laminitis in ponies kept at pasture.

A 3-year prospective study involving 446 ponies (aged ≥7 years) kept at grass was undertaken in the UK to determine risk factors for pasture-associated laminitis occurrence.[87] Biomarkers (blood and phenotypic) were only collected at entry to the study which is a major limitation. Body condition score (BCS), height, weight and crest height and thickness were measured and an overnight dexamethasone suppression test performed. Plasma or serum adiponectin, leptin, triglyceride, basal insulin, insulin post-dexamethasone, insulin-like growth factor 1 (IGF-1), IGF binding protein 1 (IGFBP-1), IGFBP-3, C-reactive protein, von Willebrand's factor, soluble E-selectin and P-selectin concentrations were assayed. Over the 3-year study, 44 ponies (9.9%) were reported to have had at least one episode of laminitis. Plasma adiponectin, and basal serum [insulin] and [insulin] post-dexamethasone levels were independently, consistently (over the 3 years) and significantly ($p \leq 0.05$) associated with laminitis (veterinary diagnosed) occurrence cumulatively after 1, 2 and 3 years with basal insulin showing a good, adiponectin a moderate and insulin post-dexamethasone a poor predictive performance by ROC analysis. Interestingly, neither obesity nor regional adiposity proved significant risk factors for laminitis in this study, possibly because the majority of the ponies in the study were obese or overweight. Nevertheless, this does indicate that not all obese ponies have ID and are at high risk of laminitis. Following the design of this study, it became clear that oral sugar tests were being used in an attempt to differentiate ponies with ID from those without this phenotypic trait and it seemed likely this would help identify ponies with ID more precisely.

A second prospective study was undertaken to overcome the limitations of the first.[88] This study involved 374 ponies followed for up to 4 years to give 891 pony years at risk, with visits to collect data being repeated every 6 months (spring and autumn). Oral sugar tests were performed at each visit and husbandry data were also collected. Time-dependent Cox's covariate models were constructed using different categories of data and then combined. Forty-three cases of laminitis occurred. Plasma basal insulin and adiponectin concentrations, divergent hoof score and plasma insulin concentration 60 min after administration of corn syrup (0.3 mL/kg) were significant univariate predictors of laminitis occurrence. Models combining these four factors did not improve the predictive power of the 60 min insulin concentration or basal insulin alone (concordance values 0.84 and 0.81 for the two models and 0.84 and 0.79 for 60 min insulin and basal insulin respectively). Risk categories were created for the three main biomarkers. Low-risk animals encompassed 70% of the population for basal insulin (<21.6 μiU/ml) and adiponectin (>10.1 μg/mL) and had a cumulative 4-year risk of developing laminitis of 6 [2–9] and 7 [3–10]% respectively. The high-risk category represented 10% of the population for basal insulin (>45.2 μiU/ml) and adiponectin (<4.2 μg/mL) and had a cumulative 4-year risk of developing laminitis of 69 [48–82] and 57 [35–72]% respectively. The high-risk category (top 10%) had 60 min post-oral sugar insulin value of >153 μiU/ml and a 4-year cumulative risk of laminitis of 73 [52–84]% whereas to be in the lowest risk category (60% of the population) the post-oral sugar insulin was <53.4 μiU/ml and the cumulative 4-year risk of laminitis was 3 [0–6]%.

Thus, the epidemiological evidence seems compelling in associating ID (giving rise to intermittent hyperinsulinaemia) and hypoadiponectinaemia with the incidence of pasture-associated laminitis. Further evidence associating hypoadiponectinaemia with a predisposition to naturally occurring pasture-associated laminitis is summarised in Table S3. Kearns et al.[89] first showed the inverse relationship between fat mass and immunoreactive adiponectin in the horse and Wooldridge et al.[90] confirmed this observation (albeit with some

obese horses having similar HMW adiponectin as the lean horses in this study) and fully validated an ELISA for HMW adiponectin in the horse. Other publications summarised in Table S3 show that the inverse relationship with adiposity only holds if that increased adiposity is associated with the development of insulin resistance; reduced plasma adiponectin concentrations have therefore been proposed as a surrogate marker of ID leading to insulin resistance.[91] Two uncontrolled studies demonstrate interventions which increase plasma high molecular weight (HMW) adiponectin also improve ID in horses and ponies.[92,93] However, the quality of the design and analysis of these studies makes the extent of the association between plasma levels of HMW adiponectin and improvement in ID difficult to determine.

Relating these findings to the models that induce laminitis (where cellular mechanisms have been studied) is difficult because most of these experiments have been done in Standardbred horses (without insulin dysregulation or hypoadiponectinaemia). The concentrations of insulin resulting from the EHC model (c1000 μiU), whilst possible to achieve in some ponies as a peak insulin concentration following a high dose of oral glucose,[94] are very unlikely to result in normal grazing behaviour. Histopathological changes associated with the EHC are evident at lower insulin concentrations induced by intravenous glucose infusion (>200 μiU/ml),[21] however, which coincides with the threshold for serum insulin (>195 μiU/ml) related to risk of laminitis in ponies challenged with a diet high in nonstructural carbohydrate.[95]

Most current hypotheses relating to the pathogenesis of EL have focused on the action of high concentrations of insulin and its ability to stimulate the IGF-1 receptor with activation of associated growth factor signalling pathways.[47] This is logical as the 60 min plasma insulin post oral sugar administration is the strongest predictor of future laminitis risk such that adding in other independent risk factors improves the risk prediction model by only a small amount. Furthermore, a direct molecular mechanism by which insulin itself could produce basal epithelial cell stress has supportive evidence, both in vitro and in vivo (see above). Consequently, little attention has been focused on how hypoadiponectinaemia would affect lamellar epithelial responses to stress associated with the different circumstances under which laminitis is known to occur. However, hypoadiponectinaemia has been repeatedly shown to be a significant risk factor for laminitis identified in horses and ponies kept at pasture. Adiponectin influences tissue sensitivity to insulin and so part of its effects on risk of laminitis are dependent on the resulting insulin dysregulation. Nevertheless, low plasma adiponectin concentrations have effects on risk of laminitis which are independent of insulin regulation so explaining additional variability in laminitis risk not explained by insulin dysregulation. In other species, adiponectin has been the focus of significant research into the adverse health consequences of metabolic syndrome and type 2 diabetes. Here the pathophysiological consequences of low circulating adiponectin (in addition to its effects on tissue insulin sensitivity) are being unravelled and the benefits of interventions which boost plasma adiponectin or directly activate its main intracellular signalling pathway (AMP-kinase) are beginning to become evident.[3]

8 | CONCLUSION

In conclusion, much progress has been made in laminitis research over the past 15–20 years. New models have been devised and new techniques applied to the study of this enigmatic equine problem. Epithelial cell stress induced by multiple factors seems to be the final common pathway leading to failure of the epidermal–dermal lamellar junction to suspend the digit within the hoof capsule and effectively support the weight of the horse. Defining those stresses and the pathways, they activate and how these combine in naturally occurring cases found could lead to ways of preventing this problem in horses and ponies that are at high risk.

AUTHOR CONTRIBUTIONS

Jonathan Elliott and Simon R. Bailey contributed equally to the production of this review.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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