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**THE PREVALENCE AND  
HISTOPATHOLOGY OF  
ENDOCRINOPATHIC LAMINITIS IN  
HORSES**

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Veterinary Medicine,  
University of Helsinki, for public examination in Walter lecture hall, EE –building,  
Agnes Sjöbergin katu 2, Helsinki, on 13th May 2016, at 12 noon.

Helsinki 2016

Published in *Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam  
Universitatis Helsinkiensis*

ISSN 2342-3161 (print)

ISSN 2342-317X (online)

ISBN 978-951-51-2122-6 (paperback)

ISBN 978-951-51-2123-3 (PDF)

Cover picture, Pedro the Pony, by Johanna Penttinen

Unigrafia  
Helsinki 2016

*To my smallest but greatest love, Hiski*

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# ABSTRACT

Laminitis is a common, debilitating condition of equids that may have substantial effects on animal welfare. Laminitis is not a disease, but a sign which may be related to endocrinopathic or inflammatory diseases, as well as uneven weight distribution. Until recently, research into the prevalence of endocrine disease amongst horses with laminitis has been overlooked, although it was anecdotally thought to be high. Most histological studies have been conducted on experimental carbohydrate overload or inflammatory models. However, more recently the histology of a hyperinsulinaemic model of laminitis was described with important differences apparent from the previous inflammatory models – however, a more detailed study had not been performed. Furthermore, the histology of chronic, naturally occurring endocrinopathic laminitis has not been described and compared to that of healthy animals.

The research presented in this thesis aimed to investigate laminitis unassociated with systemic illness or gastrointestinal disease, i.e. presenting primarily as lameness (primary laminitis). Specifically, we hypothesized that endocrine disturbances were the most common cause for primary laminitis. In addition, we aimed to provide new information on the histological appearance of experimentally induced hyperinsulinemic laminitis. Specifically, our aim was to describe the cellular changes behind the key lesion of laminitis, lamellar elongation. Lastly, the most important aim of this thesis was to describe the histology of naturally occurring endocrinopathic laminitis.

In Study I, the prevalence of endocrine disease among 36 horses that presented for laminitis at Helsinki University Equine Teaching Hospital was determined using endocrine tests and phenotypic indicators. Evidence of an endocrinopathy (either basal hyperinsulinemia or pituitary pars intermedia dysfunction, PPID) was present in 89% of cases.

In Study II, four healthy ponies were exposed to prolonged hyperinsulinemia while maintaining euglycemia - the cellular changes associated with secondary epidermal lamellae (SEL) in that experimentally induced hyperinsulinemic laminitis model were evaluated. The SEL elongation, narrowing and alteration in orientation were largely attributable to cell stretching (rather than proliferation as had been hypothesised), that occurred at the same time as an accelerated cell death-proliferation cycle.

In Study III, the histomorphometry and (macroscopic and microscopic) pathological lesions of endocrinopathic laminitis were described in 14 animals with hyperinsulinemia, with reference to age- and breed-matched controls. Lesions were largely localized abaxially within the lamellar tissue and included increased lamellar length and width, chronic abnormal keratinization, interlamellar epidermal bridging and apoptotic cell death with more acute lamellar tearing in some cases. Axially, epidermal lamellar tapering was the most frequent morphological observation. Additionally, in all the laminitis cases, there were macroscopic pathological changes (divergent rings on the outer hoof wall and/or gross separation and rotation of distal phalanx). However, both microscopic and macroscopic pathology varied in severity and

were unrelated to the reported duration of laminitis.

In Study IV, using 16 animals diagnosed with PPID, the endocrine status, histomorphometry and pathological lesions of those with laminitis (n=10) and those without (n=6) were compared. All the laminitic PPID animals also had hyperinsulinemia whereas all of the non-laminitic PPID animals were normoinsulinemic. In addition, all the animals with PPID and laminitis had histological lesions in the lamellar region, but the lamellae of PPID animals without laminitis were normal when compared to the control group.

In conclusion, endocrinopathic laminitis is by far the most common type of naturally occurring laminitis in horses and hyperinsulinemia is likely to be the primary cause in those horses, including those with PPID. In the acute phase, the pathophysiology behind the key lesion, SEL elongation, is proposed to be cellular (mechanical) compromise resulting in epidermal cellular stretching. In the chronic phase, the lamellar lesions are variable in severity in many cases allowing for a prolonged subclinical phase, and they are located predominantly abaxially close to the hoof wall.

# ACKNOWLEDGEMENTS

The research presented in this thesis was carried out at the Department of Equine and Small Animal Medicine at the Faculty of Veterinary Medicine (University of Helsinki), and University of Helsinki Veterinary Teaching Hospital during 2010–2015. Most of the work was funded by the Finnish Foundation of Veterinary Research, Finnish Veterinary Foundation, Doctoral Programme in Clinical Veterinary Medicine (DPCVM) and the Erkki Rajakoski Foundation. Several people contributed to this work and I would like to thank them all. I wish to express my special gratitude to the following people:

My friend, mentor and principal supervisor Professor Catherine McGowan to whom I am deeply grateful for guiding me through this project. Professor McGowan was a tremendous supervisor for me and taught me so much about science and equine endocrinology. Her advice on both research as well as my career has been priceless. Thank you Cathy.

My supervisor Doctor Janet Patterson-Kane for teaching me so much about histopathology. Without her supervision and constant help with histological interpretation this thesis would not have been possible.

My supervisor and director of the studies (2010–2014) Professor Emerita Riitta-Mari Tulamo, who believed in my ability to get through this project from the very beginning and gave me the opportunity to work at the Department of Equine and Small Animal Medicine. Without Professor Tulamo's positive attitude and ability to help me over the financial and technical obstacles, this thesis would have been much harder to complete.

Professor Satu Sankari for taking over the director's duties (2014–2016), for technical and administrative help as well as laboratory assistance.

My associate supervisor and co-author Doctor Ellen Singer for help in equine biomechanics and the writing process.

My co-authors Isabella Horn, Katie Asplin, Megan McNutt and Professor Dianne McFarlane for priceless help in data and sample collection.

My co-author Doctor Thomas McGowan for patience in statistical advice.

Doctor Tina Rich for advice on morphological identification and immunolabeling of apoptotic cells.

Ilpo Forsman, Kati Holmsten and all the other skillful technicians at the Department of Veterinary Pathology, Faculty of Veterinary Medicine, University of Helsinki.

Lynn Stevenson and Lynn Oxford from the Veterinary Diagnostic Services of University of Glasgow for developing the histological techniques.

All the staff at the Helsinki University Equine Teaching hospital who helped me in the sample collection and diagnostic imaging, my colleagues Johanna Penttinen and Heini Koskinen for some of the excellent photos and all the horse owners who generously donated their horses for research purposes.

All my dear friends and fellow doctoral students for their loyal support, I cannot even count how many times during this process I have gotten by 'with a little help of my friends'. I would especially like to thank my closest colleague, partner in work and friend Doctor Anna Mykkänen for just being there and unselfishly listening to my worries. In addition, a special thanks belongs to another great friend, Doctor Heli Hyytiäinen, for the daily phone discussions and priceless peer support while I was finishing this dissertation.

The official reviewers of my thesis, Professor Ken Smith and Doctor John Keen, for their valuable input, and Assistant Professor Ellen de Graaf-Roelfsema for kindly accepting to be my opponent at the public examination.

A special thanks to my family. Words cannot express how grateful I am to my mother Anne and father Jukka for all the sacrifices they have made on my behalf.

Anssi, my beloved partner, for being patient and taking care of everything while I was busy and grumpy. In addition, without your technical support in image processing as well as in linguistics this would have taken a lot longer.

Last, but not least, I would like to express my gratitude to my little Hiski, the love of my life, who has taught me what is really important.



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# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

- I           **Karikoski NP**, Horn I, McGowan TW, McGowan CM.  
Domest Anim Endocrin 2011, 41:111-117.  
The prevalence of endocrinopathic laminitis among horses presented for laminitis at a first-opinion/referral equine hospital.
  
- II           **Karikoski NP**, Patterson-Kane JC, Asplin KE, McGowan TW, McNutt M, Singer ER, McGowan CM.  
Am J Vet Res 2014, 75:161-168.  
The morphological and cellular changes in secondary epidermal laminae of horses with insulin-induced laminitis.
  
- III          **Karikoski NP**, McGowan CM, Singer ER, Asplin KE, Tulamo RM, Patterson-Kane JC.  
Vet Pathol 2015, 52:945-956.  
Pathology of natural cases of equine endocrinopathic laminitis associated with hyperinsulinemia.
  
- IV          **Karikoski NP**, Patterson-Kane JC, Singer ER, McFarlane D, McGowan CM.  
Equine Vet J 2015, Apr 13. doi: 10.1111/evj.12450, epub ahead of print.  
Lamellar pathology in horses with pituitary pars intermedia dysfunction (PPID).

Papers I-IV are included in this dissertation as reprints with the permission of their copyright holders. In addition, some unpublished material is presented.

# ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
AGE	Advanced glycation end-product
BCS	Body condition score
BM	Basement membrane
BWE	Black walnut extract
CGIT	Combined glucose-insulin tolerance test
CHO	Carbohydrate
CI	Confidence interval
COX	Cyclo-oxygenase
DP	Distal phalanx
EMS	Equine metabolic syndrome
ET-1	Endothelin-1
ETR	Endothelin receptor
HE	Hematoxylin and eosin
HPF	High-power field
IQR	Interquartile range
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloprotease
ODST	Overnight dexamethasone suppression test
PAS	Periodic acid-Schiff
PDL	Primary dermal lamella
pEHC	Prolonged euglycemic-hyperinsulinemic clamp

PEL	Primary epidermal lamella
PELL	Primary epidermal lamellar length
PELW	Primary epidermal lamellar width
PI-3 K	Phosphatidylinositol 3-kinase
POMC	Pro-opiomelanocortin
PPID	Pituitary pars intermedia dysfunction
RISQI	Reciprocal of the square root of insulin
ROS	Reactive oxygen species
RT	Room temperature
SD	Standard deviation
SDL	Secondary dermal lamella
SE	Standard error
SEL	Secondary epidermal lamella
SELA	Secondary epidermal lamellar angle
SELL	Secondary epidermal lamellar length
SELW	Secondary epidermal lamellar width

# 1 INTRODUCTION

Laminitis, defined as an “inflammation” of the hoof lamellar region, is a common debilitating condition of equids. However, while the suffix ‘itis’ refers to inflammation, it is not the primary mechanism of all types of laminitis, and, as found in this research, is not the primary lesion in endocrinopathic laminitis. Irrespective of the mechanism, in laminitis the hoof lamellar structures weaken, which may lead to mechanical failure of the suspensory apparatus of the distal phalanx (DP) and compromise the normal biomechanical function of the hoof during weight bearing. This results in foot pain with associated stance and gait alterations. Laminitis usually affects all 4 feet, although the clinical signs are often more pronounced in the fore feet. The signs vary from mild lameness to a complete inability to move. The degree of pain, poor prognosis and frequency of recurrence make this condition important from both the economic and animal welfare points of view (Hunt 1993, Pollitt 2004, Collins *et al.* 2010).

Laminitis is not a disease, but rather a clinical sign, yet for decades laminitis has been considered to be an independent disease entity. The inflammatory form of laminitis secondary to systemic infection (e.g. colitis, metritis or pleuropneumonia) was incorrectly assumed to be the most common form of laminitis because it was more commonly seen at the University hospitals where most of the laminitis studies had been conducted—and where horses with systemic inflammatory disease were more likely to be referred. Obel (1948) used both naturally occurring metritis (retained placenta) and experimental starch overload models in his original research. More recently laminitis was mostly studied using the inflammatory models of black walnut or starch/oligofructose overload (Garner 1975, Galey 1991, van Eps and Pollitt 2006). Systemic endocrine disease (insulin resistance or intolerance) was known to be clearly associated with laminitis but received relatively little attention (Coffman and Colles 1983, Jeffcott *et al.* 1986). This was most likely due to the incorrect assumption that pasture-associated laminitis was also a form of carbohydrate (CHO) overload (Longland and Byrd 2006, van Eps and Pollitt 2006, Kalck *et al.* 2009) and also the lack of appropriate epidemiological analysis of laminitis cases in general clinical populations, with studies till 2012 not involving investigation of cause (Wylie *et al.* 2012). In 2007, a new model of insulin-induced laminitis was described and this changed general thinking about laminitis remarkably. All the animals (ponies) exposed to prolonged hyperinsulinemia while maintaining euglycemia, developed laminitis in approximately 48 hours. Importantly, none of the animals became systemically ill or showed gastrointestinal disturbance during the induction of laminitis (Asplin *et al.* 2007). This hyperinsulinemic model much more closely resembled the naturally occurring clinical picture of pasture-associated laminitis, yet had been overlooked as important in the CHO overload models (van Eps and Pollitt 2006, Kalck *et al.* 2009).

In the very first studies on the histology of laminitis, the primary pathological changes were assumed to be located in the vascular tissues due to

the prominent clinical signs of bounding digital pulses and heat in the hoof (Gutenäcker 1899, Eberlein 1908, Möller 1920, Åkerblom 1934, Cohrs 1941). However, in 1948 Obel overturned this theory and showed that most of the lesions were epithelial, i.e. in the lamellae (Obel 1948). Later basement membrane (BM) pathology was claimed to be the key lesion in laminitis based on examination of specimens from three horses that developed laminitis following starch overload (inflammatory laminitis; Pollitt 1996). However, BM pathology has not been a consistent finding in all the inflammatory studies, nor has it been consistent in animals with experimental hyperinsulinemic laminitis (Morgan *et al.* 2003, Asplin *et al.* 2010, de Laat *et al.* 2011a). Elongation of the secondary epidermal lamellae (SEL) (together with increased mitotic activity and apoptosis) has been shown to be the key histological lesion of insulin induced laminitis (Asplin *et al.* 2010, de Laat *et al.* 2011a), but the induction models may not represent the naturally occurring cases very well. This is in part due to the fact that spontaneous (endocrinopathic) laminitis is seldom genuinely acute but has frequently been developing for months or even years (as compared with inflammatory laminitis which is often/always acute). However, there are few studies concerned with the histology of naturally occurring laminitis and many of them lack adequate clinical details of the sampled horses to indicate the underlying pathogenesis and, therefore, to correlate it with histological lesions (Roberts 1980, Kuwano *et al.* 2002, Kuwano *et al.* 2005, Hampson *et al.* 2012). Additionally, some studies have used horses exposed to different or several etiologies (endocrinopathic, inflammatory, mechanical) and should therefore be interpreted carefully (Carter *et al.* 2011, Engiles *et al.* 2015).

The research presented in this thesis aimed to investigate laminitis not associated with systemic illness or gastrointestinal disease, i.e. presenting primarily as lameness (primary laminitis). Specifically, we hypothesized that endocrine disturbances would be the most common cause for primary laminitis. In addition, we aimed to provide new information about the microscopic and macroscopic lesion involved in experimentally induced hyperinsulinemic laminitis. Specifically, our aim was to describe the cellular changes behind the key lesion of laminitis, lamellar elongation. Lastly, the most important aim of this thesis was to describe the histological lesions in naturally occurring endocrinopathic laminitis and to determine any relationship to lesions seen in the (acute) experimental models.



## 2 REVIEW OF THE LITERATURE

### 2.1 Laminitis

Laminitis is a common, crippling and often life threatening condition of *Equidae* which has been shown to account for up to 15% of all lameness cases in the equine population (USDA 2000). Historically laminitis has been considered to be an inflammation of the hoof lamellar region, therefore the name *laminitis*. The clinical signs include lameness involving one or multiple hooves, stiffness, weight shifting, a typical 'saw horse' stance (Figure 1) and a reluctance to move. Additional signs include increased digital arterial pulses and sensitivity to hoof tester pressure applied at the toe of the affected digit/s (Dyson 2011). Many horses with laminitis also present with gross hoof wall alterations including divergent rings (outer hoof wall horizontal lines that are wider at the heel than the toe, Figure 2), the presence of excessive cap horn (arcade-like keratin formation over the primary dermal lamellae abaxially) or a wider/separated white line, and flat or convex soles (Pollitt 2004, Collins *et al.* 2010). These gross changes have typically been considered to be associated with previous clinical episodes of laminitis or prolonged chronic laminitis (Pollitt 2004, Eustace 2010).

Diagnosis of laminitis can be made based on typical clinical signs, but diagnostic imaging (e.g. radiography or magnetic resonance imaging) is often used to confirm evidence of lamellar disruption including sinking or rotation of the DP, especially in chronic cases with structural changes (e.g. lamellar radiolucency or bone remodeling of the DP) (Figure 3; Murray *et al.* 2003, Hunt *et al.* 2010). Other techniques such as venograms have indicated disruption to the blood flow of the hoof (D'Arpe *et al.* 2010), but imaging using some of the newer emerging technologies such as 3-D and 4-D ultrasonography and imaging real-time blood flow are limited due to the hoof wall attenuating sound (Sage *et al.* 2002).

The severity of laminitis varies between animals and the phase of the disease. The severity of pain can be evaluated by using the Obel scale. **Grade 0** horses are sound. **Grade 1** horses shift weight, but lameness (stilted gait) is only noticeable at trot, whereas **Grade 2** horses are lame at walk. **Grade 3** horses move reluctantly and resist attempts to lift a hoof. **Grade 4** horses are often recumbent, but if standing, they will not move unless forced to do so (Obel 1948).



**Figure 1.** A laminitic Shetland pony with typical saw horse stance. Note the general obesity indicative of equine metabolic syndrome.



**Figure 2.** Divergent rings on the outer hoof wall of a laminitic pony.



**Figure 3.** Latero-medial radiographs of the equine distal limb. (A) A normal horse. (B) Mild distal phalanx rotation and dorsal hoof wall radiolucency (arrow) in an Estonian Coldblooded horse with equine metabolic syndrome and laminitis. (C) Severe distal phalanx rotation, bone remodelling (arrows) and hoof capsule distortion in a Shetland pony with pituitary pars intermedia dysfunction and chronic laminitis.

## 2.2 Causes of laminitis

Laminitis usually occurs in association with systemic disease or endocrine dysfunction. Less frequently it may be the result of uneven mechanical loading, for example due to lameness in the contralateral limb (support limb laminitis, SLL). Systemic diseases associated with laminitis are either endocrinopathic dysfunction or severe inflammatory conditions (e.g. colitis, metritis or pleuropneumonia). The latter are associated with severe systemic clinical signs such as alterations in demeanor, leucocyte count, pyrexia and diarrhea. Hence this type of laminitis can be termed inflammatory laminitis. Endocrinopathic dysfunction (e.g. hyperinsulinemia or pituitary pars intermedia dysfunction, PPID) is typically associated with fewer clinical signs (e.g. hypertrichosis, weight redistribution and obesity) and often presents primarily as lameness due to laminitis. This type of laminitis can be termed endocrinopathic laminitis. Inflammatory and support limb laminitis are discussed only briefly since they are not the objective of this dissertation.

### 2.2.1 Inflammatory laminitis

Historical ‘classical laminitis’, was associated with severe inflammatory conditions (inflammatory laminitis). In the time of Obel’s research (Obel 1948) inflammatory laminitis was probably the most common type of laminitis, since inflammatory conditions like post parturient metritis or colitis due to excessive amounts of grain were not as treatable as nowadays. Endocrinopathic diseases were less common since horses were lean and exercised regularly. In addition, horses did not live long enough for PPID to be a common etiological factor.

Horses with inflammatory laminitis are systemically ill (Parsons *et al.* 2007). These animals often have systemic inflammatory response syndrome (SIRS), which is manifested by two or more of the following variables: hypothermia or fever, tachycardia, tachypnea and an abnormal leukocyte count (American College of Chest Physicians/Society of Critical Care Medicine 1992). Horses with CHO - or black walnut extract (BWE) -induced inflammatory laminitis have been shown to have systemic signs of inflammation including depression, anorexia (Galey *et al.* 1991), diarrhea and fever (van Eps and Pollitt 2006). Laminitis has been shown to occur secondary to systemic inflammation and is usually acute and severe compared to animals with endocrinopathic laminitis. There is up-regulation of multiple inflammatory pathways (Noschka *et al.* 2009) and vascular dysfunction (Robertson *et al.* 2009); however, the precise pathophysiology of inflammatory laminitis has not been fully elucidated (Katz *et al.* 2012). The role of endotoxemia due to excessive amounts of carbohydrates in the hindgut, subsequent changes in bacterial population, drop in pH and degeneration of the cecal lining have been shown to contribute to the pathophysiology of inflammatory laminitis (Garner *et al.* 1978, Moore *et al.* 1979). However, lipopolysaccharides (LPS; endotoxins) did not induce laminitis when administered alone to healthy horses (Tóth *et al.* 2009). When LPS were given in conjunction with oligofructose, most of the horses developed laminitis (Tóth *et al.* 2009). Therefore, it may be suggested that endotoxins play a role in the

development of laminitis but they are not the only causative factors. In fact, insulin sensitivity decreased after oligofructose administration and, therefore, it is possible that insulin dysregulation also has a role in CHO induced laminitis (Tóth *et al.* 2009). However, in hospital settings clinical signs of endotoxemia have been shown to be the most significant risk factor in multivariate analysis for the development of laminitis (Parsons *et al.* 2007).

Inflammatory laminitis has been studied by using either CHO overload (starch or oligofructose) or the BWE models (Åkerblom 1934, Obel 1948, Garner *et al.* 1975, Minnick *et al.* 1987, Galey *et al.* 1991, Pollitt 1996, van Eps & Pollitt 2006). In these models, systemic signs, like fever and leukopenia or leukocytosis and diarrhea (only in CHO overload models) occur first and laminitis develops secondarily - on this basis, the models represent natural inflammatory cases reasonably well (Garner *et al.* 1975, Minnick *et al.* 1987, Galey *et al.* 1991, van Eps and Pollitt 2006, Loftus *et al.* 2007). Laminitis occurs in BWE models slightly earlier (8-12 h post induction) than in CHO overload models (20-44 h post induction); however, even though the majority of animals develop laminitis in these models, there are also individuals that do not (Minnick *et al.* 1987, Pollitt 1996, Morgan *et al.* 2003, van Eps and Pollitt 2006) supporting the idea that there may be multiple contributory factors in these forms of laminitis.

### **2.2.2 Support limb laminitis**

Support limb laminitis (SLL) is a relatively rare condition that occurs as a sequel of painful limb conditions including catastrophic fracture or infective bone or synovial processes. SLL occurs due to excessive unilateral weight bearing in the contralateral limb and is often a devastating condition (Baxter and Morrison 2009, van Eps *et al.* 2010, Virgin *et al.* 2011, Wylie *et al.* 2015). In a recent study the prevalence of SLL in a large equine practice in UK was 0.02% and young or middle-aged (median 6 years) Thoroughbreds were most commonly affected (Wylie *et al.* 2015). However, among horses treated with half limb, full limb or transfixation pin casts, the prevalence was 12%. Greater bodyweight and duration of casting were shown to be risk factors for SLL, but not the severity of the orthopedic condition (Virgin *et al.* 2011). In a third study only the duration of lameness, not the body weight was associated with SLL and mortality among those animals was high (Peloso *et al.* 1996).

The exact pathophysiology of SLL is unknown but according to van Eps *et al.* (2010) there are two possible theories: either SLL occurs directly due to mechanical overload or it is a sequel of hypoperfusion due to the lack of cyclic loading and unloading of the foot (van Eps *et al.* 2010). However, more research regarding the pathophysiology of SLL is warranted.

### **2.2.3 Endocrinopathic laminitis**

The major endocrinopathic disorders resulting in laminitis are equine metabolic syndrome (EMS) and/or pituitary pars intermedia dysfunction (PPID). In addition, iatrogenic glucocorticoid administration has been

associated with laminitis (McGowan 2010). Unlike inflammatory laminitis, endocrinopathic laminitis is seldom (if ever) acute but usually rather develops for months or years before the first clinical signs (Walsh 2010). In addition, inflammation is not a major factor of endocrinopathic laminitis and the animals are not systemically ill (Asplin *et al.* 2007, de Laat *et al.* 2010).

EMS is mainly a disease of young to middle-aged horses characterized by obesity, insulin resistance or dysregulation and laminitis (Frank *et al.* 2010; Frank and Tadros 2014). In addition, hyperleptinemia (Cartmill *et al.* 2003), hypertriglyceridemia (Treiber *et al.* 2006, Carter *et al.* 2009, Frank *et al.* 2006), arterial hypertension (Carter *et al.* 2009, Bailey *et al.* 2008), altered reproductive cycling in mares (Gentry *et al.* 2002, Vick *et al.* 2006) and increase in certain markers of inflammation related to obesity (Vick *et al.* 2007) may be evident in some animals. The diagnosis of EMS can be made based on phenotypic indicators (general or regional obesity, Figure 1) and disturbed insulin and glucose regulation. Insulin and glucose dysregulation can be evaluated either by measuring basal serum insulin concentration and detecting hyperinsulinemia, or using dynamic tests like the oral sugar test or combined glucose-insulin tolerance test (Frank *et al.* 2010). EMS can also affect older horses, especially those with PPID. In fact, age has been shown to be a risk factor for EMS in an epidemiological investigation of hyperinsulinemia in ponies (Morgan *et al.* 2014).

PPID is a disease of aged horses characterized by loss of dopaminergic inhibition of the pituitary *pars intermedia* and resultant overproduction of pituitary hormones: adrenocorticotropin (ACTH),  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH), corticotropin-like intermediate peptide (CLIP) and  $\beta$ -endorphin. The clinical signs include hypertrichosis (Figure 4) and abnormal shedding patterns, muscle wastage, abnormal fat distribution, laminitis, polyuria and polydipsia, increased susceptibility to infections, sweating and infertility (McFarlane 2011). PPID is diagnosed by using phenotypic indicators and diagnostic testing. Commonly used diagnostic tests are the measurement of basal plasma ACTH concentration and the overnight dexamethasone suppression test (ODST) (McFarlane 2011). Since pituitary hormones have natural circannual variation, seasonally adjusted reference ranges are used for basal plasma ACTH (Copas and Durham 2012), with increased sensitivity and specificity in late summer and autumn (McGowan *et al.* 2013). The ODST has low specificity during the autumn months and should therefore not be used from August to October (Durham *et al.* 2014). In addition to *ante mortem* tests, PPID can be diagnosed on the basis of pituitary pathology (hyperplasia, microadenomas or macroadenoma) *post mortem*, although lower grade lesions (hyperplasia and microadenomas) are not always specific for PPID (Miller *et al.* 2008).

Iatrogenic glucocorticoids have been shown to induce hyperinsulinemia and insulin resistance (French *et al.* 2000, Tiley *et al.* 2008) and therefore corticosteroid administration carries a risk of laminitis. Although iatrogenic laminitis has been shown to be relatively rare (McCluskey *et al.* 2004, Bathe 2007, Cornelisse *et al.* 2013), careful consideration should be used when glucocorticoids are administered to animals with a suspicion of an underlying endocrinopathy (obesity, long hair coat etc.), previous laminitis or current systemic inflammation (McGowan *et al.* 2016).



**Figure 4.** An aged pony with pituitary pars intermedia dysfunction in the Finnish summer (A) and winter (B). Note the long haircoat independent of season (hirsutism) and delayed or lack of shedding of the hair in summer.

### 2.3 Insulin dysregulation and laminitis

Insulin is a peptide hormone secreted by pancreatic beta cells. It is the major regulatory hormone in glucose (and fat) metabolism and the secretion is mainly regulated by the plasma glucose concentration. Compensatory hyperinsulinemia develops when there is a state of insulin resistance (the inability of cells to respond to insulin) in target cells and this can be detected basally, or as an abnormal response to dynamic glucose challenge.

The first implications of insulin resistance in the pathogenesis of laminitis were from the 1980s when glucose intolerance and reduced insulin sensitivity were noticed in ponies that had previously experienced laminitis compared to control ponies (Coffman and Colles 1983, Jeffcott *et al.* 1986). Current experimental and field studies have shown that insulin dysregulation (hyperinsulinemia and/or insulin resistance) is a key factor of EMS and endocrinopathic laminitis (Treiber *et al.* 2006, Asplin *et al.* 2007, Carter *et al.* 2009, de Laat *et al.* 2010). The horse is unique in that it can produce large amounts of insulin without hyperglycemia or pancreatic exhaustion (Reeves *et al.* 2001, McGowan *et al.* 2004) and therefore hyperinsulinemia is considered the hallmark of insulin resistance in horses. In addition to EMS related laminitis (Treiber *et al.* 2006, Frank *et al.* 2010), there is also support for the role of hyperinsulinemia in the pathogenesis of laminitis in horses with PPID. Horses with very high serum insulin concentrations at presentation ( $> 188 \mu\text{IU/ml}$ ) have been shown to be more prone to laminitis and to have a worse prognosis for survival ( $< 2$  years) than horses with normal to moderately increased serum insulin concentrations ( $< 62 \mu\text{IU/ml}$ ) (McGowan *et al.* 2004).

The pathogenesis of laminitis associated with insulin dysregulation is not fully understood. However, several theories, like impaired glucose uptake,



glucose excess or vascular and pro-inflammatory changes associated with insulin resistance have been suggested. Research regarding lamellar glucose deprivation due to decreased uptake and the following cellular death or damage has been controversial. The lamellar tissue appeared to utilize glucose exceptionally fast and when lamellar explants were incubated without glucose and with a glucose uptake inhibitor, a rapid separation of tissue layers occurred (Wattle and Pollitt 2004). However, the explant study did not provide another source of energy for the lamellae - so the rapid death of the tissue following inhibition of glucose is unsurprising. Nonetheless, this led to a prevailing theory that insulin resistance may reduce insulin mediated glucose uptake in lamellae. In another study glucose uptake in lamellar tissue was not influenced by the presence of insulin and was shown to mainly rely on insulin independent glucose transporter type 1 (GLUT1) receptors, dispelling the idea that reduction in insulin mediated glucose uptake in lamellae could be a cause of endocrinopathic laminitis (Asplin *et al.* 2011). Another theory for the lamellar damage was glucose excess, an important component in diabetic disease in humans. The accumulation of glucose in cells (glucotoxicity) promotes the formation of advanced glycation end-products (AGEs) and after binding to their receptors, AGEs increase release of reactive oxygen species (ROS) and cytokines damaging susceptible tissues like lamellae (Brownlee 2005). However, in lamellar samples acquired from horses with insulin-induced laminitis, there was no accumulation of AGEs and no increase in ROS or up-regulation of AGE receptor (RAGE) compared to controls (de Laat *et al.* 2012b). Also, naturally occurring cases of insulin resistance or dysregulation are rarely hyperglycemic (Treiber *et al.* 2006). Therefore, this theory is unlikely to explain the lamellar damage either. The activities of metalloproteases (specifically MMP-2, 9 and 14 and ADAMTS-4), that are components of the extracellular matrix capable of degrading different proteins and molecules, have been shown to increase in the developmental phases of inflammatory laminitis (Woessner 1993, Kyaw-Tanner and Pollitt 2004, Loftus *et al.* 2006, Kyaw-Tanner *et al.* 2008, Coyne *et al.* 2009). However, in horses with experimentally induced hyperinsulinemic laminitis no significant metalloprotease activity was observed in the developmental and acute stages of laminitis. The only metalloprotease that was activated at later stages was MMP-9, which is neutrophil-associated and not important in the developmental phase of laminitis (de Laat *et al.* 2011b). Therefore, it is unlikely that metalloproteases have a significant role in the development of hyperinsulinemic laminitis.

After binding to its receptor, insulin may have either metabolic effects via the phosphatidylinositol 3-kinase (PI-3K) pathway, or mitogenic and growth-related effects via the mitogen-activated protein kinase (MAPK) pathway. The metabolic effects of PI-3K pathways include, for example, the uptake and disposal of glucose and lipids and nitric oxide mediated vasodilatation. However, in a state of insulin resistance and hyperinsulinemia, the MAPK pathways dominate and convey mitogenic signals including endothelin-1 (ET-1) mediated vasoconstriction, vascular adhesion molecules and proliferation of vascular smooth muscle cells leading to endothelial dysfunction (Cusi *et al.* 2000, Kim *et al.* 2006). This dominance of signaling via the MAPK pathway has been shown to be central to insulin resistance and subsequent metabolic dysfunction including dyslipidemia, vasculopathy and coagulopathy in murine



models and human patients (Kim *et al.* 2006). The vascular and pro-inflammatory effects of insulin have been shown to be important components of human metabolic disorders like cardiovascular disease and are likely to contribute also to the development of endocrinopathic laminitis. Insulin resistance is accompanied by endothelial dysfunction and vasoconstriction in many metabolic diseases and obesity in humans (Kim *et al.* 2006). Interestingly, insulin has been shown also to affect equine blood vessel tone *ex vivo* (Venugopal *et al.* 2011, Keen *et al.* 2012, Wooldridge *et al.* 2014). In one study, vasoconstriction was noticed in insulin resistant arterial and venous rings from the palmar digital vessels in response to insulin. In addition, in explants where MAPK was blocked, the vasoconstriction was not detected, which suggests that it is MAPK dependent (Venugopal *et al.* 2011). In another study, insulin pre-incubation decreased phenylephrine and ET-1-mediated vasoconstriction (Keen *et al.* 2012) and in a third study, the pre-incubation resulted in the reduced relaxation of arteries following contraction with phenylephrine (Wooldridge *et al.* 2014). Therefore, it has been suggested, that the vascular dysfunction has role in development of hyperinsulinemic laminitis; however, the exact mechanism warrants further research.

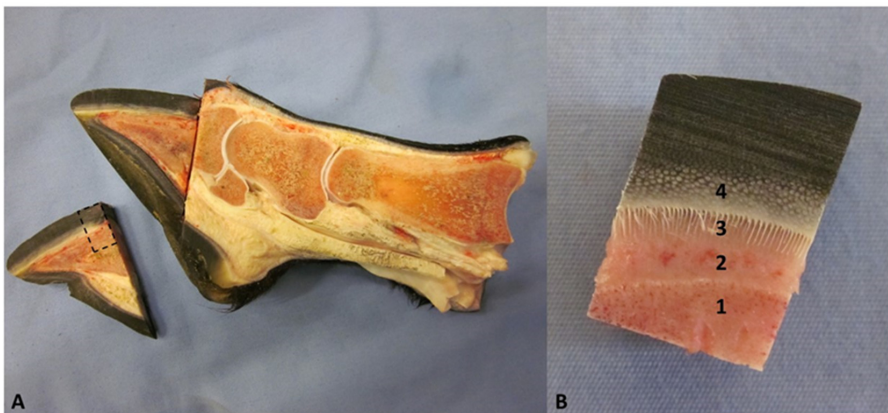
## 2.4 The relevant anatomy and histology of the hoof

The equine foot consists of a horny hoof capsule and the enclosed structures that include: the DP, the distal part of the middle phalanx, the navicular (distal sesamoid) bone, 11 ligaments and 2 tendons (common digital extensor tendon and deep digital flexor tendon) (Stump 1967). The hoof capsule is epidermal in origin and includes the hoof wall, sole, frog and heel bulbs (Figure 5). The hoof wall originates and grows down 0.143-0.48 mm per day (Reilly *et al.* 1998) from the germinal epithelium of the coronary corium. It consists of three layers: *the stratum externum*, *the stratum medium* and *the stratum internum/lamellatum* (Figure 6). The *stratum externum* is the thin outermost layer whereas the *stratum medium* is the thickest of the three layers, characterized by tubular (oriented proximo-distally) and intertubular (between the tubules) horn. The intertubular horn forms at right angles to tubules, which makes the hoof wall an extremely tough structure (Pollitt 2004). The tubule density is greatest at the abaxial edge of the wall and decreases towards the axial side, which smoothes energy transfer to the distal phalanx (Lancaster *et al.* 2013).

The lamellar region or *stratum internum/lamellatum* is the innermost layer of the hoof wall that bridges the distal phalanx and hoof capsule (Pollitt 2010). This dermo-epidermal arrangement provides a large surface area of attachment (average 0.8 m, Daradka 2000) between the bony column (via DP) and the hoof capsule, therefore enabling the DP to remain suspended within the hoof capsule by counteracting the downward forces of the animal's weight (Davies *et al.* 2007). Disruption of the mechanical stability of the lamellar region occurs at both microscopic and macroscopic levels in cases of laminitis, which may lead to pain and disruption of the normal anatomical arrangement.



**Figure 5.** A sagittal section of the distal limb of a horse showing the structures either fully or partly consisting the foot: distal phalanx (1), middle phalanx (2), navicular bone (3), hoof wall (4), lamellar region (5), deep digital flexor tendon (6), common digital extensor tendon (7), sole (8) and heel (9).



**Figure 6.** A sagittal section of an equine hoof removed from a sagittally bisected equine distal limb (A). A transverse section of the dorsal hoof which has been cut from the sagittal section of figure A (dashed line) and turned 90°, showing the distal phalanx (1), sublamellar dermis (2), lamellar region (*stratum internum*, 3) and *stratum medium* (4) (B). The thin outermost layer (*stratum externum*) cannot be distinguished in this image.

Lamellar tissue consists of primary and secondary in-foldings, called lamellae. In the normal hoof there are 550-600 primary lamellae each with 150-200 secondary lamellae, which are orientated towards the distal phalanx (Figure 7; Pollitt 2010). Lamellae have been traditionally defined as interleaved 'epidermal lamellae' (with keratinized centers) extending in from the hoof capsule and 'dermal lamellae' arising from the dermis that is attached to the DP by dense type I collagen fibers (Pollitt 2010, Engiles 2010). However, the epidermal component is actually modified and further specialized epidermal tissue, similar to skin with rete pegs. As such 'epidermal lamellae', as previously defined, are embryologically impossible (Bragulla 2003). For the purposes of this dissertation the term 'epidermal lamellae' refers to the primary or secondary lamellar structure of an epidermis overlying the dermis that it developed with. The embryological development of lamellae starts at around 4 weeks gestational age when a single layer of ectodermal cells covers the mesenchyme. The epidermis derives from these ectodermal cells and the dermis from the subjacent mesenchyme. At a gestational age of 65-70 days the epidermal cells start to invade the dermis to form microridges, and some of these enlarge to become primary (PDL) and secondary dermal lamellae (SDL). The epidermal cells proliferate and form primary (PEL) and secondary epidermal lamellae (SEL) (Bragulla 2003). Before birth, the lamellae have a homogenous appearance and symmetric distribution around the hoof. After birth, however, the lamellar region undergoes rapid changes including PEL branching, with a greater density at the toe than the quarters (Bidwell and Bowker 2006). These changes are likely a response to mechanical stress (weight bearing) and indicate that lamellae architecture can change physiologically as well as pathologically.



**Figure 7.** A histological (hematoxylin and eosin) section of normal lamellar region showing stratum medium (1), standard type of primary epidermal lamella (2), curved type of primary epidermal lamella (3), primary dermal lamella (4) and sublamellar dermis (5).

The epidermal covering of the lamellae merges with the keratin of the stratum medium, which is a product of coronary germinative epidermal cells. On the base of the epithelial cells, the epidermis attaches to the dermis via a basement membrane, which is produced by both epidermal and mesenchymal (dermal) cells (Marinkovich *et al.* 1993). The BM is composed mostly of type IV collagen, laminin, heparin sulfate proteoglycan and nidogen – the basal epidermal cells, as in normal skin, are connected to the BM by hemidesmosomes. The BM has a critical role in anchoring the epidermis and dermis together, but it also participates in other biological processes between these two tissues. Therefore, damage to the BM has been thought to have a key and possibly primary role in the pathogenesis of laminitis (Pollitt 1996). However, the BM is also reliant on the health of the epidermal and dermal cells and can be affected secondarily by cell damage (Marinkovich *et al.* 1993).

The normal morphology of the lamellar region is variable, in terms of lamellar length, width, orientation and type. Possible factors affecting the lamellar morphology may include age, weight and the mechanical environment (Kawasako *et al.* 2009, pers. observation). However, the descriptions of normal lamellar histology are few and therefore it can be challenging for the pathologist or researcher to distinguish true pathological changes from normal morphological variation. Recently, a classification system for the morphology of primary and secondary epidermal lamellae in ‘normal’ horses was described. Lamellar samples from 35 Thoroughbred cadavers showed great histological diversity, for example predominance of the so-called hyperplastic SEL type in axial regions, were detected apparently independently of clinical signs of laminitis. It was suggested that diagnosing or grading the severity of laminitis based on histological evaluation only would not be advisable. However, some of the hooves studied were from horses euthanized due to systemic inflammatory disease (e.g. colic, pleuropneumonia and placental retention). Subclinical or early lesions of inflammatory laminitis may have been present in these horses and the results should be interpreted carefully (Kawasako *et al.* 2009).

## **2.5 Histopathology of experimentally induced hyperinsulinemic laminitis**

The first clear evidence of the link between hyperinsulinemia and laminitis was provided in a study in which laminitis was induced in 5/5 previously healthy, young and lean ponies by exposing them to prolonged hyperinsulinemia (> 1000  $\mu$ IU/ml) while maintaining euglycemia (5 mmol/L); this was done using a modified (prolonged) euglycemic-hyperinsulinemic clamp technique (pEHC, Asplin *et al.* 2007). Subsequently, this experimental model was repeated in Standardbred horses with similar results. All of the exposed animals developed laminitis within 48 hours (de Laat *et al.* 2010). In these two studies, animals were euthanized and the lamellar samples were collected at the onset of Obel grade 2 laminitis that occurred at a mean of 46h in horses and at 55h in ponies. Horses developed laminitis more rapidly than some ponies, possibly due to their larger body weight, but there was greater temporal variability in ponies, with Obel grade 2

laminitis developing over a range of 37-61 hours. Since the magnitude of hyperinsulinemia is very high during pEHC and laminitis develops in approximately 48h, the pEHC model represents an exaggerated form of the naturally occurring disease. Therefore, in a later study much lower insulin concentrations (~ 200  $\mu$ IU/ml) were induced in four insulin sensitive Standardbred horses by administering continuous intravenous infusion of glucose for 48h. None of the animals developed clinical laminitis but all of them had histological evidence of lamellar disease in at least one foot (de Laat *et al.* 2012a).

The lamellar histology of both ponies and horses after 46-55 hours of hyperinsulinemia demonstrated some clear differences as well as some histological similarities to previously reported non-endocrinopathic (inflammatory) forms of the disease. Both models - hyperinsulinemic and inflammatory - had similar early histological changes. There was a loss of the normal perpendicular orientation of SEL nuclei relative to their basement membranes, with the nuclei becoming rounded, more central within the cytoplasm (rather than apical) more randomly oriented, and containing prominent nucleoli (Obel 1948, Pollitt 1996, Asplin *et al.* 2010, de Laat *et al.* 2011a, de Laat *et al.* 2012a). At the same time the SEL started to elongate, become narrower, develop tapered (rather than club-shaped or rounded) tips, and become more acutely angled to the PEL axis, with irregularity of the PEL/SEL interface. The PEL and SEL become more closely apposed and were often difficult to distinguish from each other.

In some inflammatory models (CHO overload) of laminitis, severe BM damage has been described as the predominant and perhaps primary feature of laminitis (Pollitt 1996, Nourian *et al.* 2007). Diagrams depicting a 'degloving' type of disruption were based on observations of separation of lamellar explants under certain conditions (MMP-activator aminophenylmercuric acetate; Pollitt 2004). Interestingly, BM damage has not been found in all CHO overload models, with one group noting that the BM remained intact and attached during the acute phase of laminitis (Morgan *et al.* 2003). Also in hyperinsulinemic (pEHC) models, BM damage was found to be minimal and predominantly localized to the most axial SEL only (and not in all specimens/animals) (Asplin *et al.* 2010, de Laat *et al.* 2011a, de Laat *et al.* 2012a).

## **2.6 Histopathology of naturally occurring laminitis**

Naturally occurring endocrinopathic laminitis is a chronic process and, therefore, the induction models do not represent it very well. The lamellar lesions in insulin induction models are always acute whereas in animals with naturally occurring disease the lesions may have been developing for months or even years. However, the hyperglycemic model in which moderate endogenous hyperinsulinemia is induced by administering only glucose, may be used to mimic the preclinical phase of naturally occurring hyperinsulinemic laminitis. In one study, none of the treated horses became lame 48 h after the glucose infusion; however, they all had histological evidence of lamellar disease in at least one foot, including elongation and narrowing of the SELs as

well as increased evidence of mitosis and apoptosis (de Laat *et al.* 2012a). This suggests subclinical lamellar disease, likely to not be noticed by owners or veterinarians, which, if it appears recurrently, could ultimately lead to laminitis.

Histological descriptions of naturally occurring laminitis are few and many of them lack adequate clinical details of the sampled animals, including the associated systemic disease condition (Roberts 1980, Kuwano *et al.* 2002, Kuwano *et al.* 2005, Hampson *et al.* 2012). Additionally, some studies have used horses exposed to different or several etiologies and should therefore be interpreted carefully (Carter *et al.* 2011, Engiles *et al.* 2015). Engiles *et al.* (2015) presented histological findings in a study aiming to correlate osteology and imaging in chronically laminitic hooves of different etiologies. However, the cases included laminitis cases with exposure to one or more risk factors confirmed at necropsy: endocrine (obesity, PPID), inflammatory (e.g. visceral inflammatory disease) or supportive limb overload. In addition, they included animals in the 'normal' control group of which 3/5 had also risk factors (obesity, colitis and supporting limb overload) - those animals may therefore have had subclinical lamellar disease. Histologically, SEL elongation, thinning and distortion with duplication/splitting of the BM, accumulation of suprabasilar cells and distortion of the keratinized axes were noticed in animals with mild acute or subclinical laminitis. In animals with severe chronic laminitis the lesions included epidermal lamellar necrosis with BM separation and dysplastic regeneration, including acanthosis and hyperkeratosis, corresponding to a lamellar 'wedge' (Engiles *et al.* 2015). In an earlier study Carter *et al.* (2011) used a hoof lamellar tissue bank in horses with very limited clinical histories in a study investigating the transcription factor p63, as a potential determinant of the proliferative capacity of hoof lamellar epidermal cells in laminitis. They also included cases with different or several etiologies. Additionally, they had 2/5 horses in their control group with possible PPID (pituitary adenoma) - therefore the mild axial PEL and SEL distortion noticed in these animals may have been a result of subclinical lamellar disease/preclinical laminitis. However, the animals with clinical laminitis had more severe elongation and distortion of PELs and SELs. Additionally, the histological findings included lamellar epidermal hyperplasia and acanthosis with hyperkeratosis, abaxial displacement of the keratinized axis with frequent merger of adjacent abaxial lamellae and BM pathology (separation, splitting, duplication and epidermal island formation) (Carter *et al.* 2011). Neither of the studies performed endocrine screening on all the horses and both included multiple etiologies. Therefore, as far as the author is aware, there are no previous studies on the histopathology of clearly defined naturally occurring endocrinopathic laminitis. In addition, there are no studies where the histology has been compared with clinically healthy control animals with no risk factors for laminitis/lamellar alteration.

### 3 AIMS OF THE STUDY

The aims of this thesis were

- i. To determine whether an underlying endocrinopathy (hyperinsulinemia or PPID) was present in laminitic horses presented to a first-opinion/referral veterinary teaching hospital and to compare the signalment of laminitic horses to equine hospital population during the same period.
- ii. To determine cellular changes associated with secondary epidermal lamellar (SEL) lengthening in forefeet and hind feet of experimental ponies with insulin-induced laminitis.
- iii. To describe the histomorphometry and pathological lesions in hoof lamellar tissue of horses and ponies with naturally occurring endocrinopathic laminitis, with reference to age- and breed-matched controls.
- iv. To describe the histomorphometry and pathological lesions in hoof lamellar tissue of animals that had PPID with or without concurrent laminitis, with reference to age-matched controls

## 4 MATERIALS AND METHODS

The study protocols were approved by the following authorities: The Animal Ethics Committee of the University of Queensland, Australia (Study II) and the Research Ethics Committee of the University of Helsinki, Finland with individual institutional ethical approval (University of Liverpool and University of Oklahoma) where applicable (Studies III and IV).

For more detailed information on the materials and methods, see Studies I-IV.

### 4.1 Horses

#### Study I

A total of 410 horses (229 males and 181 females) were recruited for the study between April 2007 and August 2008, representing 37 horses presented with current or historical laminitis and 373 nonlaminitic, hospitalized horses (control group) at the Helsinki University Equine Teaching Hospital, Finland.

#### Study II

Eight clinically healthy ponies (six geldings, one colt, one mare), aged  $6.3 \pm 1.7$  years old ( $244.1 \pm 32$  kg) (mean  $\pm$  s.e.), with no known history of laminitis, and no evidence of previous laminitis on inspection of the hooves were included in the study. Four of the ponies were randomly allocated to a treatment group and 4 to a control group.

#### Study III

Altogether 39 animals that had presented to the Helsinki University Equine Teaching Hospital, Finland or the Philip Leverhulme Equine Hospital, University of Liverpool, UK for euthanasia or were humanely killed at local stables or in abattoirs, were included in the study between March 2010 and September 2013. In addition, four archived samples from a previous study (control ponies) were used (Asplin *et al.* 2010). Animals were divided into 5 groups: young (<12 yrs., n=11) and old ( $\geq 12$  yrs., n=5) control ponies, young (<12 yrs., n=4) and old ( $\geq 12$  yrs., n=5) control horses and animals with endocrinopathic laminitis (n=14; 4 horses, 10 ponies). All animals under one year old and/or current evidence or a known history of systemic inflammatory disease were excluded.

#### Study IV

Twenty-six animals (10 ponies and 16 horses) that had presented to the Helsinki University Equine Teaching Hospital, Finland or the Philip Leverhulme Equine Hospital, University of Liverpool, UK for euthanasia, were



humanely killed at local stables or in abattoirs or belonged to a PPID research herd (Oklahoma State University, USA) were included in the study between March 2010 and September 2013. In addition, one archived sample from a previous study (control pony) was used (Asplin *et al.* 2010). Of the study population, 10 animals (5 ponies and 5 horses) were clinically healthy and had no evidence of endocrinopathic disease or laminitis. The remaining 16 animals (5 ponies and 11 horses) had PPID and of these, 6 had also laminitis (4 ponies, 2 horses). Only animals that were older than 12 years and had no evidence or history of systemic inflammatory disease were included.

## **4.2 Diagnosis of laminitis**

Laminitis was diagnosed by an equine veterinarian/s in all 4 studies. The diagnosis was made based on physical examination identifying lameness in both front hooves or all 4 hooves associated with weight shifting, a typical saw horse stance and/or reluctance to move. Supportive signs included increased digital arterial pulses, sensitivity to hoof tester pressure applied at the toe of the affected foot/feet and macroscopic hoof alterations in chronic cases (divergent rings in the outer hoof wall, flat or convex soles, widened white line). Radiographs were taken, when indicated, to confirm evidence of lamellar disruption including DP sinking and/or rotation.

## **4.3 Induction of laminitis with prolonged euglycemic-hyperinsulinemic clamp (Study II)**

In Study II, archived samples from a previous experiment were used (Asplin *et al.* 2007). In this experiment laminitis was induced in 4 ponies using the prolonged euglycemic-hyperinsulinemic clamp (pEHC) technique. These ponies received recombinant insulin (Humulin R™, Eli-Lilly Australia, West Ryde, New South Wales, Australia) and glucose IV while control ponies (n=4) received an equivalent volume of saline solution (0.9% NaCl) IV. For treatment group ponies, one jugular venous catheter was used for the simultaneous administration of insulin at a fixed rate and glucose at a variable rate to maintain euglycemia (blood glucose concentration, 5 mmol/L). A catheter in the other jugular vein was used to collect blood samples for monitoring of blood glucose and insulin concentrations. After collection of baseline blood samples, insulin (45 mU/kg in 50 mL of saline solution as a bolus followed by 6 mU/min/kg for the duration of the experiment) was administered IV. Intravenous administration of 50% glucose solution (Baxter Healthcare Pty Ltd, New South Wales, Australia) was initiated at a mean  $\pm$  SE rate of  $24.4 \pm 3.0$  mmol/min/kg. The glucose administration rate was adjusted when blood glucose concentrations differed from the target concentration (5 mmol/L) by  $> 1$  mmol/L. Control ponies received saline solution (14.7 mL/min/kg for 72 hours, which was the mean rate of fluid administration for treatment group ponies during the same period). Infusions were continued until Obel grade 2 (Obel 1948) laminitis occurred or for a maximum of 72 hours.

## **4.4 Collection of lamellar and blood samples**

### **4.4.1 Lamellar samples (Studies II-IV)**

For each horse/pony, the distal aspect of all 4 limbs (Study II) or right forelimb (Studies III-IV) was disarticulated at the metacarpophalangeal or metatarsophalangeal joint within 1 h of death and sectioned with a band saw, as soon as possible. In Study II, the hooves were sectioned as previously described (Pollitt 1996). Briefly, the sole on the base plate of the band saw, the first cut was made medial to lateral just palmar to the coronary band. Then a 15 mm sagittal section was obtained from the dorsal midline by cutting on either side of the frog and after that, most of the hoof wall and DP were removed. Then a 15 mm wide sample was cut from the mid-wall region; a scalpel blade was used to cut the sublamellar dermis from the distal phalanx. Finally, the sample was dissected to obtain 5 mm square explants, extending from the inner hoof wall to the dermal connective tissue. All samples were fixed in 10% neutral buffered formalin for 24h, processed by routine methods, embedded in paraffin wax and sectioned at 5 µm. In Studies III and IV, Pollitt's method was slightly modified. First, the foot was bisected sagittally. Then, a 1 cm thick parasagittal section was cut from one half of the hoof; this specimen included the hoof wall, lamellar tissue and most of the DP. The midpoint between the coronary band and the ground-bearing surface was identified on the dorsal aspect of this section. A square or rectangular segment including the dorsal cortex of the DP was excised, and then a 0.5 mm thick transverse section cut from its lower face (at the previously described mid-point). From that transverse section (in which lamellar detail should be seen), the dense hoof wall (often pigmented) was removed to within approximately 1 mm of the abaxial aspect of the lamellar tissue using a single edge razor blade (scalpel blades are not sufficient for this step). The phalangeal bone was then removed, by running a scalpel blade along its dorsal edge. Each specimen was divided into 5 mm wide (square) blocks that were fixed in 4% paraformaldehyde for 24-72 h before processing by routine methods and embedding in paraffin wax. These blocks were oriented with the longitudinal axes of the PEL parallel to the cutting edge; if oriented perpendicularly (i.e. incorrectly) the remaining horn at the abaxial aspect of the lamellar tissue was likely to fold over during section cutting.

### **4.4.2 Blood samples (Studies I-IV)**

Blood samples were collected from the jugular vein for the measurement of insulin, ACTH, cortisol and glucose concentration. In Studies I, III and IV blood samples for basal serum insulin concentration were collected in serum sample tubes following at least 2 hours of fasting (Vacuette®, Greiner bio-one, 2 serum clot activator). In Study II the ponies had access to lucerne hay during the blood collection for basal insulin. After the collection, the samples were centrifuged and the serum was separated within 3 hours. In studies I, III and IV serum samples were frozen to -20°C and sent by courier to the laboratory

by next day delivery; however, in Study II the samples were stored at  $-80^{\circ}\text{C}$  until analyzed.

Blood samples for ACTH concentration were collected in 10 mL plastic EDTA tubes (Vacuette®, Greiner bio-one, EDTA K3). Plasma was separated and frozen in plastic tubes within 1 hour and sent frozen by courier to the laboratory. The samples were taken, when possible, in the morning when the stable environment was quiet and the horse was calm. Samples were not taken during the acute phase of pain or from abattoir horses, to reduce the possibility of stress-related elevation in plasma ACTH concentration.

Blood samples for cortisol measurements for the dexamethasone suppression test were collected in 6-mL serum sample tubes (Vacuette®, Greiner Bio-One, 2 serum clot activator). After collection, the serum was separated and sent by mail to the laboratory.

Blood samples for basal glucose measurement in Studies III and IV were collected in 2 mL plastic lithium heparin tubes following at least 4 hours of fasting (Vacuette®, Greiner bio-one). In Study II glucose concentrations were measured in 1 mL blood samples.

## **4.5 Analysis of blood samples**

In Study I insulin analysis was performed with the DiaSorin S insulin radioimmunoassay (RIA) validated for use in horses (Cambridge Specialist Laboratories, UK). In Study II the analysis was performed with another RIA kit validated for use in horses (Diagnostic Systems Laboratory; Charles Sturt University, Wagga Wagga, Australia). In Studies III and IV insulin analysis was performed using a chemiluminescent immunoassay (Immulite®, Liphook Equine Hospital, UK or Laboratory of Comparative Aging Research, Center for Veterinary Health Sciences, Oklahoma State University, Oklahoma, USA).

Quantitative plasma ACTH analysis was performed in Study I by a commercial two-site, solid-phase immunoradiometric assay (Mitsubishi, Japan) and in Studies II-IV by using a chemiluminescent immunoassay (Immulite®). In Study I, the analyses were performed at Cambridge Specialist Laboratories, UK; in Study II at VETPATH laboratory services, Ascot, Western Australia; in Study III at the Liphook Equine Hospital, UK; and in Study IV either at the Liphook Equine Hospital, UK or at the Laboratory of Comparative Aging Research, Center for Veterinary Health Sciences, Oklahoma State University, Oklahoma, USA.

In Study I, cortisol analysis (dexamethasone suppression test) was performed using a chemiluminescent immunoassay (Siemens Immulite 2000; Vetlab, Tampere, Finland).

In Studies II-IV glucose concentrations were measured using a portable glucometer. In Study II an Advantage (Roche Diagnostics) glucometer was used and in Studies III and IV either Ascensia Contour® (Bayer HealthCare, Germany) or AlphaTRAK® (Abbott Laboratories, Abbott Park, Illinois, USA) glucometers.

## 4.6 Diagnosis of hyperinsulinemia and PPID

Basal fasting ( $\geq 2$  hours) hyperinsulinemia indicative of insulin resistance was diagnosed in Studies I, III and IV in animals having basal serum insulin values  $> 30 \mu\text{IU/ml}$  (Study I) or  $> 20 \mu\text{IU/ml}$  (Studies III and IV). In addition, in Study I the reciprocal of the square root of insulin (RISQI,  $[\text{basal insulin } \mu\text{IU/ml}]^{-0.5}$ ) was calculated as a proxy measurement of insulin sensitivity and values of  $\leq 0.18$  were considered indicative of insulin resistance (lowered insulin sensitivity).

PPID was diagnosed on the basis of clinical signs and a diagnostic test (Studies I, III and most cases in Study IV) or on the basis of clinical signs and pituitary pathology (hyperplasia, microadenomas or macroadenoma; Miller *et al.* 2008) (2 animals from Oklahoma subgroup of Study IV). Clinical signs included hypertrichosis, muscle wastage, hyperhidrosis, polyuria/polydipsia and abnormal fat distribution. Diagnostic tests included basal plasma ACTH measurement and/or the dexamethasone suppression test. A plasma ACTH value of  $> 300 \text{ pg/mL}$  (immunoradiometric assay; Study I) or  $> 29 \text{ ng/L}$  from November to July and  $> 47 \text{ ng/L}$  from August to October (chemiluminescent immunoassay; Studies III and IV) were considered positive for PPID. The dexamethasone test was considered positive for PPID if there was a failure to suppress cortisol to values  $< 27.6 \text{ nmol/L}$  in 20 to 24 h after dexamethasone administration (Dybdal *et al.* 1994). In Study I diagnostic tests for PPID were not performed during the autumn months (August to October) to avoid seasonal variation in blood hormone concentrations.

## 4.7 Evaluation of obesity

The body condition was scored in all 4 studies using the Carroll and Huntington 0 to 5 body condition score (BCS; Carroll and Huntington 1988). In Study I, laminitic horses were considered overweight if their BCS was  $> 3$  and nonoverweight if their BCS was  $\leq 3$ . A cresty neck score of  $\geq 3/5$  (Carter *et al.* 2009) and the presence of bulging supraorbital fat were also considered indicative of obesity and EMS.

## 4.8 Histomorphometry and categorization of lamellae

Lamellar histomorphometry was described in Studies II-IV. First, histological sections were stained using hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) methods and then examined using a light microscope. The HE sections were used for detection of lamellar lesions, and the PAS sections for morphometry. Digital images were taken using image capture software (Cell<sup>^</sup>D version 2.8, Olympus Soft Imaging Solutions, Münster, Germany) and a microscope-attached digital camera (Olympus DP71, Olympus Imaging America Inc., Center Valley, Pennsylvania, USA). For PEL measurements, images were taken at 40x magnification and then merged (to image the complete PEL) (Adobe Photoshop CS5 version 12.0 x64, Adobe Systems Incorporated, San Jose, California, USA). For SEL typing, images were taken

at 200x magnification from abaxial (adjacent to keratinized hoof wall), middle, and axial (adjacent to DP) regions of 10 randomly selected PEL (<http://www.randomizer.org/form.html>). These regions were defined as 10%, 50% and 90% of the length of the PEL, respectively.

All of the measurements were made using a drawing tool (Wacom Intuos4 or Bamboo, Wacom Europe, Krefeld, Germany) and image analysis software (ImageJ version 1.4.3, National Institutes of Health, Bethesda MD, USA. Available at: [rsbweb.nih.gov/ij/index.html](http://rsbweb.nih.gov/ij/index.html)) from 10 randomly selected PEL. PEL length (PELL) was measured by drawing a continuous line from the axial tip of the PEL to the level of the base. The width of a PEL (PELW) was measured in Study II at a location 50% along the length of the measurement line for that lamina; this measurement was determined from the tips of the SEL on one side to the tips of the SEL on the other side. In Study IV PELW was measured from the bases of the SELs on one side to the bases of the the SELs on the other.

The SEL length, width, and angle were measured in Study 2 for 10 randomly selected SELs (5 from the left side and 5 from the right side of the PEL) at 200X magnification from each of the 3 aforementioned regions of randomly selected PEL. The lengths of the SELs were measured from the tip to the base of such lamina at the junction with the keratinized axis of the associated PEL. The widths of the SELs were measured from basement membrane to basement membrane at the longitudinal midpoint of each SEL (identified along a straight line). The angle between the axes of the PELs and those of SELs was measured with the angle tool in the image analysis software.

In Study 2, the number of epidermal cell nuclei per SEL was counted from an SEL base to the tip for 10 randomly selected SELs from each of the 3 regions of a randomly selected PEL (as for measurement of the other SEL variables). The number of epidermal cell nuclei per micrometer of an SEL was calculated by dividing the number of cell nuclei by the SEL length (for an individual SEL). Percentage differences between the control and treatment group ponies were calculated for each variable (PEL length and width; SEL length, width, and angle; cell number per SEL; and cell number per micrometer of an SEL).

In Studies III and IV, the PEL type was defined as standard (straight) or curved (with 2 or more bends), and bifurcations were reported when present (Figure 7). The axial region (tip) of PEL was defined as standard, tapered, sharp or bifurcated; and the abaxial region (base) as standard, sharp, bifurcated, 'proliferative' (epidermal cells bridging dermal tissue), separated (i.e. epidermal and dermal lamellae had torn apart), or keratinized (dyskeratosis of the bridging epidermal cells). The SEL types were defined as standard, tapered, club-shaped, suprabasal layer hyperplasia, bifurcated, fused, separated or keratinized. The degree of abaxial dyskeratosis (increased keratinization), i.e. in the 'cap horn' region was graded as normal, slightly (1-10 extra layers), moderately (10-30 layers), or markedly increased (>30 layers). The degree of epidermal tissue bridging a PDL at its abaxial aspect (i.e. joining the adjacent PEL) was defined as none, mild (1-2 epithelial bridges), moderate (> 2 epithelial bridges) or extensive (abaxial region completely filled with epidermal cells). Lakes within the cap horn or bridging abaxial epidermal tissue were defined as small to large cavities that were either empty (most likely due to processing artifact) or filled with homogeneous eosinophilic (proteinaceous) material. Epithelial islands were defined as isolated round or

ovoid structures adjacent to a PEL; their presence in the different regions was noted (abaxial, middle, axial).

## 4.9 Quantification of apoptotic cells

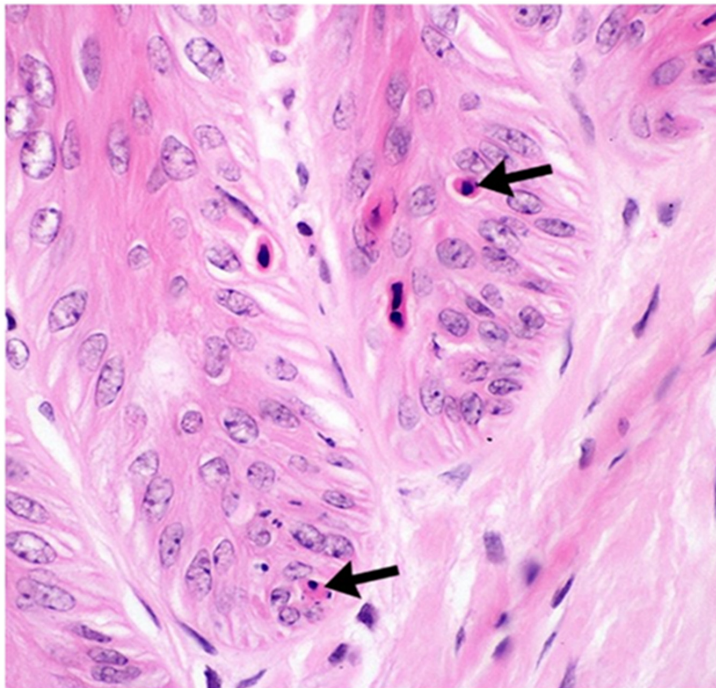
In Studies II-IV, apoptosis of SEL cells was confirmed on the basis of morphological criteria, including cytoplasmic rounding, nuclear shrinkage, chromatin condensation and fragmentation, and formation of apoptotic bodies (Meyers *et al.* 2007, Figure 8). Some of the apoptotic bodies had been phagocytosed by neighboring epithelial cells. In Study II, the numbers of apoptotic cells per 200X field were counted for each of the 3 regions (axial, middle, and abaxial) of randomly selected PEL (n = 10/hoof) in HE-stained sections. In Studies III and IV, the numbers of apoptotic cells were visually evaluated for each PEL region in HE sections and apoptotic cell death was further confirmed by immunolabeling for gamma-H2AX, a marker of double-strand DNA breaks. First, the sections were deparaffinized (Histo-Clear, National Diagnostics, Hull, UK) and rehydrated using graded alcohols. Antigen retrieval was required for anti-gamma-H2AX immunolabeling; this was performed by placing slides in sodium citrate buffer (pH 6.0) for 1 minute and 40 seconds using a pressure cooker (Antigen Access Unit, A. Menarini Diagnostics Ltd., Wokingham, UK). All the slides were then loaded into a Dako-Autostainer (Dako UK Ltd., Ely, UK). Endogenous peroxidase and nonspecific staining were blocked using 3% hydrogen peroxide (in PBS) for 5 minutes. All sections were incubated with a primary mouse monoclonal antibody against gammaH2AX (clone JBW301 (1:500); Millipore, Watford, UK) for 30 minutes at room temperature (RT). An anti-mouse secondary antibody conjugated to an HRP-labeled polymer ((K4003) Dako Envision™ + System-HRP, Dako UK Ltd.) was then applied for 30 minutes at RT. Visualization was achieved using 3,3'-diaminobenzidine (DAB+ Substrate Chromagen system, Dako UK Ltd.), 20 µl/ml for 10 minutes. Tissues were counterstained using Gill's hematoxylin (made in-house) for 27 seconds, then dehydrated, cleared, and mounted (DPX mounting medium, Cellpath, Newtown, Powys, UK).

## 4.10 Quantification of mitotic cells

In Study II, immunolocalization of proliferating cells was performed with a mouse monoclonal antibody (Ki-S2) to the TPX2 protein (TPX2/ab32795, Abcam, Cambridge, United Kingdom); TPX2 is a nuclear protein (repp86/p100) associated with cell proliferation that is specific for the S, G2, and M phases of the cell cycle. Unlike the more commonly used immunohistochemical proliferation markers Ki-67 and topoisomerase-II $\alpha$  antigens, TPX2 does not label cells in the G1 phase of the cell cycle, thereby avoiding inclusion of cells in damage-induced G1 arrest (Rudolph *et al.* 1998). In addition, because TPX2 is required for mitotic spindle formation, the antibody to that protein can label dividing cells that do not contain mitotic figures, which makes it superior to conventional staining techniques for the

detection of mitosis (Heidebrecht 1997). The TPX2 immunolabeling was performed with an automated stainer and a horseradish peroxidase system (Dako, Glostrup, Denmark). Antigen retrieval was achieved by heating sections in a digital electric pressure cooker (Menarini Antigen Access Unit, A. Menarini Diagnostics Ltd, Berkshire, United Kingdom) in sodium citrate buffer (pH, 6; 125°C for 1 minute and 40 seconds). This antibody to the TPX2 protein has been validated for use with equine tissue samples by means of Western blot analysis and immunolabeling of positive control (epidermal) tissues (personal communication, Dr. Janet Patterson-Kane). Primary antibodies were diluted at an optimal ratio (1:800) in antibody-dilution solution (Dako, Glostrup, Denmark). The positive control tissue samples were human and equine skin samples. For negative reagent control samples (to exclude nonspecific binding identified by the detection system), mouse IgG was used instead of the primary antibody. Sections were stained with chromagen 3,3'-diaminobenzidine tetrahydrochloridem (Dako, Glostrup, Denmark) twice for 5 minutes (with an intervening Tris-buffered saline with Tween wash) followed by counter-staining with Mayer hematoxylin (30 seconds). Five PELs on each slide were randomly selected, and the total number of cells with positive results for TPX2 in each region (axial, middle, and abaxial) was counted with the assistance of image analysis software (ImageJ version 1.4.3, National Institutes of Health, Bethesda MD, USA. Available at: [rsbweb.nih.gov/ij/index.html](http://rsbweb.nih.gov/ij/index.html)).

In Studies III and IV, mitotic cells were assessed by observing mitotic figures from HE sections with a light microscope.



**Figure 8.** Rounded, hypereosinophilic apoptotic epithelial cells in secondary epidermal lamellae. HE.

## **4.11 Statistical analyses**

### **4.11.1 Study I**

For the comparison of groups (laminitis and hospital group) Chi-squared analyses were used for categorical parameters (breed and gender) and Mann-Whitney tests (after testing for normality) for age. Results were presented as mean  $\pm$  standard deviation (SD) or median and interquartile range (IQR) where applicable. Significance was set at  $P < 0.05$ .

### **4.11.2 Study II**

Comparisons between groups (treatment and control group) were made for PELL, PELW, SELL, SELW, SELA, cell number per SEL, cell number per  $\mu\text{m}$  of SEL and apoptotic and proliferative cells. All variables, except PELW, were not normally distributed; therefore, a non-parametric Kruskal-Wallis test with Bonferroni adjustment was used for pairwise comparisons. Results were presented as medians and IQR and significance was set at  $P < 0.05$ .

### **4.11.3 Studies III and IV**

Comparisons between groups were made using ANOVA for normally distributed parameters and a Chi-square test, Chi-square tests of independence, the Mann-Whitney test or Kruskal-Wallis test with Bonferroni adjustment were used for the parameters that were not normally distributed. The normality of the parameters was tested using the Shapiro-Wilk test. The significance level was set at  $P < 0.05$ , and results were presented as medians with IQR or means with confidence intervals (CI).



## 5 RESULTS

The main results of Studies I-IV are presented below. For more detailed results, please see the original publications.

### 5.1 Prevalence of endocrinopathic laminitis among horses presented for laminitis at a first-opinion/referral hospital (Study I)

Altogether 37 horses with current or historical laminitis and 373 nonlaminitic, hospitalized horses were recruited to the study at the Helsinki University Equine Teaching Hospital between April 2007 and August 2008. One laminitic horse was excluded because of missing endocrine results and therefore the final number of laminitic animals was 36. Evidence of endocrinopathy (either hyperinsulinemia, PPID or both) was present in 89% (32 of 36 horses; 95% CI, 74% to 97%) of laminitic horses. One third of the horses (11 of 32) with endocrinopathy had PPID while two thirds (21 of 32) had hyperinsulinemia indicating insulin dysregulation. However, 10/11 (91%) horses with PPID also had hyperinsulinemia. Most of the horses with hyperinsulinemia had phenotypic signs of EMS. All except one (20/21) were considered to be overweight and the mean BCS was  $4.2 \pm 0.65$ . A cresty neck score of  $\geq 3$  was noted in 16/21 (76%), bulging supraorbital fat in 13/21 (62%) and laminitic rings in 18/21 (86%) of horses with hyperinsulinemia. Of the horses with laminitis and not diagnosed with an endocrinopathy ( $n=4$ ), two had divergent rings in their hooves and phenotypic signs of EMS (BCS  $\geq 4/5$ , cresty neck score  $\geq 3$  or bulging supraorbital fat).

Horses with endocrinopathic laminitis were significantly older (15 vs. 9 yrs.,  $p < 0.001$ ) and more likely to be pony breeds ( $p = 0.002$ ) than the hospital population during the same period. However, no association was observed between sex and diagnosis of endocrinopathic laminitis ( $p = 0.29$ ).

### 5.2 Morphological and cellular changes in secondary epidermal laminae of horses with insulin-induced laminitis (Study II)

The histomorphometry and number of apoptotic and mitotic cells were evaluated in the epidermal lamellae of four ponies with insulin-induced laminitis and four control ponies. The treatment group ponies were euthanized at the onset of Obel grade 2 laminitis, which occurred  $50.3 \pm 5.3$  hours after initiation of insulin and glucose infusion. The control ponies were euthanized after 72 hours of saline solution administration. The PEL length was significantly longer in the treatment group ponies than in the control ponies ( $p < 0.001$ ; Table 1). In addition, PEL were significantly longer in the

forefeet than in the hind feet in both groups ( $p=0.015$ ). The SEL in treated ponies were longer, narrower and had a smaller angle relative to PEL compared to the control ponies in all three PEL regions (axial, middle, abaxial) of both the fore and hind feet; however, these changes were most prominent in the middle region ( $p < 0.001$ ). In the treatment group ponies, the number of epidermal cell nuclei per SEL was typically higher and the number of cells per micrometer of SEL lower than in the control ponies. In the treatment group, numbers of apoptotic cells were significantly higher ( $p < 0.001$ ) than in the control group in the abaxial and middle regions in the forefeet and hind feet. In addition, the numbers of proliferating cells were higher in the treatment group in the axial lamellar regions in the forefeet and all lamellar regions in the hind feet, versus the control group ( $p < 0.05$ ).

**Table 1.** Median (interquartile range) primary epidermal lamellar length (PELL) and width (PELW) in the fore and hind hooves in ponies with insulin-induced laminitis (insulin,  $n=4$ ) and normal control ponies (control,  $n=4$ ). Measurements were made in all four feet of each pony. Ten PELLs and PELWs per foot were measured and recorded. Statistically significant  $p$ -values ( $<0.05$ ) are bolded.

Fore feet			
	Control	Insulin	p-value
PELL ( $\mu\text{m}$ )	3566 (2940-4060)	4105 (3754-4504)	<b>&lt;0.001</b>
PELW ( $\mu\text{m}$ )	295 (263-341)	294 (249-330)	N.S.

Hind feet			
	Control	Insulin	p-value
PELL ( $\mu\text{m}$ )	3267 (2929-3587)	3769 (3225-4278)	<b>&lt;0.001</b>
PELW ( $\mu\text{m}$ )	302 (271-347)	314 (270-366)	N.S.

### 5.3 Pathology of natural cases of equine endocrinopathic laminitis associated with hyperinsulinemia (Study III)

Lamellar morphology and pathology were described in 14 laminitic animals (4 horses and 10 ponies) that had hyperinsulinemia (median serum basal insulin concentration 32.0 mIU/L, IQR 27.5–58.1 mIU/L), with reference to 25 age- and breed-matched controls. The median age of the laminitic animals was 15.5 years (IQR 8.75–18.25), median BCS 4.0 (4.0–4.1) and there were 9 mares and 5 geldings. Breeds included 5 Mixed-breed ponies, 2 Shetland ponies and one each of the following: Welsh section A, Russ pony, Welsh section D, Finnhorse, Standardbred, Irish Draft cross and Cob. Three of the hyperinsulinemic animals also had PPID based on elevated plasma ACTH concentration and clinical signs. In addition, all the laminitic animals had gross pathological changes (divergent rings and/or gross separation and rotation of DP) in their hooves (Figure 9).

In control animals (9 horses and 16 ponies), PEL were significantly longer in horses than in ponies ( $p < 0.001$ ). Old horses had longer PEL than young horses ( $p = 0.04$ ), a finding that was not seen in ponies ( $p = 0.99$ , Table 2). The most common PEL and SEL types, as well as PEL axial and abaxial type were standard, with no increase in keratinization at the abaxial aspects. Occasional epithelial islands were noted in axial regions in some ponies (in 3/5 of old ponies and 2/11 of young control ponies) but these were not noted in horses of any age or in abaxial or middle regions in any animal. Additionally, the number of apoptotic cells was multifocally increased in 2/5 old ponies; up to 5 to 6 apoptotic cells were noted per high power field (400x).

The histological lesions noted in laminitic animals were variable between individuals. The PEL were significantly longer in laminitic animals than in controls ( $p < 0.001$ , Table 2). Most of the lesions noticed were localized abaxially (close to the hoof wall) within the lamellar tissue and included apoptotic cell death, lamellar fusion, hyperplasia, and partial replacement with aberrant keratin containing nucleated debris and proteinaceous lakes. The lesions resulted in irregular margins between the inner horn and the lamellar tissue. Acute lamellar separation was seen in one-third of laminitic cases and it originated from the abaxial region, with minimal associated inflammation. In some cases, the separation was accompanied by vacuolar swelling of many SEL cells and marked increases in numbers of apoptotic cells. However, in sections where the separation was not observed, increased and often large numbers of apoptotic cells (up to 30 hpf) were still noted abaxially.

**Table 2.** Median (interquartile range) primary epidermal lamellar length (PELL) in animals with naturally occurring endocrinopathic laminitis ( $n=14$ ) and control animals ( $n=25$ ). Measurements were made in the right fore foot of each animal. Ten PELLs per foot were measured and recorded. <sup>a</sup>Significantly longer than in other 4 groups ( $p<0.05$ ), <sup>b</sup>significantly longer than in young control horses ( $p<0.05$ ).

Group	PELL ( $\mu\text{m}$ )
Young control ponies (n=11)	2563 (2189-3368)
Old control ponies (n=5)	2960 (2824-3456)
Young control horses (n=4)	3039 (2793-3738)
Old control horses (n=5)	3837 <sup>b</sup> (3384-4413)
Animals with endocrinopathic laminitis (hyperinsulinemia) (n=14)	7288 <sup>a</sup> (5534-8506)



**Figure 9.** Three sagittal sections of hooves from animals with endocrinopathic laminitis showing macroscopic lesions. The red lines are showing the outer hoof wall and the dorsal aspect of the distal phalanx (DP) (A) Mild DP rotation in a 15 years old New Forest pony mare with 3 weeks duration of laminitis, (B) Moderate DP rotation (and abnormally long toe) in an 8-year-old Shetland pony mare with 2 months duration of laminitis, (C) Severe DP rotation in a laminitic pony with unknown history.

Axially, epidermal lamellar tapering was the most frequent morphological observation. Additionally, the numbers of apoptotic cells were increased in sections with no separation, but more variably (between horses) than in abaxial regions. However, the mitotic figures were most frequently observed in axial regions, especially in sections where separation had occurred or there was extensive abaxial fusion (1-3 hpf).

The median reported duration of laminitis (reported by the owner or treating veterinarian) was 120 days (range 5-730 days); however, the type and severity of macroscopic or microscopic lesions had no correlation with duration.

#### **5.4 Lamellar pathology in horses with pituitary pars intermedia dysfunction (PPID) (Study IV)**

The clinical pathology, histomorphometry and pathological lesions in lamellar tissue were described in PPID animals with (n=6; 4 ponies and 2 horses) or without (n=10; one pony and 9 horses) concurrent laminitis, with reference to age-matched controls (n=10; 5 ponies and 5 horses). PPID animals without laminitis were significantly older than control animals (median age 22 years vs. 16.5 years,  $p=0.007$ ) and the animals with PPID and laminitis had a higher median BCS than the animals with PPID without laminitis (4.0 vs. 2.5,  $p<0.001$ ). All animals with PPID and laminitis had fasting hyperinsulinaemia (median serum insulin concentration 74.1 mIU/L, IQR 49.9-349.5 mIU/L) whereas PPID animals without laminitis (and control animals) had serum insulin concentrations below the upper limit of the reference range ( $< 20$  mIU/L). In addition, all the laminitic PPID animals had macroscopic changes (divergent rings and/or DP deviation) in their hooves whereas the PPID animals without laminitis (and control animals) did not have any macroscopic evidence of previous episodes of laminitis.

The lamellar morphology in nonlaminitic PPID animals was normal and not different from that of the control animals. Axially the most common PEL type was sharp whereas in control animals it was standard; however, there was no significant difference between the groups.

Lamellar pathology was observed in all of the PPID animals with laminitis. However, alterations in lamellar morphology and pathological lesions were variable in severity and not related to the reported duration of laminitis (median 12 months, range 2 months-5 years). PEL were significantly longer and wider in laminitic PPID animals than in the other two groups ( $p<0.05$ ). Most lesions were located abaxially within the lamellar tissue and included increased keratinization, fusion (interlamellar epidermal bridging) and dermoepidermal separation. Axially, increased tapering was noticed. In 2/6 PPID animals with laminitis the number of apoptotic SEL cells was generally (at all levels) increased; in 2/6 they were increased only in the abaxial and middle regions. However, increased numbers of apoptotic cells were noticed also in 3/10 of the nonlaminitic PPID horses (at all levels) and 2/10 of the control animals (in one at all levels and in another axially). The number of mitotic figures in SEL cells was increased in 4/6 laminitic PPID animals, but they were significantly less frequent than the numbers of apoptotic cells. Most

of the mitotic figures were seen axially but some were noticed in other regions. In 2/6 laminitic PPID animals, acute separation mostly through the BM region was noticed in abaxial and middle regions.

Occasional epithelial islands were noted mostly in axial regions of the PEL in all other groups but the control horses. However, in the control ponies the islands were only seen in small numbers compared to PPID animals. In PPID animals (both with and without laminitis), however, some islands were noted in addition to axial region, also in the middle and abaxial regions.

## 6 DISCUSSION

### 6.1 The prevalence of endocrine diseases among laminitic horses

The first aim of this thesis was to determine the prevalence of horses presented primarily for laminitis with a diagnosis of underlying endocrine disease (Study I). Endocrinopathic and pasture-associated types of laminitis have been known to occur commonly in field (USDA 2000, Geor 2009), yet most of the research has focused on laminitis arising secondary to inflammatory disease (Garner *et al.* 1975, Galey *et al.* 1991, Pollitt 1996, van Eps and Pollitt 2006). During the last few decades there has been a growing body of evidence that pasture-associated laminitis is also of endocrine origin. This hypothesis was supported in 2006 by the study of Treiber *et al.* where they showed that ponies with prelaminitic metabolic profiles (including reduced insulin sensitivity) on pasture in early spring (March) were 10 times more likely to have clinical laminitis than ponies with normal profiles in late spring (May), when the carbohydrate concentration in grass was high. In addition, ponies with clinical laminitis in late spring had significantly higher basal insulin concentrations than non-laminitic ponies providing more evidence of laminitis being caused primarily by an endocrine disease (hyperinsulinemia and/or insulin resistance) rather than pasture alone (Treiber *et al.* 2006).

In our study the prevalence of an underlying endocrinopathy was not just the majority, but almost 90%, and of those that were negative in endocrine tests (4/36), most had phenotypic changes typical for EMS and/or PPID. One previous study showed similar results: the prevalence of endocrinopathies among laminitic horses in a primary care setting was shown to be 70% (Donaldson *et al.* 2004). However, Donaldson's study was published before EMS was well known and only animals with PPID were reported in that 70% figure. If laminitic animals with hyperinsulinemia with no PPID had been included, the prevalence would have also been approximately 90%. The results of this work suggest the focus of laminitis research and treatment in preceding decades should be now revised from inflammatory etiologies to endocrine laminitis. More research on prevention, treatment and management of endocrine laminitis and the endocrine disorders associated with it, will have the greatest impact on equine welfare.

### 6.2 Diagnosis of hyperinsulinemia

Different tests have been developed for diagnosing EMS and insulin dysregulation. In this dissertation, elevated basal serum insulin concentration was used in diagnosing hyperinsulinemia indicative of insulin dysregulation. This test is less sensitive for diagnosis of individual cases of insulin resistance than dynamic tests, such as the combined glucose-insulin tolerance test (CGIT; Kronfeld *et al.* 2005). Therefore, some of the cases of hyperinsulinemia

may have been missed and classified as falsely negative. In Study I there were 4/36 horses with normal endocrine values among laminitic horses; however, 2/4 of these had phenotypic indicators of EMS. Therefore, it may be that the prevalence of endocrinopathic laminitis in this study was actually even higher than presented. Another commonly suggested problem in insulin measurements is the possible effect of pain or stress induced cortisol release on insulin values. Cortisol has antagonist effects on insulin via its gluconeogenic actions and has therefore been suggested to promote insulin resistance and hyperinsulinemia (Johnson *et al.* 2004). However, when Obel grade  $\geq 2$  laminitis and colitis (moderate to severe pain) was induced experimentally with oligofructose overload, the horses showed only a slight increase in serum insulin concentration (van Eps and Pollitt 2006). In addition, no alteration in insulin regulation was seen in a CHO overload model using lower doses of oligofructose, despite alterations in glucose regulation (Kalck *et al.* 2009). It has been known from studies of humans and laboratory animals since the 1960's that catecholamines associated with pain and stress actually inhibit insulin secretion (Porte *et al.* 1967). In addition, although acute pain or stress may increase insulin resistance at the tissue level, they have not been shown to affect basal insulin levels at the same time (Greisen *et al.* 2001). Therefore, it is unlikely that the increased cortisol levels due to pain or stress would have induced the hyperinsulinemia observed in laminitic animals in this thesis.

### **6.3 Hyperinsulinemia and laminitis associated with PPID**

It has long been known that PPID is associated with laminitis; however, the exact pathogenesis of how laminitis develops in animals with PPID has not been explained. The results of this dissertation and previous research support the hypothesis that it may actually be hyperinsulinemia that is the key causative agent of laminitis in PPID horses. In Studies I and IV all except one horse with PPID and laminitis also had hyperinsulinemia. However, all the PPID horses without laminitis, in which insulin was measured, had normal insulin concentrations (3 horses in Study I, 10 horses in Study IV). In addition, in Study IV, all the laminitic PPID horses (with hyperinsulinemia) had pathological changes in the lamellar region while the lamellae of nonlaminitic (and normoinsulinemic) PPID horses were not different from normal aged horses and ponies.

It has been suggested that insulin resistance and hyperinsulinemia may occur in PPID horses as a result of uncontrolled ACTH and related pituitary peptide release from the pituitary gland and the resultant antagonizing effects of cortisol on insulin. However, it is known that not all the horses with PPID have impairments in insulin sensitivity. In 2013, McGowan *et al.* showed that only 32% of horses with a diagnosis of PPID (using basal ACTH) had hyperinsulinemia (McGowan *et al.* 2013). Later, Mastro *et al.* showed that there was no difference in insulin sensitivity between old horses with PPID and old control horses (Mastro *et al.* 2015). Therefore, it may be suggested that



PPID alone may not cause the insulin insensitivity in those PPID animals with hyperinsulinemia. In addition, if hyperinsulinemia does occur due to PPID related hormone release, it could be assumed that the PPID horses without clinical laminitis would have had at least early or mild histological changes in their lamellar region, which they did not have. In addition, usually horses with PPID have resting cortisol concentrations within the reference range, which does not support the aforementioned theory either (Hillyer *et al.* 1994, Couëtil *et al.* 1996, Eiler *et al.* 1997, van der Kolk 1998, Reynolds and Walker 2003).

It is possible that all PPID animals with laminitis have also either subclinical or clinical EMS. The prevalence of PPID in horses  $\geq 15$  years old has been shown to be 21% (McGowan *et al.* 2013) and the prevalence of hyperinsulinemia as an indicator of EMS (in a population where PPID animals have been excluded) in ponies (mean age 9 years) was 27% in Australia (Morgan *et al.* 2014). In addition, age has been shown to be a significant risk factor for hyperinsulinemia (Morgan *et al.* 2014). Therefore, the probability of having PPID and EMS together in the same animal may be considered quite high, especially in older horses ( $\geq 15$  yrs.). It may be that the pituitary peptides released in PPID exacerbate underlying insulin resistance in horses with EMS, allowing for the clinical expression of laminitis and PPID. Therefore, it is possible that in animals with subclinical EMS, the development of PPID later in life makes them more insulin resistant and more susceptible to laminitis.

#### **6.4 Epidermal cellular changes in insulin-induced laminitis**

The second article of this dissertation (Study II) supported the hypothesis that the histology of insulin-induced laminitis is different from that in the previously described inflammatory laminitis. Increased inflammation (defined as increased leukocyte infiltration), metalloprotease activity and BM degradation have been shown to have role in the development of inflammatory laminitis modeled with CHO overload or BWE (Pollitt 1996, Kyaw-Tanner and Pollitt 2004, Black *et al.* 2006, Loftus *et al.* 2006, Kyaw-Tanner *et al.* 2008, Faleiros *et al.* 2011a, Faleiros *et al.* 2011b). BWE studies have shown that leukocyte infiltration is a key element in the development of laminitis lesion, but in CHO overload studies the leukocytes have been shown to arrive only after the tissue (BM) pathology is underway (Visser 2008, Faleiros *et al.* 2009, Faleiros *et al.* 2011a). In studies on insulin-induced laminitis (including this dissertation), leukocytes have not been shown to increase significantly compared to controls and the minor extravasation noticed has been shown to follow (similar to CHO overload models) the development of BM lesions and alterations in SEL architecture (Asplin *et al.* 2010, de Laat *et al.* 2011a, de Laat *et al.* 2011b). Therefore, it is unlikely that leukocytes would initiate the lamellar epithelial damage in CHO overload or insulin models. Interestingly, the extent and severity of inflammation in both CHO overload and insulin models (but not in BWE models) are significantly less than would be expected in many other tissues subjected to similar levels of cellular stress and

mechanical compromise, including the skin. However, the reason for this finding is not known.

BM separation (possibly related to increased metalloprotease activity) has been suggested to be a key element in inflammatory laminitis (Pollitt 1996). However, in hyperinsulinemia models, as well as in this dissertation, BM lesions have been absent to limited and have followed SEL elongation and cellular degradation (Asplin *et al.* 2010, de Laat *et al.* 2011a, Study II). These findings indicate a spectrum of severity of BM separation in insulin-induced laminitis that occurs after the SEL elongation phase and cellular damage. Another important point is that in ponies with insulin-induced laminitis (Study II), the BM damage appeared to occur mostly axially, whereas in animals with naturally occurring endocrinopathic laminitis (Study III) the separation occurred abaxially, indicating that the BM lesion hypothesis cannot fully explain the lesions occurring naturally.

Even though increased leukocyte infiltration, metalloprotease activity and BM separation do not seem to be key elements of insulin-induced laminitis, increased numbers of apoptotic cells and autophagocytosis were noticed in the hyperinsulinemic ponies in Study II 50 h after the onset of pEHC. Therefore, it is possible that inflammatory events also occur in hyperinsulinemic laminitis, but within the epithelium itself, not from the infiltration of leukocytes. Apoptotic cells were rare in normal lamellar tissue, but significantly increased in the abaxial and middle regions of PEL in all 4 feet of the hyperinsulinemic ponies, indicating some form of cytokine signaling resulting in the dramatic cellular response in those regions.

The numbers of proliferating cells (confirmed by TPX2 immunolabeling) were also significantly increased in ponies with hyperinsulinemic laminitis at 50 h: only axially in their fore feet but in all regions in their hind feet. In one previous study it was hypothesized that aberrant proliferation of lamellar epidermal cells, potentially due to alterations in cell signaling and a predominance of insulin-like growth factor 1 (IGF1) or MAPK pathways which favor mitogenesis and growth, might be the key lesion of insulin-induced laminitis causing SEL elongation (de Laat *et al.* 2013a, de Laat *et al.* 2013b). However, in Study II, after measuring the length of SELs, we found a reduction of epidermal SEL cells per micrometer, despite elongation of the SEL in ponies with insulin-induced laminitis, and concluded that elongation was probably secondary to cell degradation/damage and loss of structural integrity under the biomechanical forces of weight bearing, rather than aberrant proliferation. Additionally, epidermal cell proliferation has not been shown to be an important element in naturally occurring laminitis either: In one previous study the lamellae of animals with naturally occurring laminitis (even though different etiologies) had reduced amounts of epidermal stem cells suggesting rather decreased than increased proliferation capacity (Carter *et al.* 2011).

## 6.5 The histology of naturally occurring endocrinopathic laminitis

Even though the histology of experimentally induced hyperinsulinemic laminitis has been well described (Asplin *et al.* 2010, de Laat *et al.* 2011a, de Laat *et al.* 2012a, de Laat *et al.* 2013a, Study II), the experimental models only show us the effect of hyperinsulinemia and indicate what structures alter in response in the acute phase. Naturally occurring endocrinopathic laminitis is seldom, if ever acute, but develops gradually with time. Therefore, not all the experimental models represent the naturally occurring endocrinopathic laminitis very well.

The normal histology and morphometry of lamellar tissue had not been properly described previously and therefore it may have been challenging to differentiate pathological lesions from normal ones. For this reason, in Studies III and IV we described the histology and histomorphometry of the lamellar regions of young and old, clinically healthy animals with no evidence of endocrinopathic disease. Recently the histology of the lamellar region has been described in animals without clinical or history of clinical laminitis (Kawasako *et al.* 2009). However, these animals were not healthy otherwise but were euthanized because of some other disease (e.g. infection, colic, bone fracture) and may, therefore, have had conditions that predispose to subclinical or early (in this case either inflammatory or mechanical) laminitis. The predominance of tapered SEL type abaxially and detection of hyperplastic changes in the aforementioned study are findings that we did not observe in our healthy control animals and they may be considered as possible laminitis lesions. In addition, in Kawasako's study also foals (1-9 months old, n=9/35) and yearlings (n=5/35) were included whereas we included only animals that were over one-year-old. The morphology of the lamellar region has been shown to change during the first year of life and therefore only animals of a similar age can be compared with each other (Bidwell and Bowker 2006). It is also likely that the weight of the animal as well as the mechanical environment affect the lamellar morphology, but as far as the author is aware, no proper research on this has been published. Kawasako *et al.* (2009) suggested that the dominant PEL type would depend on the mechanical stimulation. Only in the racehorse group in their study was the predominant PEL type standard (straight) and in the other groups it was curving. However, in our study the most common PEL type was standard in all the control groups. Therefore, it is likely, that also other factors than the mechanical environment affect the PEL type. Interestingly, the most common axial PEL type in Kawasako's oldest group (mares 7 to 22 years) was sharp. In our studies old horses (old control horses in Study III and PPID horses without laminitis in Study IV) seemed to have more sharp axial PEL ends than animals in other groups although no statistical significance was found. In addition, old control horses in Study III had significantly longer PEL than young control horses. However, whether the PEL elongation or sharpness of PEL axial end is related to aging, warrants more research.

The third article (Study III) was the first to describe the histology of naturally occurring endocrinopathic laminitis. We hypothesized that the lamellar histology of naturally occurring cases would resemble that of horses

and ponies with experimentally induced hyperinsulinemic laminitis, but show more advanced lesions based on chronicity. The hypothesis was shown to be correct with lamellar elongation, altered SEL angles and cellular changes with limited to no inflammation and lack of widespread BM degradation. A new finding was that the microscopic lesions in laminitic animals were largely localized abaxially (close to the hoof wall) in most cases; however, the cellular changes including apoptosis and mitosis were noticed also axially (although less than abaxially). The abaxial lesions included apoptotic cell death, as well as lamellar fusion, hyperplasia, and partial replacement with aberrant keratin containing nucleated debris and proteinaceous lakes. These changes could be interpreted as representing the following sequence of events: 1. fusion of the tips of SELs across the PDL resulting in isolation of rounded areas of vascular dermal tissue that continued to keratinize peripherally (centrifugally) and produce increasing amounts of cap horn (Figure 10); 2. reduction in the area of this dermal 'circle' with complete isolation from the more axial (epidermal and dermal) tissue by keratin, and keratinization of most of the immediately surrounding epidermal tissue (Figure 11); 3. degeneration of the blood vessel(s) and completion of keratinization of the remaining (central) epidermal cells (Figure 12); and finally, 4. filling of the area with keratin or proteinaceous fluid (Figure 13). The aforementioned lesions would all in theory, predispose the lamellar tissue to tearing. When tearing was seen histologically in Study III, it was always abaxial and involved BM separation, tearing of SELs, and/or separation of the PEL by ripping through the SEL bases - in acute inflammatory cases (induction models) tearing has been shown to be mostly axial and involving exclusively BM separation (Pollitt 1996, de Laat *et al.* 2011a). In cases of naturally occurring endocrinopathic laminitis, we observed leukocytes, although surprisingly minimal, mostly related to epidermal-dermal separation and consider that to be a normal reaction (secondary) after tissue damage.

The reason why lesions in these chronic natural cases are mostly located in the abaxial region is unknown but there are a few suggested theories including hypocirculation due to longer distance from the primary vascular supply of the dermis, higher metabolic rates or susceptibility to toxins (e.g. insulin) or greater mechanical stress in abaxial epidermal cells vs. axial cells (Morgan *et al.* 2003, Study II). Another hypothesis is that the lesions start axially from the origin of the blood supply that delivered factors inciting the lamellar cell damage and then extend abaxially. Therefore, in a naturally affected animal with typically chronic lesions, as opposed to (acute) experimental cases, these lesions will be more likely to be observed abaxially. However, more research with better and humane models of the disease is warranted to fully document the temporal changes in naturally occurring laminitis.

Epithelial islands (often completely unconnected to their respective PEL) have been described previously in horses with naturally occurring chronic laminitis ('keratin pearls', Roberts *et al.* 1980), and also in experimental animals seven days post-induction of laminitis with oligofructose (i.e. inflammatory laminitis; van Eps & Pollitt 2009). In Studies III and IV, epithelial islands were commonly observed mostly axially in laminitic animals; however, occasional axial small epithelial islands were also noted in some

control ponies but not in control horses. This relatively frequent occurrence of axial epithelial islands in pony hooves, perhaps represents subclinical damage ('normal wear and tear') caused by some factors not directly related to laminitis (e.g. mechanical overloading) clearly not experienced in horse hooves. In some laminitic animals the epithelial islands were not confined to axial regions (i.e. adjacent to PEL tips) - they were also seen in middle and abaxial regions of the lamellar tissue. This might be directly related to laminitis pathophysiology as previously noted in models of inflammatory laminitis, however the extent of island formation was extreme in that model and not necessarily comparable (van Eps and Pollitt 2009). It could also represent a spatial shifting of mechanical forces (that exceed SEL 'safety limits') within the damaged hoof wall. Interestingly this expansion of the area affected by island formation was also seen in some PPID horses that did not have laminitis, perhaps again indicating a change in hoof wall dynamics not directly associated with hyperinsulinemia or with noticeable lameness.

## **6.6 Macroscopic changes of the hoof in naturally occurring endocrinopathic laminitis**

Macroscopic structural changes of the hoof wall, like divergent rings, have been previously anecdotally known as indicative of chronic laminitis. However, Study III is the first to document macroscopic changes (either divergent rings and/or ventral DP deviation) corresponding with the histology in (all) clinical cases of naturally occurring laminitis. The exact mechanism of divergent ring formation and why they are divergent (wider at the heels than the toe) is not known. A healthy hoof grows faster from the dorsal part than from the heels, but in laminitis this growth pattern no longer applies. It may be that the cellular changes (e.g. stretching and apoptosis) in the laminitic hoof are worse on the dorsal part of the hoof wall than in the heels and therefore growth is compromised more severely there. Another explanation might be that the blood supply to the dorsal hoof wall is reduced or that altered biomechanical forces occurring along the dorsal hoof wall allow greater elongation of the PELs and SELs there relative to the heels. Only dorsal hoof wall samples were obtained in this dissertation; however, sampling would be required also from other parts of the hoof wall to better understand the mechanism behind the divergent ring formation.

The severity of both macroscopic and microscopic lesions was not correlated with the reported duration of clinical lameness due to laminitis. Many animals (6/14) had divergent rings in their hooves but a reported duration of laminitis shorter than the rings would need to form (<3 months). Therefore, it may be suggested that clinical laminitis was preceded by a prolonged subclinical phase. Additionally, it may also be suggested that if the divergent rings are noted before the clinical signs, laminitis may be possible to prevent e.g. by changing the diet and lifestyle to reduce the hyperinsulinemia. In another study from Australia all ponies with a history of laminitis also had

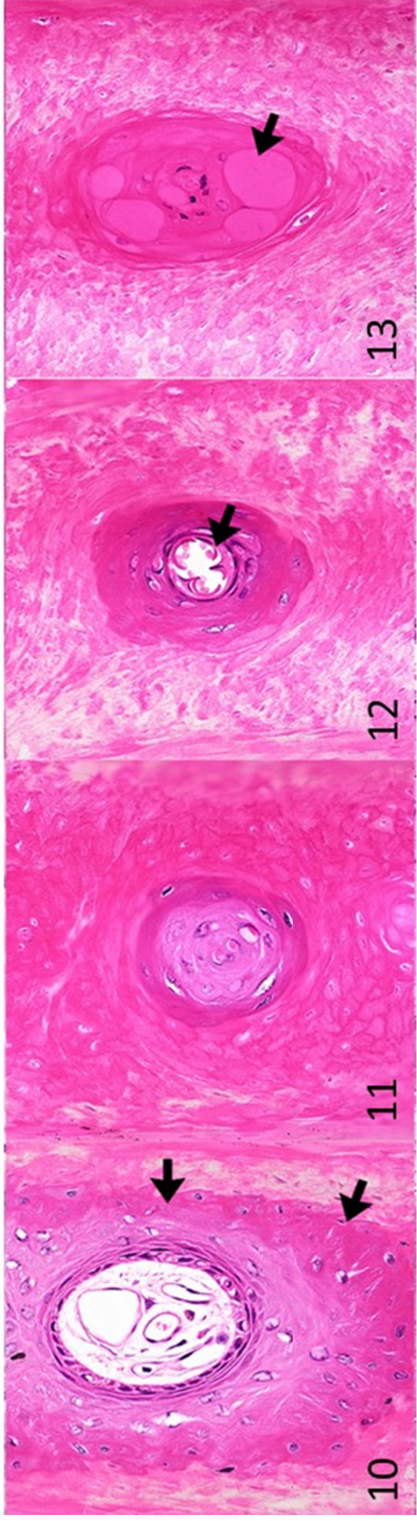
divergent rings, but there were also some animals (ponies) with divergent rings without an owner-reported history of laminitis (Morgan *et al.* 2014); therefore, it is possible that a long phase of preclinical laminitis is common. However, the rate at which natural endocrinopathic lamellar pathology occurs in subclinical episodes ahead of the acute episode is not known. Due to the chronic nature of endocrine disease development, it is possible that many animals with endocrinopathic laminitis suffer from repeated episodes of lamellar alteration before clinical signs are noted. However, it is also likely that there is significant individual variation in the severity and frequency of these subclinical episodes where factors like weight, mechanical environment, hoof shape and duration of hyperinsulinemia may have an effect on the development of hoof capsular changes.

## **6.7 Treatment and prevention of endocrinopathic laminitis**

Despite a large amount of research, the mechanism by which hyperinsulinemia causes laminitis is still only partly understood. As far as the treatment options are concerned, a deeper understanding of the actions of insulin and disease pathogenesis would be required. However, one possible mechanism that has been suggested is an insulin-induced imbalance between ET-1 and nitric oxide, leading to over-expression of ET-1 and vasoconstriction, which has been shown to occur in humans and rodents (Kim *et al.* 2006). Interestingly, short-term hyperinsulinemia was recently shown to cause increased vascular resistance and ET-1 expression in equine cadaver limbs (Gauff *et al.* 2013). Later the same group showed that hyperinsulinemia causes significant changes in endothelin receptor (ETR) expression in equine limbs and suggested that ETR antagonists might be beneficial in the treatment of laminitis (Gauff *et al.* 2014). However, the use of ETR antagonists in the treatment of hyperinsulinemic laminitis requires further investigation *in vivo*.

Based on findings of other studies and this dissertation, veterinarians are recommended to concentrate on preventive measures for the development of hyperinsulinemia; especially the identification, prevention and treatment of the endocrine dysfunction. In many cases with endocrinopathic laminitis tissue damage in the lamellar region appears to be well under way before the onset of clinical signs and therefore early diagnosis and treatment of endocrine diseases is of crucial importance. Horses with obesity (either general or regional), delayed hair shedding or long hair coats should be tested for an endocrine disorder, especially hyperinsulinemia. In addition, observations for changes in the hoof wall should be included in veterinary check-ups and if divergent rings or cap horn are noticed that should prompt endocrine evaluation. Anti-inflammatory agents like cyclooxygenase (COX) inhibitors have been traditionally used for the treatment of laminitis; however, more

work on the most appropriate anti-inflammatory is warranted. Based on our results, treatment of inflammation in the classic sense is not necessary in cases of endocrinopathic laminitis, but the vascular effects of non-steroidal anti-inflammatory agents (NSAIDs) may well be warranted. More COX-2 selective anti-inflammatory drugs have become popular recently, but COX-2 has been shown to be constitutive in the hoof lamellae and therefore COX-2 selective drugs may not be ideal (Blikslager *et al.* 2006). It is clear, however, that if prevention is not achieved, effective analgesia and biomechanical support of the hooves would be advisable.



**Figures 10-13.** The proposed sequence of pathological changes in the abaxial aspects of primary dermal lamellae (PDL) in chronic laminitis. Fig. 10. Isolation of a circular region (in 2 dimensions) of PDL tissue containing blood vessels, with continuing peripheral (centrifugal) keratinization (arrows) that is adding to the cap horn and filling in the space between adjacent primary epidermal lamellae (PELs). At this stage the surrounding epidermis is still attached to more axial lamellar tissue (at top margin of the photomicrograph). HE. Fig. 11. Continued keratinization has now isolated the small area of dermis from more axial lamellar tissue (which now sits within the cap horn), and much of the immediately surrounding epidermis is now keratinized. HE. Fig. 12. The walls of the blood vessels degenerate (arrow) and the surrounding epidermis is almost completely keratinized. HE. Fig. 13. The blood vessels are necrotic (with loss of cellular detail) and surrounding tissue is filled with lakes of proteinaceous fluid (arrow). No viable epidermal cells remain. HE.



## 7 FUTURE PERSPECTIVES

Studies III and IV of this dissertation are first to describe the histopathology of naturally occurring endocrinopathic (hyperinsulinemia with or without PPID) laminitis and compare the histology with healthy animals. Since the sample sizes were relatively small, more samples would be beneficial in future to strengthen the results and conclusions of this dissertation. In addition to the traditional dorsal hoof wall samples, lamellar samples should be collected also from other parts of the hoof. The normal hoof growth differs in different parts of the foot and samples collected around the hoof wall might give us more information of the cellular events associated with e.g. divergent ring formation.

We also described the normal histology of lamellar region in this dissertation. However, the control animals were mostly pleasure horses from Europe (Finland or UK) and therefore the use and mechanical environment the horses were living in were quite similar. The future work could compare the normal histology of animals that are used for racing purposes (in which the mechanical stimulation of the lamellar region is much more severe than in pleasure animals) and that of the pleasure animals to find out the effect of mechanical stress in the lamellar region. Kawasako *et al.* (2009) suggested that certain PEL types might be more common in racehorses compared to pleasure animals (Kawasako *et al.* 2009), but more research regarding the normal morphological variation is warranted. In addition, different environments may influence the lamellar morphology and therefore also samples from horses kept on harder and drier surfaces (compared to Finland and UK) might increase our knowledge of morphological variation.

The lamellar samples of this dissertation were collected from dead animals. Ideally, the development of protocols for minimally-invasive techniques to collect biopsies from live horses with endocrinopathic laminitis would help in the prognosis of horses. Morgan *et al.* (2003) collected lamellar samples by drilling a hole through the dorsal hoof wall and then removed lamellar tissue with scalpel blades and biopsy knives (Morgan *et al.* 2003). However, this procedure is highly invasive and potentially harmful for the horse, although all the biopsied horses in Morgan's study recovered completely and did not have any adverse effects attributable to the biopsy procedure. Since lamellar tissue may be considered histologically as highly specified skin, it is possible that hyperinsulinemia affects also other epithelial structures. In horses with endocrinopathic laminitis e.g. skin. These tissues might show changes during hyperinsulinemia and offer some clues about the possible lesions in lamellae. In addition, the aforementioned sites would be much easier and safer to biopsy than the hoof.

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