Exercise induced pulmonary haemorrhage (EIPH) and exercise associated fatal pulmonary haemorrhage (EAFPH) in racing horses. Two different diseases?

'Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Master in Philosophy by Guido Rocchigiani

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Contents

Guido Rocchigiani - Exercise induced pulmonary haemorrhage (EIPH) and exercise associated fatal pulmonary haemorrhage (EAFPH) in racing horses. Two different diseases?

Exercise-induced pulmonary haemorrhage (EIPH) is a common condition of Thoroughbred (TB) racehorses usually responsible for reduced performance, while exercise-associated fatal pulmonary haemorrhage (EAFPH) is characterized by severe pulmonary bleeding of unknown pathogenesis resulting in sudden death during strenuous exercise. One aim of the study was to describe and compare anamnestic data, pulmonary gross, histologic, and ultrastructural findings in racehorses with EIPH (n = 10), EAFPH (n = 10), and control horses (n = 5). No differences in anamnesis were identified. Grossly, cranial lobe reddening and oedema were significantly more prevalent and severe in the EAFPH group compared with the EIPH and control groups. Histologically, haemorrhage scores were higher in the EAFPH group, while hemosiderophages, iron encrustations, and vascular remodelling scores were significantly higher in EIPH group compared with the EAFPH and control groups. Ultrastructural analysis of perivascular collagen showed fibrils with significantly larger diameters in the EAFPH group compared with the EIPH group. The other aim was to compare pulmonary fibrosis, haemosiderin, pulmonary (TTF-1, α-SMA, SP-C) and cardiac (cardiac troponin T, connexin 43, cleaved caspase 3) IHC markers through image analysis software in non-TB control (N=10), TB control (N=7), EIPH (N=11) and EAFPH horses (N=17). The analysis of the lungs showed that haemosiderin was significantly higher in the EIPH group compared to controls, whereas α-SMA was lower in the EAFPH group compared to the TB control. For cardiac markers, the EAFPH group showed significant lower expression of connexin 43 compared to both control groups. This study demonstrates that the lungs of horses that died of EAFPH show significantly less vascular remodelling and pulmonary abnormalities then horses with EIPH; furthermore, EAFPH horses seem to have lower amounts of Connexin43 compared to control groups.

Preface

The present manuscript represents the original work of the candidate Mphil Guido Rocchigiani. This study was fully funded by the Horserace Betting Levy Board (HBLB) from 2018-2022 as a senior clinical scholarship (Reference VET/CS/027). All the material presented here has been produced thanks to the Veterinary Anatomy, Physiology and Pathology Department, Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool.

Literature review

Thoroughbred (TB) horses are a notorious racehorse breedwhose English origins date back to the 18th century, when three Arabian sires, namely Darley Arabian, Byerley Turk and Godolphin Barb were used to develop the whole dynasty. One of the foals born from Darley Arabian was Bartlett's Childers, who, although never trained for racing, foaled multiple generations of first-rate runners, including Eclipse. Nevertheless, Bartlett's Childers was probably more famous for frequent nasal bleeding, making it worth the name "bleeding Childers" that representsthe first reported case of EIPH in history. The first demonstration that nasal bleeding originated from the lung was proven in the early 80s: Pascoe and colleagues revealed bloody streaks over the bronchial lumen using flexible fibreoptic endoscopes and coined the term "exercise induced pulmonary haemorrhage (EIPH)", after finding out the source of bleeding (e.g. lung) and the association of the condition with training (Pascoe, Ferraro, Cannon, Arthur, & Wheat, 1981). Pascoe also established a four-level grading scheme based on trachea-bronchoscopic findings, which has been widely used to measure the severity of EIPH. Beyond TB horses, this disease has been described in other racehorse breeds, such as Standardbred (Lapointe, Vrins, & McCarvill, 1994), and in other equine disciplines, including jumping shows (Bonomo, Michelotto, Viccino, Barussi, & Fernandes, 2019), barrel racing (Gold, Knowles, Coffey, & Bayly, 2018), endurance (Tarancon et al., 2019) and polo (da Silva et al., 2017). This condition has also been reported in camels (Akbar, Derksen, Billah, & Werney, 1994) and dogs (Epp et al., 2008), after running efforts. The trigger that usually causes pulmonary bleeding is the exercise: indeed, most of these horses do not show clinical signs at rest. Therefore, establishing how EIPH affects horses constitutes a real challenge. The ACVIM consensus statement published in 2015 specified that there is not enough evidence that EIPH can induce clinical abnormalities, apart from epistaxis after racing, which is only reported in 1.1-3.5% of affected horses. Similarly, no evidence indicates that EIPH can provoke blood/gas exchange disruption (Hinchcliff et al., 2015). Despite the ACVIM consensus paper, multiple studies have underlined some correlations between EIPH and reduced

performance. One study published in 2017 that examined 3794 tracheo-bronchoscopic data results from 1567 TB racehorses found a statistically significant relationship between grade 4 EIPH and lower finishing positions, reduced amount of money earned and reduced speed in the last 600m when compared to horses with no EIPH. In the same study, horses showing EIPH grade >3 also showed a similar trend when compared with horses showing EIPH grade < 2 (Crispe, Lester, Secombe, & Perera, 2017). EIPH also seems able to affect the long-term performance of racehorses: a prospective study analysing tracheo-bronchoscopic data after a single flat race from 744 TB horses indicated that EIPH grade 1-3 was not affecting the horse athletic career significantly, apart from fewer earning; conversely, EIPH grade 4 horses were likely to display worse long term performance, with shorter racing carrier duration and reduced earnings (S. L. Sullivan, Anderson, Morley, & Hinchcliff, 2015).

It is difficult to establish the prevalence of EIPH in racehorses, as its detection depends on the diagnostic methods used for the investigation. If we consider epistaxis as the only diagnostic criterium in TB racehorses, the prevalence would be as low as 1.1-3.5% (S. Sullivan & Hinchcliff, 2015); however, epistaxis has low specificity, as it can present in other diseases, including progressive ethmoidal haematoma or guttural pouch mycosis. Tracheo-bronchoscopy within 2 hours since race completion is a tool widely used to diagnose EIPH, as it also helps to evaluate the degree of EIPH intensity (Pascoe et al., 1981). The EIPH prevalence with this diagnostic assay oscillates between 43% and 75% in TB horses with a single examination, but this percentage can go to 85% if multiple tracheo-bronchoscopy measurements are made (S. L. Sullivan et al., 2015). Radiography and ultrasonography are diagnostic tests showing limited diagnostic value for this disease as these techniques cannot distinguish extravasated blood from any pulmonary exudates, which could be elicited by any pulmonary inflammatory conditions (Ferrucci et al., 2009) (Doucet & Viel, 2002). The most sensible and specific technique for the diagnosis of EIPH is the cytology of bronchoalveolar lavage (BAL) fluid. This method allows collecting, evaluating and quantifying cells within the lower airways lumina. This

technique makes it possible to detect extravasated erythrocytes, and the product of the erythrocytic breakdown, such as haemosiderin, usually observed within alveolar macrophages (haemosiderophages) as a sign of previous bleeding. Removal of blood and its catabolite from the airways is considered relatively slow in horses and other animals: erythrocytes and haemosiderophages are present in lavage fluid up to 21 days after experimental intra-bronchial inoculation of autologous blood (Slocombe & Mckane, 2000). In mice, haemosiderophages are observed two months after similar experiments (Epstein, Elidemir, Colasurdo, & Fan, 2001). BAL analysis has the advantage of not being time-restrained to running activity and can quantify the number of haemosiderophages present in the specimen. Using BAL cytology, prevalence of EIPH can reach almost 100% in TB racehorses (McKane, Canfield, & Rose, 1993). Despite the high sensitivity of the BAL analysis, there are still some controversies regarding how many haemosiderophages should be present to diagnose EIPH (Gold et al., 2018).

The first study on EIPH pathology was published in 1987 by a team led by Dr. O' Callaghan with a multi-disciplinary approach, including scintigraphy, CT scan and microradiography (O'Callaghan, Pascoe, Tyler, & Mason, 1987a). The study was carried out on 26 Thoroughbred horses retired from racing with previous EIPH history. Macroscopically, EIPH manifests as bilateral dark blue to dark brown discolouration of caudo-dorsal pleura and lung, usually confined to the caudal compartment. These areas showed increased consistency and were inflating slowly during experimental inflation compared to unaffected airways. Furthermore, a large proliferation of pleural vessels was evident, mostly co-localised with the abovementioned pulmonary lesions.

Before following with the description of the histological lesions, a little revision of average equine pulmonary anatomy is needed. The lung in horses displays well-defined and disorganised interlobular septa, which usually originate from a markedly vascular pleura, from which it deepens within the pulmonary parenchyma. The distal airways are mainly composed of terminal bronchioles and fewer, less distinct, respiratory bronchioles, between which collateral air ventilation seems plausible

due to the haphazardly arranged septal organisation. Understanding the vascularization of the equine lung is pivotal to understanding EIPH pathogenesis. As in other species, three are the vessels that allow blood circulation throughout the lung and are almost always located near the bronchi and bronchioles (see below).

: The pulmonary artery originates from the right ventricle and distributes high-flow low-pressure blood directly to the pulmonary capillary bed. As for other arteries, it shows a prominent tunica media, whose thickness progressively is reduced; with specific stains (e.g. Verhoeff Van Gieson), it reveals an internal (between tunica intima and media) and external (between T. media and T. adventitia) elastic circumferential laminas.

The bronchial artery is a part of the systemic circulation (characterised by low flow and high resistance) and supplies blood to the pulmonary artery and pulmonary vein *vasa vasorum*, bronchi and terminal bronchioles. In the horse, the bronchial artery supplies blood to the pleura, the interlobular septa and bronchioles. A small number of anastomoses are also present at the bronchiolar level connecting bronchial and pulmonary arterioles. Morphologically, it resembles the pulmonary artery, but is usually smaller and displays only an evident external elastic lamina with an absent/indistinct internal one.

Pulmonary veins receive entirely the venous blood drained from the lung; theyre satellites of the pulmonary arteries following the bronchioles topography. However in proximity of the lung centre, these vessels deviate from the airway direction and follow a more independent path towards the hilum. They usually are much thinner than arteries, and only the larger ones display an external elastic lamina (McLaughlin, Tyler, & Canada, 1961).

Lymphatic system is still not fully characterised and understood.

O' Callaghan characterised EIPH histologically individuating three hallmark lesions mostly restricted to the dark blue discoloured areas:

1. Accumulation of intralveolar, peri-bronchiolar and peri-vascular haemosiderophages

2. Thickened bronchioles showing distorted anatomy

3. Increased connective tissue showing increased vessel density and occasional basophilic staining.

Areas showing infiltration of eosinophils were also observed but separated from the other three processes. In the most severely affected location, few arteries, especially the ones with increased T. adventitia, exhibited fragmentation of the internal and external elastic membranes associated with disruption of the vascular smooth muscle. The latter lesion was interpreted as an arterial degenerative change, probably due to hypertension, differently from the following studies.

With all these findings, supported by other examinations, O Callaghan described convincingly that the primary lesion of EIPH consisted of chronic pulmonary haemorrhages, generalised fibrosis, angiogenesis and distorted bronchioles, which was interpreted as a potential sequela of bronchiolitis. Bronchiolitis of some sort was therefore hypothesised as the possible cause of EIPH: he believed that pulmonary inflammation stimulated the angiogenesis and induced less compliant alveoli due to partial airway obstruction which could have led to higher transmural pressure at the alveolar capillary interface, eventually resulting in capillary haemorrhage. Nevertheless, this hypothesis was not verified in that paper.

Another key paper on EIPH pathogenesis was the article by West et al. published in 1993 (West et al., 1993). The authors used a treadmill to make three TB horses gallop before examining their lungs through histology and electron microscopy. Several changes were observed: erythrocytes were frequently observed within the interstitium and the alveolar lumina, interruption of both endothelial and alveolar lining was recorded but with intact basement membrane, in addition to oedema and fluid-filled protrusion within the capillary lumen were also observed. All these changes are strong evidence of stress failure due to high pressure. Leukocytes and platelets frequently plugged the endothelial and alveolar gaps, indicating that these changes were secondary to *intra-vitam* capillary injuries. These defects were likely responsible for the extravasated erythrocytes and oedema observed in the alveolar lumina;

the fluid-filled protrusion in the capillary lumina are not well-understood changes but are also described in experimentally induced pulmonary hypertension in a rabbit model (Tsukimoto, Mathieu-Costello, Prediletto, Elliott, & West, 1991). West et al. suggested that a high transmural pressure at capillary level was at the base of EIPH pathogenesis. This theory was finally confirmed in the following years with two important papers. The first one aimed to measure the mean pulmonary arterial pressure in horses exercising, which could be greater than 95 mm Hg during maximal exercise on a treadmill. Similarly, also wedge arterial pressure increased consistently (Manohar, Hutchens, & Coney, 1993). Despite such high hydrostatic pressure, oedema was not observed in these horses. Its absence was probably due to the lymphatic system, which can remove excess fluid production, and interrupting pulmonary oedema formation. The capillary pressure value alone looked pretty high but acquired even more importance after 1997. In that year, an elegant *ex vivo* study measured the transcapillary pressure required to break down the pulmonary capillary wall (i.e. 75 -100 mm Hg), which was lower than the actual pressure in galloping horses (Birks, Mathieu-Costello, Fu, Tyler, & West, 1997). These data didn't consider the alveolar (pleural) pressure, which usually becomes negative during exercise up to -64 mm Hg, further increasing the transmural pulmonary capillary pressure (Jones et al., 2002). Upper obstruction of the airways has also shown to increase negative intra-pleural pressures during inspiration, further worsening the difference of pressure between alveolar and capillary lumina. The latter could explain why some horses not subjected to maximal exercise, hence not developing such a dramatic rise in pulmonary arterial pressure, can still suffefrom EIPH (Ducharme et al., 1999). In light of these findings and supported by histology and electron microscopy changes consistent with capillary stress failure, EIPH was thought to be fully understood; nevertheless, some features of this disease went undetected until another key study was published in 2008 (Williams et al., 2008). Williams and colleagues described all the changes described by O' Callaghan almost twenty years before in seven thoroughbred horses. However, they found that something was happening to small calibre veins: these vessels showed larger amount of

collagen within the T. adventitia, circumferentially restricting the lumen. Such morphology was similar to the “small arteries showing fragmented and duplicated elastic membranes” described by O’ Callaghan, potentially suggesting the misidentification of small venules for small arteries. Such change was highly similar to a veno-occlusive remodelling, as described in human pulmonary veno-occlusive diseases, which manifest with septal fibrosis, marked haemosiderin accumulation and proliferation of bronchial vessels (Pietra et al., 1989); such vascular changes were therefore called vascular remodelling (VR). This change was a striking new finding in EIPH. Its distribution was further studied in a following study a few years later, and its distribution was further studied in a following study in 10 EIPH racehorses (Williams et al., 2013). Williams discovered that VR and haemosiderin were significantly more frequent in the dorsal lung when compared to the ventral and significantly higher in number in the cranio-caudal direction. Interestingly, interstitial fibrosis never occurred without haemosiderin and VR. VR was most of the time associated with haemosiderin or fibrosis and itwas observed alone, without haemosiderin, only when VR severity was mild. Only a small number of samples displayed haemosiderin in the absence of VR. These findings indicate that VR could play a key role in the EIPH pathogenesis. Its distribution also mirrors the blood flow distribution during horse exercise, where blood doesn’t follow gravity and is mostly redistributed throughout the dorsal areas (Bernard et al., 1996). Williams also described other lesions: pleural vessels displayed multifocally T. media and T. intima hyperplasia often without any detectable elastic lamina (Williams et al., 2008); furthermore, areas of interstitial oedema and fibrosis were also present in areas with concomitant severe EIPH (Williams et al., 2013). In conclusion, the hypothesis regarding EIPH’s pathogenesis claims that VR occurs in the same areas where the blood flow is higher, suggesting that VR ensues secondarily to the intermitting rise of pulmonary pressures, as occurs in strenuous exercise. Such a change (i.e., collagen deposition around venules) makes the vessel less compliant and stiffer, raising the pressure backwards and leading to rupture in the locus minoris resistentiae (i.e., the capillary). The continuous and frequent capillary

bleeding is likely responsible for the accumulation of haemosiderin, which probably provokes interstitial fibrosis.

All the newest articles focus on vascular pathology and capillary stress, relegating inflammation to a marginal role in EIPH pathogenesis, unlike many of the 80s articles were hypothesizing. More recent studies have shown some controversies: a possible correlation between EIPH and inflammation has been hypothesised, claiming that EIPH induce inflammation (Michelotto et al., 2011) or that inflammation could be the cause of EIPH (O'Callaghan, Pascoe, Tyler, & Mason, 1987b). It is now widely accepted that the cause of EIPH is the VR, as many racehorses show EIPH without any inflammatory changes. Nevertheless, horses also experiencing a pre-existing pulmonary inflammatory condition are observed to bleed more under experimental conditions (McKane & Slocombe, 2010), possibly indicating that with a not yet fully understood mechanism, inflammation can play a role in EIPH; nevertheless, inflammation is not necessary to promote EIPH.

Other predisposing factors for EIPH have been discovered, although some controversial data are present in the literature (Hinchcliff et al., 2010). Considering its pathogenesis, it is reasonable to think that the greater the exercise intensity, the greater the severity of the bleeding. A positive correlation has been found between EIPH prevalence and the type of race/exercise [with racing (compared with breezing) (Preston, Riggs, Singleton, & Troedsson, 2015) hurdle races (compared with flat racing) (Newton, Rogers, Marlin, Wood, & Williams, 2005), and jump height (Bonomo et al., 2019)]. Older age is considered a risk factor associated with EIPH (Takahashi, Hiraga, Ohmura, Kai, & Jones, 2001). Still, this condition could be due to a higher number of training sessions and races rather than an independent risk factor. Indeed,EIPH lesions have also been describedin horses younger than 22-month-old after low-intensity exercise (Oikawa, 1999), suggesting that exercise and age are not the only risk factors involved. A surprising predisposing factor for epistaxis is a cool environmental temperature, lower than 20°C, ,but the exact mechanism responsible for this predisposition has not been clarified yet (Hinchcliff et al., 2015). Genetic predisposition and

hereditability of EIPH are fields not yet explored. A recent work on 72 thoroughbred horses highlighted an association between cell stiffness and the blood flow linked gene to EIPH risk (Blott, Cunningham, Malkowski, Brown, & Rauch, 2019).

Blood within the airways lumina is not only a feature of EIPH. It is well known that widespread pulmonary haemorrhage is a common cause of sudden death in horserace industries, as reported in previous studies (Gunson, Sweeney, & Soma, 1988). This condition has quite recently been renamed "Exercise associated fatal pulmonary haemorrhage" (EAFPH) but has been for a long time also been called "fatal EIPH". EAFPH certainly seems more appropriate as the old name as EIPH refers to a widespread condition of a racehorse and is not necessarily linked with sudden and fatal outcomes. EAFPH is responsible for the sudden death of horses while running or within a short timeframe after the race, without any premonitory clinical signs. The carcasses of these horses are often in advanced decomposition when the *post-mortem* examination takes place, for the high body temperature, due to the intense exercise, or to high environmental temperature. EAFPH is characterised by multifocal to coalescing areas of pulmonary haemorrhage and oedema, often fill the lower airways and trachea and pour out from the nostrils. These lesions are more severe in the caudal lobe. However, they can spread more cranially and involves a large part of the pulmonary parenchyma, with common raised haemorrhagic areas occurring along the margins of the lungs. Histologically, this disease manifests with a large accumulation of extravasated erythrocytes expanding multiple locations (e.g. septa, alveolar spaces) in conjunction with markedly congested vessels. Due to the usually autolysed carcase and severed widespread haemorrhage, evaluation of differentanatomical structures (i.e. vessels, alveoli) is hampered, making this disease quite challenging to investigate thoroughly (Caswell & Williams, 2015).

In a study by Lyle et al., (2011), 268 sudden deaths cases were used, and a definitive diagnosis was reached in slightly more than half of them (53%). Severe pulmonary haemorrhage was deemed cause of sudden death in 18% of the cases. Nevertheless, the authors highlighted that the

interpretation of pulmonary haemorrhage, pulmonary oedema and congestion was variable within the centres included in the study. These differences are related to the variation in sampling protocol and the almost impossible way to quantify the amount of pulmonary haemorrhage and oedema objectively; in fact, all these changes (oedema, haemorrhage, and congestion) could also be interpreted as secondary to cardiac pathologies rather than to represent primary lung disease. Furthermore, pulmonary congestion, haemorrhage and oedema are often amplified by euthanasia after musculoskeletal severe impacts, further complicating the achievement of a final diagnosis. At last, EIPH and EAFPH present overlapping features, apart from the overwhelming and primary role of alveolar bleeding in the latter.

So far, no relationship has been reported between these two pathologies. Horses who died from severe pulmonary bleeding have shown inconsistent presence of haemosiderophages, suggesting that the two diseases do not share an identical pathogenesis (Lyle et al., 2011). While the pathogenesis EIPH has been largely and deeply studied, the cause of EAFPH is still not understood. Someone says that such pulmonary haemorrhages are due to cardiac arrhythmias (Kiryu et al., 1999), while other studies believe that they are the expression of cardio-vascular failure related to exercise (Gelberg, Zachary, Everitt, Jensen, & Smetzer, 1985). Acute cardiac failure appears unlikely to some authors as such a dramatic increase in pressure should lead to a hypothetic pulmonary oedema phase (which has not been reported) before such massive bleeding could occur. It has also been hypothesized that the contraction of post-capillary sphincterscould explain such a sudden rise in pulmonary capillary pressure; nevertheless, such anatomical structure has never been confirmed in horses (Caswell & Williams, 2015).

Pulmonary bleeding in racehorses: a gross, histological and ultrastructural comparison of exercise induced pulmonary haemorrhage and exercise associated fatal pulmonary haemorrhage.

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Introduction

Pulmonary haemorrhage is a common clinical condition and post-mortem finding in equine athletes. The term “exercise induced pulmonary haemorrhage” (EIPH) was coined by Pascoe et al. in 1981 to describe epistaxis of pulmonary origin, especially after exercise (Pascoe et al., 1981). Since the original paper, many studies have characterised EIPH in flat or jump racehorses, as well as in horses participating in other sports, such as barrel racing and endurance(Gold et al., 2018) (Tarancon et al., 2019). EIPH is believed to be an important cause of reduced athletic performance, especially in cases with severe bleeding (S. Sullivan & Hinchcliff, 2015).

Typical post-mortem findings in horses with EIPH is bilateral dark blue to light brown discoloration of the caudo-dorsal pleura and lung. These discoloured areas are microscopically characterized by variable accumulation of hemosiderophages and fibrosis of multiple pulmonary micro-compartments, including the interstitium, the pleura, and small (100-200 μm in diameter) intralobular veins, the latter known as vascular remodelling (VR) (Caswell & Williams, 2015). More specifically, vascular remodelling is characterized by transmural accumulation of collagen with occasional narrowing of the lumen. In a study on Thoroughbred (TB) horses with EIPH, the distribution of VR in small intralobular veins was most prevalent in the caudo-dorsal pulmonary parenchyma, where EIPH lesions were more frequent (Williams et al., 2013). Additionally, accumulation of hemosiderophages and interstitial fibrosis almost never occurred in the absence of VR. All these histological findings suggested a central role for the VR of small intralobular veins in the pathogenesis of EIPH. It was hypothesized that the concentric rings of collagen affecting small intralobular pulmonary veins (i.e. VR) developed in response to the high blood pressure during exercise, leading to reduced vascular compliance and higher blood pressure in the capillaries, with consequent capillary breakdown (Williams et al., 2008). This hypothesis seems to be

reinforced by the co-localization of VR in the same locations where the blood is redistributed during exercise (i.e. caudo-dorsal areas) (Williams et al., 2013). Other histologic features reported less frequently are bronchiolar distortion, eosinophilic infiltration, basophilia and Perls Prussian blue positivity of collagen and elastic fibres and interstitial oedema (O'Callaghan et al., 1987b), (Williams et al., 2013), (Williams et al., 2008). Ultrastructurally, extravasated erythrocytes and oedema within alveolar wall, and gaps between type I pneumocytes and endothelial cells, with basal membrane preservation, were observed (West & Mathieu-Costello, 1994).

In comparison with EIPH, exercise associated fatal pulmonary haemorrhage (EAFPH) is the term first coined in the reference book in 2015 to describe a condition characterized by fatal pulmonary haemorrhages in racehorses, and was previously listed amongst the leading causes of sudden death under the term “pulmonary hemorrhages” (Caswell & Williams, 2015) (Lyle et al., 2011). EAFPH is characterized by sudden death during or immediately after the end of exercise (Caswell & Williams, 2015). *Post-mortem* features of this condition include widespread bilateral pulmonary oedema and haemorrhage, that is more evident in caudo-dorsal regions, accompanied by occasional subpleural pulmonary infarcts and copious blood-tinged froth and frank blood pouring out from both nares when the cadaver is rested on a side. Histologically, EAFPH is characterized by severe haemorrhages involving multiple pulmonary micro-compartments, including, but not limited to, alveoli and lobular septa and diffuse pulmonary congestion. Fatal pulmonary haemorrhage is one of the most frequent causes of sudden death in racehorses and such lethal pulmonary bleeding has been reported long before the acronym EAFPH was coined (Lyle et al., 2011) (Gunson et al., 1988). The occurrence of acute cardiac failure or spastic contraction of pulmonary post-capillary sphincters have been listed as possible pathogenetic mechanisms for the occurrence of EAFPH, but this has not been proven (Caswell & Williams, 2015).

It is not fully understood whether EAFPH horses show concomitant EIPH lesions (i.e. hemosiderophages accumulation, VR and fibrosis). The aim of the present study was to compare anamnestic data, gross, histologic, and ultrastructural findings of racehorses that died and had lesions compatible with EAFPH, of racehorses that died of causes not related to pulmonary pathology but had incidental EIPH pulmonary lesions (under light microscopy), and of horses that died without pulmonary lesions (control). We hypothesized that racehorses with EAFPH would show significantly less chronic changes and VR than racehorses with EIPH, suggesting, if confirmed, that histopathological lesions of EIPH are not predisposing to fatal pulmonary haemorrhagic events (EAFPH).

Materials and Methods

All horses included in the study were submitted for post-mortem examination to the Laboratory of Veterinary Pathology, University of Liverpool, for diagnostic purposes. Three groups of horses were included in this study. The EIPH group was composed of TB racehorses that were euthanized or died naturally from non-cardiopulmonary conditions (e.g. catastrophic fractures), but showed characteristic histologic lesions of EIPH. The microscopic inclusion criterion for this group was the presence of at least one cluster of three hemosiderophages within the bronchial or bronchiolar lumen in 10 fields of view with a 10X objective (31.4 mm2). The EAFPH group was composed of TB racehorses that died during or a few (~ 0-4) hours after a competition with gross and microscopic lesions compatible with EAFPH and in the absence of other potentially fatal lesions. The macro and microscopic criterion for this group was the presence of large volume of uncoagulated blood within the airways and widespread pulmonary haemorrhage confirmed histologically. The control group was composed of horses (TB and other breeds) that were euthanized due to or that died from non-pulmonary related causes and showed no microscopic lesions compatible with EIPH.

Twenty-five horses were included in the study. Five horses were included in the control group: two TBs, one Irish draft, one Welsh Cob and one Arabian. EIPH and EAFPH groups were composed of 10 TB racehorses each.

For each racehorse, anamnestic data including race type (i.e. flat, jump), total number of races run, age, sex and days passed since last race, were recorded and compared between EIPH and EAFPH groups. Local environmental humidity and temperature at the time of each race were recorded. Race data were retrieved from the Racing Post website (https://www.racingpost.com), while the weather data were retrieved using the closest meteorologic station, on the weather underground website (https://www.wunderground.com).

Each horse underwent a thorough post-mortem examination, involving all body systems conducted by GR with another senior board-certified pathologists (LR, ER, RV). Systematic measurement of the heart/body weight ratio, cardiac ventricular diameters, and wall thicknesses (i.e. left ventricular free wall, interventricular septum and right ventricular free wall) were also included. Measurements were conducted on a transverse section at one third of the heart height, measured from the cardiac apex to the cardiac base. The larynx was fully evaluated for the presence of potential post-surgical scars, muscular atrophy and for any other abnormalities. Samples for histology were collected from every horse, and included: brain (frontal cortex, hippocampus, cerebellum, and choroid plexi), stomach (*margo plicatus* area), small intestine, large colon, liver, epiglottis, lungs, heart, ascending aorta, kidney, and spleen. From both lungs, samples of caudal, cranial, dorsal, and ventral locations (Fig. 1) were collected.

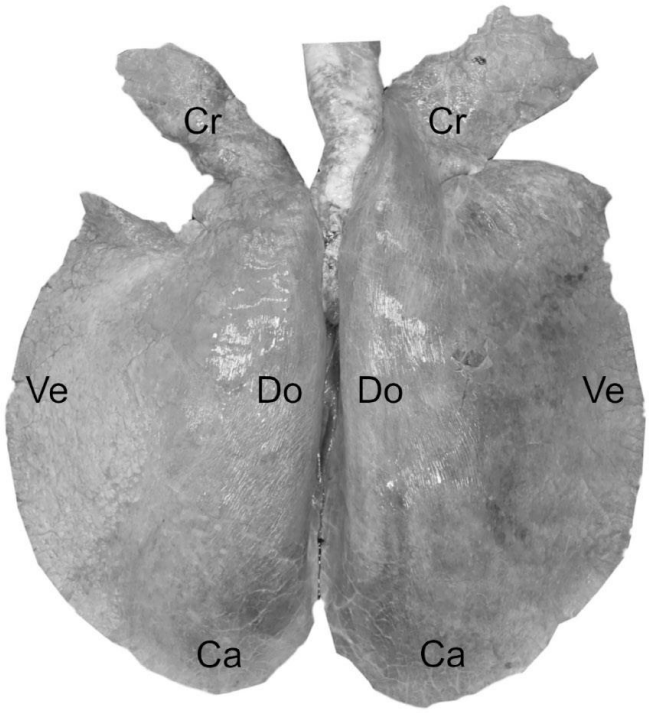

Figure 1: Schematic representation of the lung sampling protocol, illustrating the anatomical locations. Cr, cranial; Ve, ventral; Do, dorsal; Ca, caudal.

From the heart, full thickness slices from the right and left ventricular free walls, interventricular septum, and myocardium adjacent to the fibrous trigone (atrio-ventricular node) were collected. All tissue samples were fixed by immersion in 10% formalin, pH 7.4 for at least 48 hours, paraffin embedded, and cut to produce 4 µm thick sections, before staining them with haematoxylin & eosin (H&E), as per standard protocol. To assess the presence of hemosiderin and VR in the pulmonary sections, Perl's Prussian blue and picrosirius red staining, respectively, were also performed.

Ultrastructural analysis using transmission electron microscopy was performed on the dorsal part of the left lung of horses from each group, with special emphasis on the alveolar wall and intralobular veins. Cubes of lung tissue (1 mm^3) were fixed first in 2.5% glutaraldehyde and then in osmium tetroxide 1%, followed by uranyl acetate staining. After dehydration the sections were embedded in epoxy resin that was polymerized at 60^oC overnight. Semi-thin, 0.5 µm thick, toluidine blue 1% stained sections were produced to assess target areas for ultrastructural

analysis. Ultra-thin sections (75 nm) were then mounted on copper grids and examined under a Philips EM208S (FEI UK, Cambridge, UK) transmission electron microscope (TEM).

For the pulmonary gross pathology findings, a scoring system ranging from 0 (absent) to 3 (severe) was applied to each type of finding, including blue-brown caudo-dorsal discoloration, rib imprints, fibrous tags, pleural haemorrhages, pleural plaques, airway oedema and haemorrhage, cranial lobe reddening and oedema, and laryngeal haemorrhages. Pleural haemorrhages were defined as raised, red to black, well-demarcated lesion restricted to the pleura. Pleural plaques were defined as raised, well-demarcated, pink to white opaque lesions restricted to the pleura and obscuring the underlying pulmonary parenchyma. Laryngeal haemorrhages included variable degrees of laryngeal reddening and raised mucosal haemorrhages (Table 1).

	0	1	2	3	Notes
Blue-brown caudo-dorsal discolouration	None	Multifocal to coalescing, occurring in one or two lung, restricted to the caudal third of the lungs	Multifocal to coalescing, forming large continuous areas of discolouration extending not exceeding the cranial half of the lungs	Extension exceding the cranial half of the lungs, bilateral and also exteding to the ventral areas.	
Rib imprints	none	barely appreaciable	mostly monolateral, visible on the surface of the caudal lung	bilaterally evident over both lungs, up to the cranial half of the lung	
Pleural hemorrhages	none (< 5% of total lung surface)	Mild, mostly scattered and restricted to the lateral margins of one lung (5-20% of total dorsal pleural surface)	Moderate, forming larger coalescing foci (20-40% of total dorsal pleural surface)	Multifocal to coalescing confluent into a single large plaque covering a vast area of one or both lungs (More than 40 % total dorsal pleural surface)	
Pleural plaques	none (< 5% of total lung surface)	Mild, mostly scattered and restricted to the lateral margins of one lung (5-20% of total dorsal pleural surface)	Moderate, forming larger coalescing foci (20-40% of total dorsal pleural surface)	Multifocal to coalescing confluent into a single large plaque covering a vast area of one or both lungs (More than 40 % total dorsal pleural surface)	
Fibrous tags	None	Rare isolated small tags on margin of a single lung lobe	Multiple, discontinuous segments of the lung ventral margins	Large and continuous areas of ventral margins on both lung showing tags	
Airway hemorrhages	None	Small volume of frank blood, not extending past the bronchial bifurcation	Moderate volume of frank blood extending to the trachea	Large volume of frank blood extending to the nostrils	In case of concomitant pulmonary edema, score is 1, 2 and 3 if the oedema is, respectively, pink, red, dark red
Airway edema	None	Small volume (up to the bronchial bifurcation) of white froth	Moderate volume (up to the larynx) of white to pale red pulmonary oedema. A small volume of frank blood is possible	Large volume (up to the nostrils) of white to pale red pulmonary oedema.	
Cranial lobes reddening and edema	Cranial lobes shows minimal thickened interstitial pattern and unilateral mild reddening	Cranial lobes show mild unilateral, dark reddening or increased interstitial thickening	Cranial lobes display moderate bilateral dark reddening with unilateral dark red raised pleural lesions and/or increased interstitial thickening.	Cranial lobes display severe bilateral dark reddening with occasional dark red raised pleural lesions and increased interstitial thickening extending also to the cranial part of the caudal lobes	
Laryngeal hemorrhages	The larynx shows none or mild congestion (<50% larynx mucosa showing reddening)	The larynx is extensively congested (> 50% larynx mucosa showing reddening)	The larynx is extensively congested and multiple mucosal raised hemorrhagic foci are present.	The larynx is diffusely congested and multiple raised haemorrhagic foci forming large coalescing haemorrhagic plaques are present on the mucosa or in the surrounding soft tissue.	

Table 1: Macroscopic respiratory finding scoring system

Scoring of the gross changes was conducted by one of the authors (GR, blinded to sample identity). For the pulmonary microscopic findings, a scoring system ranging from 0 (absent) to 3 (severe) was applied to each type of finding (Table 2).

		0	1	2	3	Notes
Pleura	Hemorrhage	none	Scattered erythrocytes not forming clusters are observed in at least 2 10X HPFs.	Moderate hemorrhages forming clusters are observed in 2-3 10X HPFs.	Large hemorrhages expanding the pleura are observed in 4-5 10X HPFs.	
	Hemosiderophages	none	Scattered hemosiderophages not forming clusters are observed in< 2 10X HPFs.	Moderate number hemosiderophages forming rare clusters are observed in 2-3 10X HPFs.	Numerous hemosiderophages forming common clusters are present in 4-5 10X HPFs.	
	Iron encrustation	none	Scattered collagen fibers display iron encrustation in < 2 10X HPFs	Multiple collagen fibers display iron encrustation in 2-4 10X HPFs.	Numerous fibers showing iron encrustation are present in more than 4 10x HPFs.	
Septa	Hemorrhage	none	Scattered isolated erythrocytes are present in<2 10 HPFs.	Multiple erythrocytes forming clusters are observed within the septa in 2-3 10X HPFs.	Large amount of erythrocytes expanding the septa are present in 2-3 10X HPFs.	
	Hemosiderophages	none	Scattered hemosiderophages not forming clusters are observed in < 2 10X HPF.	Moderate number of hemosiderophages forming rare clusters are observed in 2-3 10X HPF within the septal collagen.	Numerous hemosiderophages forming common clusters are present in 4-5 10X HPFs.	
	Iron encrustation	none	Scattered collagen fibres display iron encrustation in < 10% of the septa	Multiple collagen fibres display iron encrustation in 10-30 % of the septa	Numerous collagen fibres display iron encrustation in > 30% of the septa.	
Bronchioles	Intraluminal hemorrhage	none	Small number of erythrocytes are observed within the lumen of at least 3 bronchioles.	Moderate number of erythrocytes partially occluding the lumen is present in > 3 bronchioles.	Large number of erythrocytes occluding the lumen are present in more than 3 bronchioles.	
	Peri-bronchiolar hemosiderophages	none	Scattered Hemosiderophages are observed in < 2 bronchioles.	Moderate number of hemosiderophages forming rare clusters are observed in 2-3 bronchioles.	Numerous hemosiderophages forming common clusters are present in 4-5 bronchioles, occasionally spilling into the lumen	
	Peribronchiolar inflammatory cells (I.C.)	Very rare I.C. are observed in not more than 2 bronchioles.	Scattered I.C. are observed, forming clusters extending to the submucosa present in at least two bronchioles.	Moderate number of I.C., forming up to 1 continuous layer within the airway stroma/l.p. in at least two bronchioles.	Large number of infl. cells, forming > 1 layers within airway stroma/l.p. in at least two bronchioles.	focal clusters of lymphocytes and/or plasma cells (BALT) are not considered I.C.
	Peri-bronchiolar iron encrustation	none	Scattered peri-bronchiolar collagen fibers display iron encrustation in 1 bronchiole.	Multiple peri-bronchiolar collagen fibers display iron encrustation in 2-3 bronchioles.	Numerous peri-bronchiolar collagen fibers display iron encrustation in 4-5 bronchioles.	
Alveolar parenchyma	Hemorrhage	Present in less than 10% of parenchyma.	Present in 10-30% of parenchyma. Usually, composed of sparse, loosely packed erythrocytes.	Present in 30-50% of parenchyma. Usually, forming occasional clusters and separated by the septa from empty alveoli.	Present in > 50% of parenchyma, forming large accumulation expanding the alveoli.	
	Hemosiderophages	none	Scattered hemosiderophages (not forming clusters) are observed in < 2 10X HPF. Perls usually needed to confirm their presence.	Moderate number of Hemosiderophages forming clusters are observed in 2-4 10X HPF. Perls not needed to observed them	Large clusters of hemosiderophages are commonly present in 4-5 10X HPF.	
	Proliferation of type II pneumocytes	none	Scattered individual subpleural alveoli lined by single cordon in < 2 10X HPFs.	Cluster of more than 3 subpleural alveoli lined by discontinuous cordons of type 2 pneumocytes	Large coalescing foci of subpleural alveoli entirely lined by type 2 pneumocytes in more than 2 10X HPFs.	
	Iron encrustation	none	Isolated collagen bundles display iron encrustation in 1 10X HPF	Multiple collagen bundles display iron encrustation in 2-3 10X HPF	Multiple collagen bundles display iron encrustation in >3 10X HPF	
Vessels	Vascular remodeling	none	Rare, detected in 1-2 vessels mainly characterised by mild thickening.	Multiple, detected in 3-6 vessels and in 2-3 10X HPF with evident marked adventitial thickening	Common, detected in more than 7 vessels and/or in more than 4 10 X HPF	
	Adventitial iron encrustation	none	Rare, detected in 1-2 vessels in less than 2 10X HPF.	Multiple, detected in 3-6 vesssels and in 2-3 10X HPF.	Common, detected in more than 7 vessels and/or in more than 4 10 X HPF	
	Adventitial Hemosiderophages	none	Scattered hemosiderophages (not forming cluster) are observed in 2 vessels.	Moderate hemosiderophages forming rare clusters are observed in 3-6 vessels.	Large hemosiderophages forming common clusters are present in more than 6 vessels	

Table 2: Microscopic respiratory finding scoring system

For each individual pulmonary location (cranial, caudal, ventral, and dorsal locations), the histological scores were recorded as mean score of the left and right lung. For each tissue section, evaluation of the pulmonary parenchyma was performed by analysing 5 random, non-

overlapping 10x fields (15.7 mm^2), whereas every portion of interlobular septa and pleura present in the slides was analysed for these micro-compartments. Bronchiolar evaluation was performed by analysing 5 random bronchioles in each tissue section. Vessels were classified into pleural vessels (vessels within the pleura), pulmonary artery branches (larger vessels adjacent to the bronchioles or bronchi), and intralobular small veins (100-200 µm calibre vessels not adjacent to any septa, pleura, bronchioles, or bronchi) according to their morphology and localization. For each type of vessel, a maximum of 10 random non-overlapping vessels were examined at 10x (31.4 mm^2). Basophilic discoloration of collagen and elastin coupled with positivity to Perl's Prussian blue staining was defined as iron encrustation. Vascular remodelling (VR) was defined as larger amount of adventitial collagen around small intralobular pulmonary veins (Williams et al., 2008) Bronchiolar inflammation was evaluated according to the presence of neutrophils, eosinophils, macrophages, lymphocytes, and plasma cells (the latter two when not forming lymphoid follicles) around bronchioles. An individual score (ranging from 0 to 3) for each parameter was calculated for each horse. A combined haemorrhage score was obtained by calculating the mean of the pleural, septal, airway, and alveolar haemorrhage scores for each pulmonary location. A combined hemosiderophage score was obtained calculating the mean of the pleural, septal, peri-bronchiolar, alveolar, and peri-vascular hemosiderophage scores for each pulmonary location and vessel type. A combined iron encrustation score was obtained calculating the mean of the pleural, septal, peribronchiolar, alveolar, and perivascular iron encrustation scores for each pulmonary location and vessel type. Vascular remodelling, airway inflammation and type II pneumocyte hyperplasia scores were evaluated individually (i.e. not combining scores from other micro-compartments) by calculating the mean score of each pulmonary location. Histological scoring was performed by one of the authors (GR, not blinded to sample identity).

Electron microscope images were evaluated qualitatively by searching for ultra-structural changes of alveolar septa (at least 2 areas per horse) and intralobular small veins (at least one vessel per horse). Alveolar septa were evaluated for morphological changes in the endothelial cells, type

I and type II pneumocytes, interstitial fibroblasts, and alveolar lumina. Small intralobular veins were evaluated for morphological changes occurring in the tunica intima, external elastic lamina, tunica muscularis, or tunica adventitia. In addition, cross and longitudinal sections of collagen bundle around small intralobular veins were examined in order to characterize both fibril diameter and D periodic band length. D periodic band length was calculated as the length of the polar, electron dense, segments visible on longitudinal sections of collagen fibrils. ImageJ software (https://imagej.nih.gov/ij/)(Collins, 2007) was used to calculate fibril diameter and D periodic band length of 30 representative images of cross and longitudinal sections of fibrils, coming from every horse evaluated with TEM.

GraphPad Prism version 9.3.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) was used for statistical analyses.

Normality of distributions was verified by means of Kolmogorov and Smirnov tests, and Bartlett's tests were applied to identify if standard deviations were significantly different among groups. Then, to compare scores obtained in the three examined groups, one way ANOVA followed by Tukey's multiple comparisons tests was performed for parametric results, whereas, for non-parametric data Kruskal-Wallis tests were applied, followed by Dunn's multiple comparison tests. Differences were considered significant for $P < 0.05$. Histological scores were also compared according to their pulmonary locations (i.e. cranial vs. dorsal vs. ventral vs. caudal), regardless of the horses' groups. For categoric variables (e.g. race type, and sex), correlation was assessed using a Pearson's chi square test.

Results

Anamnestic data are reported in Table 3 and Figure 2.

Horse ID	Group	Sex	Breed	Age (years)	Race type	Humidity (%)	Temperature ©	Euthanasia	Season	HW/BW ratio
1	Control	Gelding	Thoroughbred	3	N/A	N/A	N/A	yes	Winter	N/A
2	Control	male	Arab	7	N/A	N/A	N/A	yes	Summer	N/A
3	Control	Female	Thoroughbred	2	Flat	87	10.7	yes	Autumn	0.97
4	Control	Female	Irish Draughtx	17	N/A	N/A	N/A	no	Spring	0.66
5	Control	Male	Welsh	1	N/A	N/A	N/A	no	Spring	N/A
6	EIPH	Female	Thoroughbred	4	Flat	76	14	yes	Summer	0.94
7	EIPH	Gelding	Thoroughbred	8	Jump	73	20.5	no	Autumn	0.87
8	EIPH	Gelding	Thoroughbred	8	Jump	66	10	yes	Autumn	1.04
9	EIPH	Gelding	Thoroughbred	8	Jump	N/A	6.6	no	Autumn	N/A
10	EIPH	Gelding	Thoroughbred	12	Jump	92	10	yes	Spring	1.01
11	EIPH	Female	Thoroughbred	7	Jump	70	7	yes	Spring	1.19
12	EIPH	Gelding	Thoroughbred	8	Jump	47	15	yes	Spring	0.91
13	EIPH	Gelding	Thoroughbred	7	Jump	50	14	yes	Spring	1.00
14	EIPH	Female	Thoroughbred	7	Jump	90	9.1	yes	Winter	0.93
15	EIPH	Gelding	Thoroughbred	6	Jump	98	7.5	yes	Winter	0.94
16	EAFPH	Gelding	Thoroughbred	6	Flat	88	7.7	no	Autumn	0.99
17	EAFPH	Gelding	Thoroughbred	8	Jump	95	1.6	no	Winter	N/A
18	EAFPH	Gelding	Thoroughbred	8	Flat	75	3.9	no	Winter	0.97
19	EAFPH	Female	Thoroughbred	6	Jump	51	14.7	yes	Winter	0.88
20	EAFPH	Gelding	Thoroughbred	4	Jump	49	11	no	Spring	0.89
21	EAFPH	Female	Thoroughbred	9	Jump	89	9	no	Spring	1.00
22	EAFPH	Female	Thoroughbred	8	Jump	55	19	no	Summer	1.10
23	EAFPH	Gelding	Thoroughbred	7	Jump	89	7.6	no	Summer	1.07
24	EAFPH	Gelding	Thoroughbred	3	Flat	78	8.7	No	Winter	0.90
25	EAFPH	Gelding	Thoroughbred	5	Jump	96	8.5	No	Winter	N/A

Table 3. Anamnestic data for each horse.

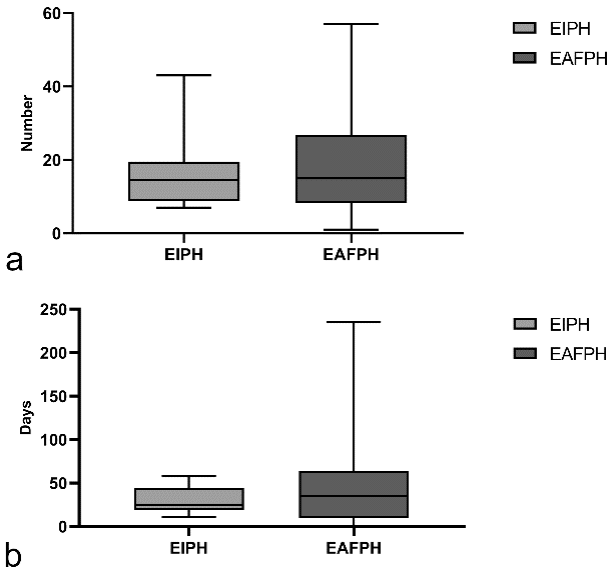

Figure 2: Comparison of race data between exercise-induced pulmonary hemorrhage (EIPH) (N = 10) and exercise associated fatal pulmonary hemorrhage (EAFPH) (N = 10) racehorses. a: number of total starts. b: days passed since last race. No significant difference observed.

Age and sex were not significantly different among all experimental groups. Racing and weather data were not significantly different between EIPH and EAFPH group. Heart/body weight ratios were available for 2, 9 and 8 horses from the control, EIPH, and EAFPH groups, respectively. Heart/body weight ratios were not significantly different among groups and ranged from 0.66-0.97 (mean 0.81), 0.87-1.19 (mean

0.98) and 0.88-1.1 (mean 0.97) in control, EIPH, and EAFPH groups, respectively.

Dark blue-light brown caudo-dorsal discoloration and airway haemorrhage scores were significantly higher in EIPH and EAFPH groups compared to the control group ($P = 0.0049$ and $P < 0.0001$, respectively). Histologically, pleural plaques were characterized by accumulation of collagen expanding the pleura and variable numbers of newly formed vessels, haemorrhage, hemosiderophage, and spindle cells (likely myofibroblasts). Pleural plaques were present in 0/5, 9/10, and 8/10 horses from the control, EIPH, and EAFPH groups, respectively. Pleural plaque scores were significantly higher in EIPH horses compared to the control group ($P = 0.0049$). Airway oedema was significantly greater in the EAFPH group compared to control group ($P = 0.0069$), but not in comparison to the EIPH group. Cranial lobe reddening and oedema scores were significantly different among all three groups ($P = 0.0002$), with the control group showing the lowest and the EAFPH group the highest scores. Laryngeal evaluation did not detect post-surgical scars nor muscular atrophy in any horse. The other macroscopic finding scores were not significantly different among groups (Fig. 3).

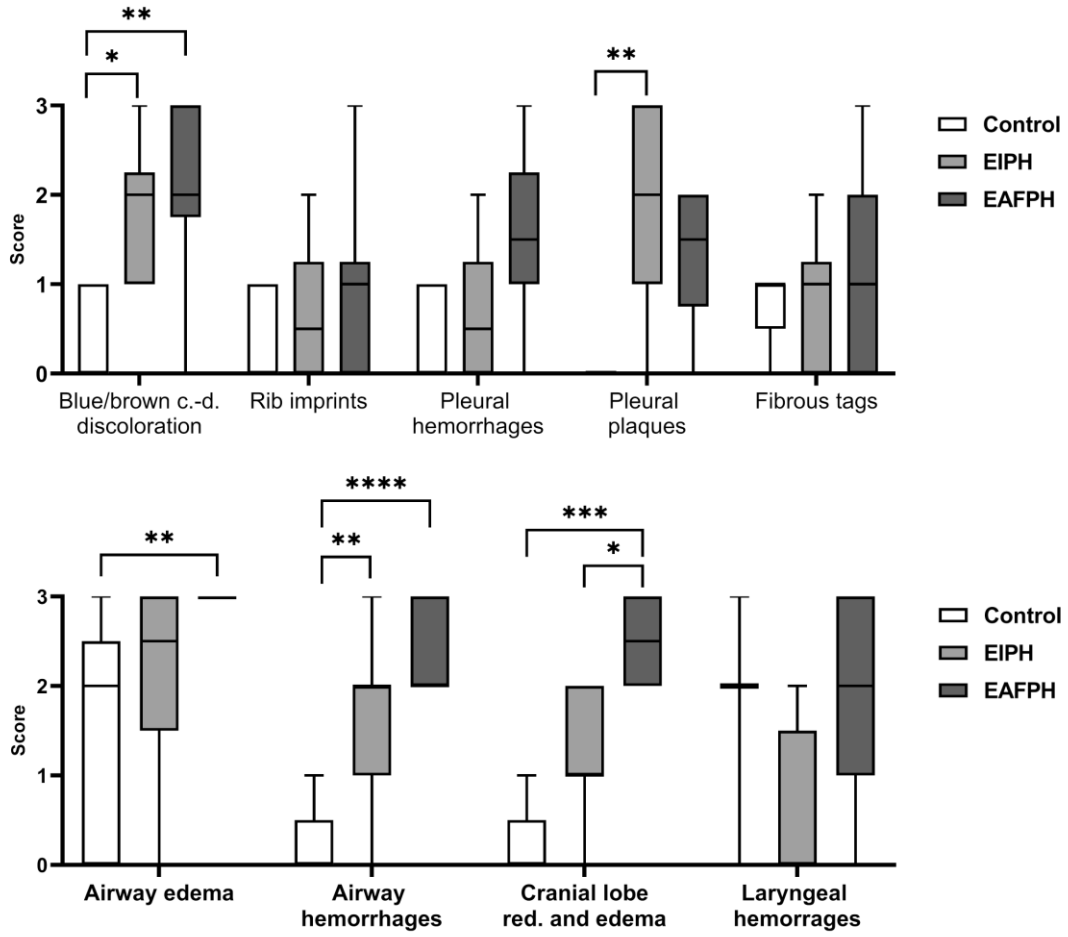

Figure 3. Comparison of macroscopic mean scores between control (N = 5), exercise-induced pulmonary haemorrhage (EIPH) (N =10), and exercise-associated fatal pulmonary haemorrhage (EAFPH) (N = 10) horses. EIPH and EAFPH horses showed higher scores for blue/brown discolouration, airway haemorrhages, pleural plaques, and cranial lobe reddening and oedema than control horses. EAFPH horse showed higher cranial lobe reddening and oedema score compared to EIPH horses. c.-d., caudo-dorsal; red., reddening. *P < .05. **P < .01. ***P < .001. ****P < .0001.

Regarding the histopathological examination, combined haemorrhage scores were significantly different among all groups (P < 0.0001), with the control group exhibiting the lowest and the EAFPH group the highest scores (Fig. 4).

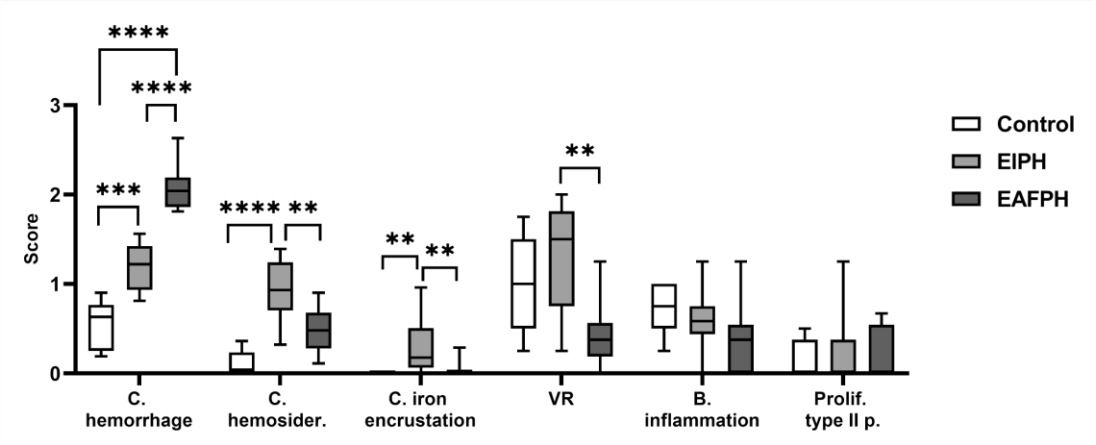

Figure 4. Comparison of microscopic mean scores between control (N = 5), exercise-induced pulmonary haemorrhage (EIPH) (N = 10) and exercise-associated fatal pulmonary haemorrhage (EAFPH) (N = 10) groups. EIPH horses exhibited higher scores of hemosiderophages, iron encrustation and VR compared to EAFPH horses. EAFPH showed higher haemorrhage score compared to all other groups. C., combined; hemosider., hemosiderin; VR, vascular remodelling; B., bronchiolar; Prolif., proliferation; p., pneumocyte. **P < .01. ***P < .001. ****P < .0001.

A similar trend was observed in all micro-compartments (Fig. 5).

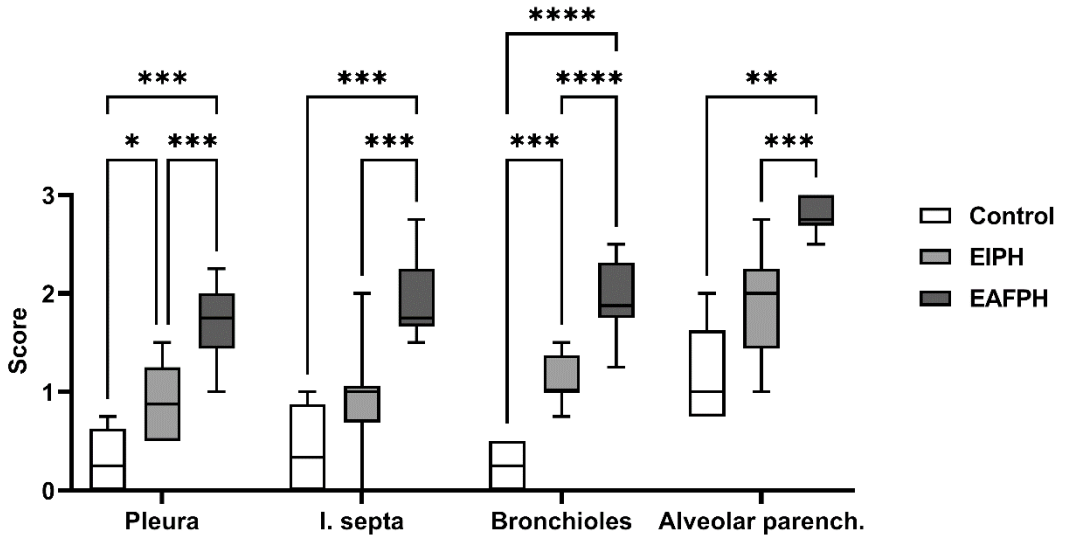

Figure 5. Histological haemorrhage mean scores divided by micro-compartment and group. Control (N = 5), EIPH (N = 10), and EAFPH (N = 10) horses. I = interlobular; parench. = parenchyma. * = P< 0.05; ** = P< 0.01; *** = P< 0.001; **** = P< 0.0001.

Combined hemosiderophage scores were significantly higher in the EIPH group compared to the other two groups (P < 0.0001). Aggregates of alveolar hemosiderophages (i.e. at least one) within the alveolar lumina were observed in 3/5, 10/10, and 10/10 horses from the control, EIPH and EAFPH groups, respectively. In the control group, hemosiderophages were more frequently observed in alveolar lumina while hemosiderophages were fewer to absent in the pleura, septa, peribronchiolar collagen and vessel adventitia. In the EIPH and EAFPH

groups, hemosiderophages were more frequently encountered in all the micro-compartments examined compared to control groups, although not always simultaneously. Hemosiderophages in EIPH horses were also more frequently observed within the pulmonary arteries and intralobular small veins adventitia than in EAFPH horses (Fig. 6).

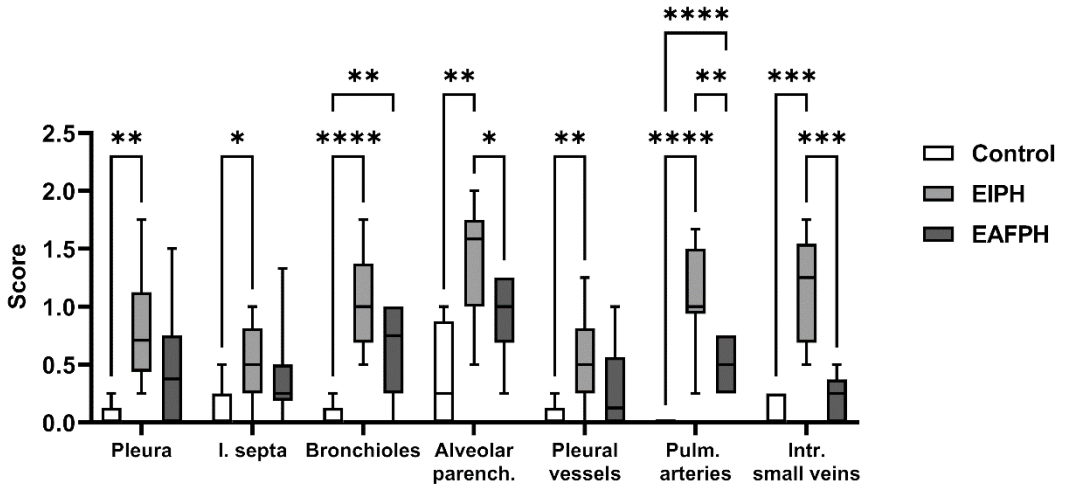

Figure 6. Histological hemosiderophage mean scores divided by micro-compartment and group. Control (N = 5), EIPH (N = 10), and EAFPH (N = 10) horses. I = interlobular; parench. = parenchyma; Pulm. = pulmonary; Intr. = intralobular * = P< 0.05; ** = P< 0.01; *** = P< 0.001; **** = P< 0.0001.

Iron encrustation was diffusely Perl's Prussian blue positive and Von Kossa negative (data not shown). Iron encrustation of, at least, a single micro-compartment (e.g. pleura or interstitium) was present in 0/5, 9/10, and 3/10 horses from the control, EIPH, and EAFPH groups, respectively. Combined iron encrustation scores were significantly higher in EIPH group compared to EAFPH and control groups (P = 0.0011), with no statistical difference between control and EAFPH groups. Iron encrustation of intralobular small veins was more frequently observed in EIPH horses compared to all other groups (Fig. 7).

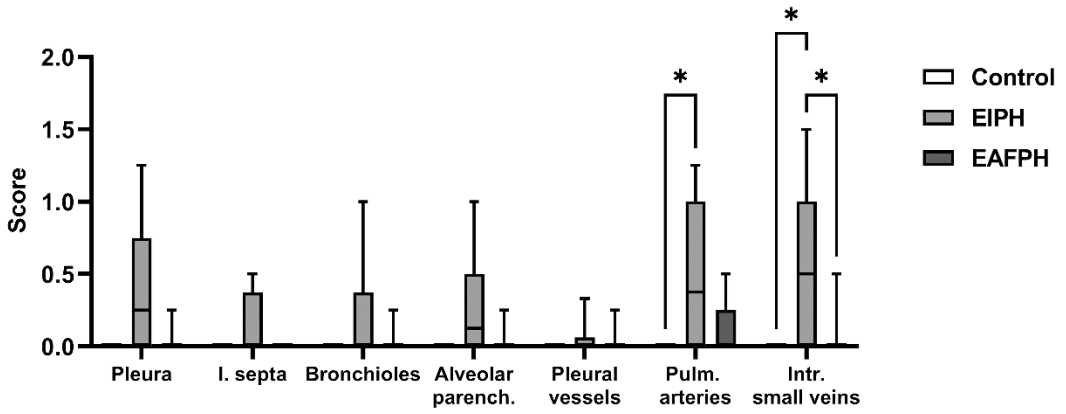

Figure 7. Histological iron encrustation mean score divided by micro-compartment and group. Control (N = 5), EIPH (N = 10), and EAFPH (N = 10) horses. I = interlobular; parench. = parenchyma; Pulm. = pulmonary; Intr. = intralobular; * = P< 0.05

VR was significantly higher in the EIPH compared to the EAFPH group (P = 0.002), but not compared to the control group. Type II pneumocyte hyperplasia and bronchiolar inflammation were not significantly different among the groups (Fig. 4).

Histopathological scores were compared between pulmonary locations (caudal, ventral, cranial and dorsal) for all groups in combination. Total hemosiderophage scores were significantly higher in the caudal location compared to all other locations (P < 0.001), while the dorsal location also showed significantly higher scores compared to the ventral and cranial locations. Total iron encrustation scores were significantly higher in the caudal location compared to the other three locations (P = 0.001), while no iron encrustation was detected in any micro-compartment of the cranial lobes. Vascular remodelling was significantly higher in the caudal location if compared to cranial and ventral regions (P = 0.006), but not if compared to the dorsal location. Type II pneumocyte hyperplasia scores were significantly higher in the caudal region than in all other locations (P = 0.007) (data not shown).

Ultrastructural analysis was conducted on two control, three EIPH, and three EAFPH horses. In the perivascular (adventitial) collagen of intralobular small veins, collagen fibril diameters were significantly larger in the EAFPH group compared to the EIPH group (P = 0.03), with the EAFPH group showing also higher variance (133.9 compared to 54.1 nanometres, respectively) but not compared to controls; no significant

differences regarding the length of D periodic bands were observed (Fig. 8).

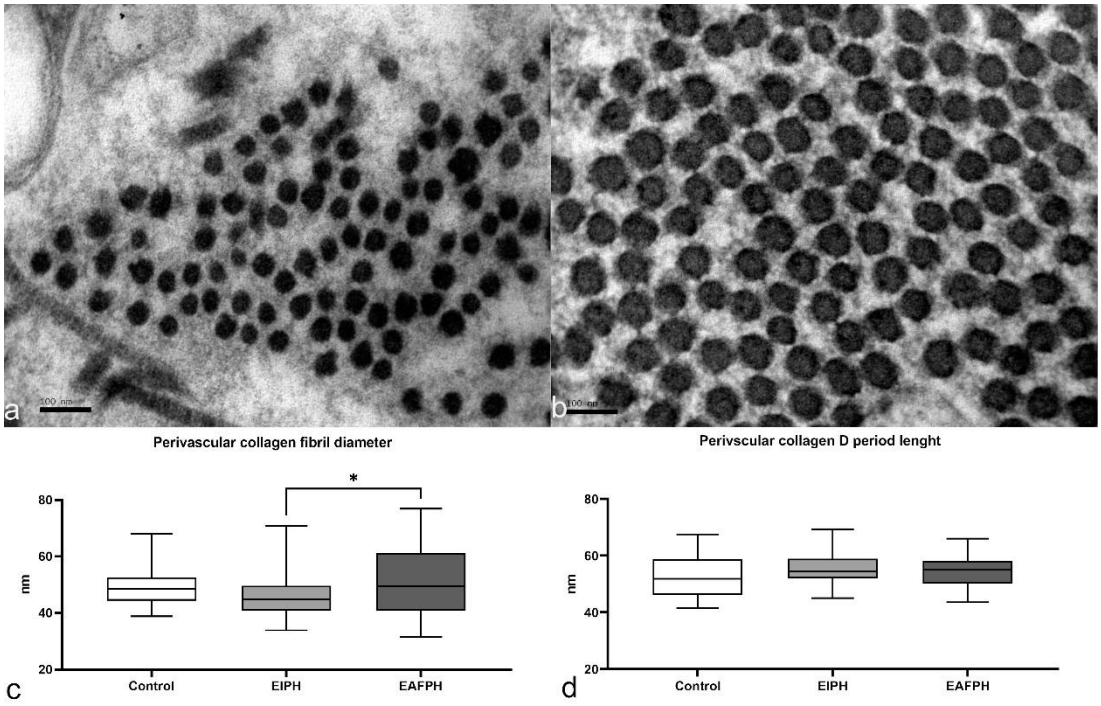

Figure 8. Transmission electron microscopy (TEM) comparison of perivascular collagen measures of intralobular pulmonary veins between EIPH and EAFPH horses. a). Case 10, Cross-sectioned collagen fibrils of an EIPH horse. Bar = 100 nm, TEM. b) Case 19, Cross-sectioned collagen fibrils of an EAFPH horse. Bar = 100 nm, TEM. c) comparison of collagen fibrils diameter between control (N = 2), EIPH (N = 3) and EAFPH (N = 3) horses. * = P< 0.05. d) Comparison of collagen D period length between control (N = 2), EIPH (N = 3) and EAFPH (N = 3) horses.

Small intralobular veins and interalveolar septa did not show any other morphological differences among groups.

Gross and histopathological analysis of the other organs revealed occasional abnormalities of incidental nature or negligible relevance in the pathophysiology of the pulmonary changes at the core of the present study (e.g. distal limb fracture, intestinal torsion with infarction).

Gross and histological features of EIPH and EAFPH are highlighted in figures 9 and 10.

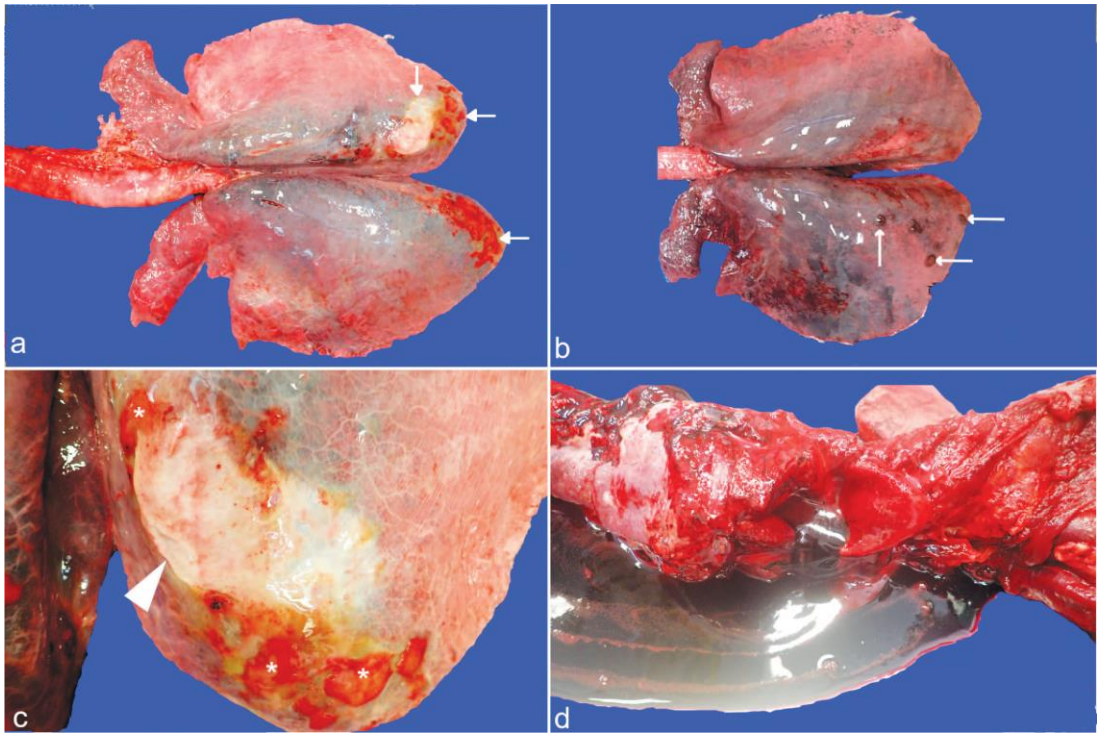

Figure 9. Exercise-induced pulmonary haemorrhage (EIPH) and exercise-associated fatal pulmonary haemorrhage (EAFPH), macroscopic changes in racehorse respiratory system: (a) Case 10, lung, EIPH, bilateral dark blue dorso-caudal discoloured areas, with multifocal to coalescing red and white pleural plaques (arrows) on both caudal locations. Cranial lobes are within normal limits. (b) Case 22, lungs, EAFPH, widespread dark red discoloration extending bilaterally from the caudal lung tip to the cranial and lateral regions. Multiple raised dark red elements suggestive of infarcts (arrows). The cranial lobes show bilateral reddening and interstitial oedema. (c) Case 10, lung, EIPH, closer view of the pleural surface with a focal white pleural plaque (arrowhead), together with multiple pale red smaller plaques (asterisks). (d) Case 21, larynx and trachea, EAFPH. Large volume of uncoagulated blood draining from the lung after gentle laryngeal handling.

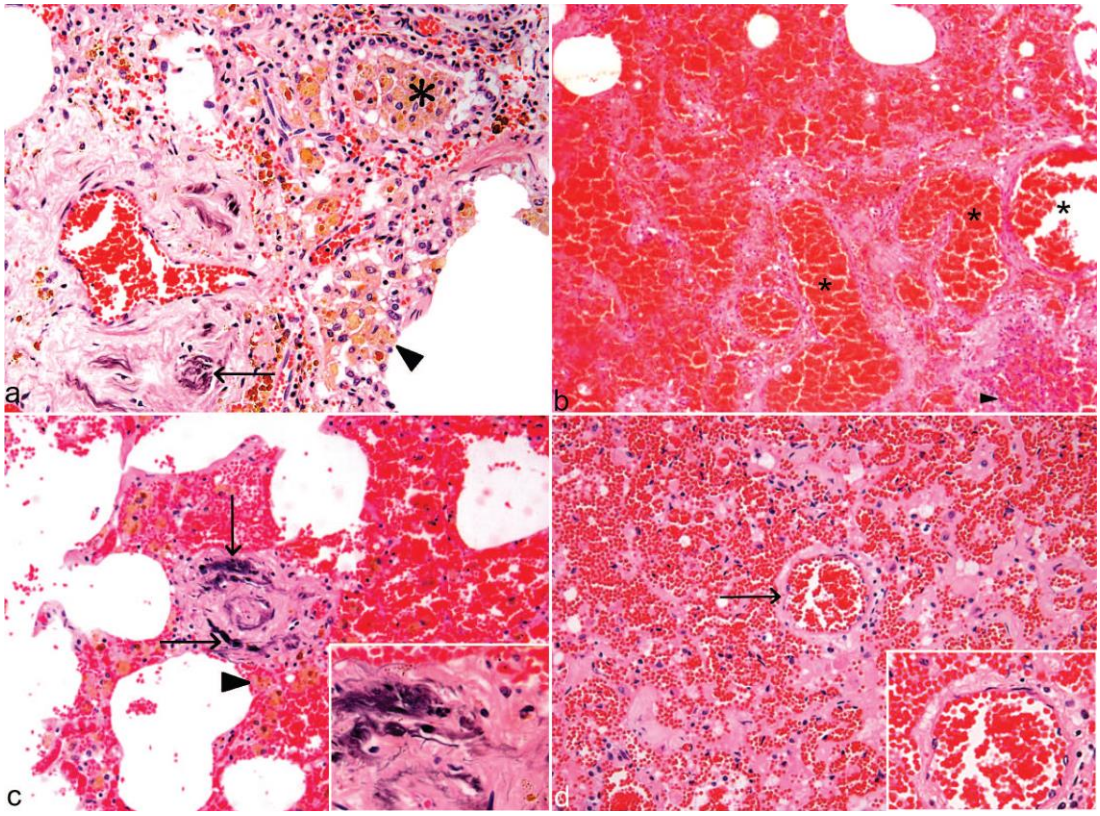

Figure 10. Exercise-induced pulmonary haemorrhage (EIPH) and exercise-associated fatal pulmonary haemorrhage (EAFPH), microscopic pulmonary changes in racehorses: (a) Case 7, EIPH, multiple hemosiderophages within the bronchiolar lumen (asterisk), as well as alveolar lumina (arrowhead) with perivascular iron encrustation (arrow). Haematoxylin and eosin (HE). (b) Case 16, EAFPH, diffuse alveolar haemorrhage expanding all the alveolar spaces with markedly congested vasculature (asterisks) and haemorrhage within bronchiole (arrowhead). HE. (c) Case 9, EIPH, small intralobular vein with severe circumferential intramural collagen deposition which narrows the lumen together with multifocal basophilic discoloration of the collagen (i.e. iron encrustation—arrows); alveolar haemorrhage is surrounding rare hemosiderophages (arrowhead). Inset: higher magnification of the remodelled vessel wall. HE. (d) Case 24, EAFPH: Small intralobular vein within normal thickness (arrow) and with surrounding congestion and haemorrhage. Inset: higher magnification of the within normal limits vessel wall. HE.

Discussion

The present study characterized and compared the gross, histopathological, and ultrastructural pulmonary changes of racehorses that died during or soon after racing competitions with lesions consistent with either EIPH or EAFPH. We documented distinctive changes that help to differentiate the two conditions, providing insight into their characteristic pathologic features.

Our findings support the initial hypothesis that EAFPH horses show significantly less VR (of small intralobular pulmonary veins) than EIPH horses, which is considered hallmark for EIPH pathogenesis (Williams et al., 2008). The absence of severe and frequent VR in most of EAFPH horses suggests that EAPFH doesn't need long-standing EIPH to take

place. This finding suggests that the severe pulmonary haemorrhage observed in EAFPH racehorse does not represent the chronic exacerbation of EIPH, but a more extensive and acute process; furthermore, this finding might also suggest that EIPH horses are not at greater risk to develop EAFPH. Considering the relative “non-lethal” role of EIPH in equine pathology (supported by our findings), it seems even more fundamental to distinguish EIPH from the EAFPH, even if they both manifest as pulmonary haemorrhages.

Our study also reveals that VR, which was originally hypothesized to be responsible of EIPH, was also noted with relative frequency in control horses, challenging the view of a crucial role for VR in the pathogenesis of EIPH. The control group exhibited surprisingly high scores for intralobular VR with three out of five horses exhibiting intralobular small veins with VR score > 1. Except for one control TB racehorse, the other horses were an Irish draught cross and a Welsh horse who died due to a gastric rupture and a *Strongylus vulgaris* associated aortic rupture, respectively. The VR in these control horses, showed morphological similarity (i.e. dense collagen within intralobular vein adventitia) with previously reported grade II VR(Williams et al., 2013). Further studies are needed to properly characterize the VR and pathological relevance in non-TB horses. Thus, in the present study, VR of small intralobular pulmonary veins have been detected in control group animals, indicating that such a lesion is not specific to EIPH or exclusive to racehorses.

Another commonly observed histological change in EIPH and in some EAFPH horses was iron encrustation. This basophilic discoloration affected pleural, adventitial, peribronchiolar and interalveolar collagen and elastin, and had a similar appearance to dystrophic mineralization observed in routine diagnostic pathology. Nevertheless, Perl’s Prussian blue positivity and the frequent association with surrounding hemosiderophages seems suggestive of a pathogenesis most likely linked to the breakdown pathway of the haemoglobin or at least indicating that iron (confirmed with Perl’s staining, data not shown) constitute such basophilic encrustations. The regional distribution of iron encrustation mirrored the distribution of hemosiderophages, showing the highest

score in the caudal lungs, where VR was also most severe. Although the exact pathogenesis by which recurrent haemorrhages cause basophilic discoloration of collagen and elastin in the lung is poorly understood (Bal, Bhalla, & Joshi, 2008), a similar histological appearance is reported in people with pulmonary veno-occlusive disease(Szturmowicz et al., 2018). In veterinary literature, iron encrustation is described in one case report in a cat (Yang, Rivers, & Baumgartner, 2021), while in horses, iron encrustation is cited in few EIPH studies, without attributing any particular importance to its presence. Since we found iron encrustation in at least one micro-compartment in the majority of EIPH horses (9/10), with none present in the control group, we propose that iron encrustation should be included in the list of the EIPH lesions, together with VR, fibrosis, and hemosiderophage accumulation. Iron encrustation of at least one micro-compartment was observed only in 3 EAFPH horses and with lower intensity compared to the EIPH horses, a finding which might be useful to distinguish these two entities.

The significantly higher score for total hemosiderophages in the EIPH group was expected because EIPH manifests commonly with hemosiderophage accumulation across multiple micro-compartments. Nevertheless, small numbers of alveolar hemosiderophages were present in all EIPH and EAFPH horses investigated and, occasionally in some control group horses (3/5). This finding seems in agreement with previous clinical studies, in which almost all TB horses in training showed numerous hemosiderophages in the bronchoalveolar lavage cytology (McKane et al., 1993). These findings suggest that small number of hemosiderophages are commonly present in equine alveolar lumina and the diagnosis of EIPH should not solely rely on their presence/absence but on their relative proportion.

Our data support the widespread evidence that hemosiderophages are more consistently present in the caudal and dorsal locations compared to cranial and ventral ones, irrespective of the group to which the horse belonged. In terms of micro-compartment distribution, our results highlight that few differences were present between EIPH and EAFPH horses, with higher amount of hemosiderophages in the adventitia of

pulmonary arteries and intra-lobular small veins in the EIPH group but not in the pleural vessels (Fig 6); such finding can be interpreted as a result of a blood flow alteration present in the pulmonary circulation (i.e. pulmonary arteries and small intralobular pulmonary veins) but absent in the systemic circulation (as pleural vessels are served from the systemic circulation).

Another interesting histological finding was the presence of type II pneumocyte hyperplasia in multiple TB racehorses. The type II pneumocyte hyperplasia was randomly distributed but with higher predilection for the subpleural alveoli, where hemosiderophages, pleural haemorrhages, and fibrosis (pleural plaques) also colocalize. This finding led to the hypothesis that type II pneumocyte hyperplasia, a widely accepted expression of alveolar repair after mechanical damage (Edwards, 2001) occurs in areas exposed to greater mechanical tension and stretching determined by high alveolar transmural pressure following alveolar over-distension during athletic peak performance.

Regarding the macroscopic findings, the gross lesion that significantly diverged between EIPH and EAFPH groups was the cranial lobe reddening and oedema. According to our results, bilateral reddening of the cranial lobes indicates that haemorrhagic foci and interstitial oedema consistently affected the cranial lobes and that such a change is highly characteristic of EAFPH. This finding is in accordance with the literature where lesions of EIPH are mostly restricted to the caudo-dorsal lungs, rarely extending to the cranial lobes, unlike EAFPH.

Examination of pleural plaques revealed that they were absent in the control group; supporting the hypothesis that such lesions, which are extremely common in racehorses (i.e. 17/21 racehorses exhibited these lesions), are likely linked to the racing activity, and remain uncommon in non-racing horses (unpublished observation of GR and ER). Pleural plaques are likely the result of localized remodelling of previous acute sub-pleural bleeding, since the plaques are often intermixed with areas of acute and subacute pleural haemorrhages and are more prevalent and more obvious in EIPH horses. Furthermore, pleural plaques co-localized with underlying parenchymal changes such as acute and chronic alveolar

haemorrhage and type II pneumocyte hyperplasia. Thus, it is possible that the aforementioned cohort of pathological changes (pleural haemorrhages and plaques, and type II pneumocyte hyperplasia) share a similar pathogenetic mechanism, which appears to induce alveolar and pleural damage contemporaneously in the same area.

Laryngeal evaluation did not reveal any lesions that would predispose to a possible static occlusion of the larynx. It has been suggested that any type of obstruction in the upper airways could be a contributing factor in the pathogenesis of EIPH and sudden death (Cook et al, 1988). Our findings present no evidence to support laryngeal static obstruction as cause of neither EIPH nor EAFPH, such as evidence of postoperative laryngeal scars or atrophy of cricoarytenoid muscles. The only changes affecting the larynx in our experimental groups of horses are ascribable to the cranial "reflux" of abundant red froth and frank blood associated with intense mucosal haemorrhages.

All other macroscopic changes appeared as poor discriminators for distinguishing EIPH from EAFPH. What is surprising is that even horses that died or were euthanized for fractures can show moderate volume of uncoagulated blood in the airways; therefore, any pathologist should resist the temptation of making an EAFPH diagnosis when a small to moderate volume of blood is present within the trachea/major bronchi, unless diffuse acute alveolar haemorrhages are confirmed by histopathology and/or profuse bleeding fills the upper airways draining from markedly haemorrhagic lungs.

The TEM analysis, which focused on the perivascular adventitial collagen, revealed no differences in the length of the D-period, whereas significant differences were observed regarding the diameter of collagen fibrils that were significantly smaller in diameter in EIPH horses when compared to EAFPH horses. This finding has to be analysed with consideration that newly formed collagen, in the context of repair and regeneration, is first achieved by deposition of smaller diameter fibrils which, subsequently are remodelled to "normal size"(Birk & Trelstad, 1986). Despite the reduced statistical power of such an observation, due to smaller sample size and variability in specimen orientation and preservation, collagen

fibrils of smaller diameter in EIPH may be due to regeneration, in comparison to the larger diameter fibrils in EAFPH that have significantly less chronic alterations in these vessels. Interestingly, collagen fibril diameter did not differ between control and EAFPH horses, possibly suggesting that control horses were likely undergoing active regeneration of collagen fibres of intralobular veins; alternatively, it is also possible that EAFPH and control horses showed “normal” perivascular collagen, unlike EIPH. To further complicate the scenario, collagen fibril diameter can be regulated by a plethora of other molecules, including small leucine rich proteoglycans, and fibril diameter can vary with aging (Sorushanova et al., 2019), which was not evaluated in the present study. Understanding the exact cause of collagen fibril diameter variation in the horses in this study and the role of fibril diameter in vascular pathology remains to be elucidated.

The main limit of this study is the small number of horses evaluated. Moreover, some sampling was limited due to the degree of autolysis, which rendered some tissues unsuitable for inclusion in the study. In particular, autolysis was consistently more advanced in EAFPH horses than in EIPH horses (data not shown). Possibly, despite an equal or even shorter post-mortem interval, the high body temperature of the horses who died suddenly at the peak of an exhausting athletic performance, coupled with the large volume of extravasated blood within the alveoli, could have provided favourable ground for the quicker onset and progression of autolysis in EAFPH horses.

Comparing EIPH and EAFPH in TB racehorses, EIPH horses are dominated by diffuse chronic changes, encompassing large number of hemosiderophages, iron encrustation, and VR, mostly in the caudal and dorsal locations. On the other hand, EAFPH horses were characterized by acute abnormalities, such as widespread and severe haemorrhages, which were evident throughout all lung locations, including the cranial lobes.

In conclusion, this paper highlights several features of EIPH and EAFPH in relation to both anamnestic data, gross, histological, and ultrastructural morphology, paving the way for novel pathogenetic theories. Since the

triad of obvious histopathological changes that characterized EIPH (VR, iron encrustation and hemosiderophages) are rare or absent in EAFPH, the results of the current study indicate that advanced EIPH lesions do not predispose the racehorse to EAFPH, in line with ACVIM (American College of veterinary Internal Medicine) EIPH consensus statement, in which EIPH is not considered a predisposing factor to other pulmonary diseases (Hinchcliff et al., 2015). Whereas the cohort of histopathological alterations observed in EIPH horses is interpreted as the consequence of repeated previous episodes of bleeding with tissue organization, the conspicuous alveolar haemorrhages of EAFPH are the morphological expression of an acute process, differentiated from EIPH by clinicopathological severity, chronicity, and extent of tissue involvement. The present study shows significant divergence in the lesions of these haemorrhagic pulmonary syndromes, suggesting potentially different pathogeneses. In other words, it is possible that the two conditions (EIPH and EAFPH) are clinicopathological manifestations of the same pathogenetic mechanism leading to either a chronic and mild or acute and fatal pulmonary bleeding or the morphological expression of two separated pathogenetic mechanisms, whose identification remains elusive.

Image analysis software and equine pulmonary bleeding: an un-biased whole slide imaging study of multiple pulmonary antigens and features in non-runner and runner horses

Introduction

The previous chapter focused on semiquantitative analysis of multiple features of equine lungs, with the most significant ones observed histologically. Although histological analysis performed through human eyes is an invaluable tool able to characterise semi-quantitatively any tissue, a genuine quantitative analysis is often arduous to achieve. Technological advances such as slide scanners and image analysis software aid in enhancing the “objectivity” for any histological analysis. For example, a recent paper has been published regarding the

implementation of image analysis software in the evaluation of BAL in horses with EIPH (Bertram et al., 2022). In this study, image analysis software showed a greater consistency and accuracy than the trained human eye.

For these reasons, we used image analysis software to compare histological features for the present and the following chapter. In addition to histological features such as the amount of collagen and haemosiderin, we decided also to analyse via immunohistochemistry multiple proteins commonly expressed in the lung, and, namely, α-smooth muscle actin (α-SMA), surfactant protein C (SP-C) and thyroid transcription factor 1 (TTF-1).

α-SMA is a protein present in any smooth muscle, including airway and vascular ones; α-SMA has been used in horses to study the pulmonary vessels (Williams et al., 2008). In addition to airway and vascular smooth muscle cells, α-SMA is also expressed by pulmonary myofibroblasts, which are vital players in the fibrosis and extracellular matrix remodelling of all tissues, lungs included (Pakshir et al., 2020).

SP-C is one of the proteins secreted by pneumocytes type II and responsible for co-adjuvating surfactant to decrease intra-alveolar surface tension, preventing alveoli from collapsing during breathing (Christmann et al., 2006). Defective production of SP-C has been associated with a variety of pulmonary disorders in human (Peca et al., 2015), and SP-C implementation have also shown therapeutic properties in multiple animal experimental models of lung disease (Cattel et al., 2021): such studies suggest a relevant role of surfactant proteins for a healthy lung.

TTF-1 is a transcription factor whose expression is restricted to pneumocytes type II and non-ciliated airway epithelium and, when expressed, promotes surfactant protein secretion. Pneumocyte type II are cuboidal cells lining a relatively small area of the alveoli but presiding to a variety of functions, including production of surfactant and surfactant proteins, regeneration of pneumocyte type 1, phagocytosis of apoptotic type II pneumocytes and synthesis of pro-fibrotic factors (Fehrenbach, 2001). TTF-1 IHC has already been used to evaluate the number of pneumocytes type II in experimental animal models (Balka et al., 2013).

The main hypotheses evaluated in this chapter is that EAFPH and EIPH horses could express significantly different amounts of collagen, haemosiderin, and other pulmonary antigens.

Material and methods:

Equine *post-mortem* examinations submitted for diagnostic purposes to the Laboratory of Veterinary Pathology, University of Liverpool, between 2017 and 2022 were considered for this study. The inclusion criteria in this study consisted of lung samples from caudal or dorsal locations (see previous chapter) and not showing extensive and severe autolytic changes. Four groups of horses were issued. The non-TB control group comprised non-TB horses euthanised or dead naturally for non-pulmonary pathologies. The TB control group was composed of non-runner TB horses euthanised or dead naturally for non-pulmonary pathologies and did not show intra-bronchial clusters of at least three haemosiderophages. The EIPH group was composed of TB racehorses that were euthanised or died naturally from non-cardiopulmonary conditions (e.g. catastrophic fractures) but showed at least one cluster of three hemosiderophages within the bronchial or bronchiolar lumen. The EAFPH group was composed of TB racehorses that died during or a few (~ 0-4) hours after a competition with gross and microscopic lesions compatible with EAFPH and without other potentially fatal lesions. All lung samples were fixed by immersion in 10% formalin, pH 7.4 for at least 48 hours, paraffin-embedded, and cut to produce 4-µm-thick sections before staining them with haematoxylin & eosin (H&E) and picrosirius red, as per standard protocol; furthermore, TTF-1, SP-C, and α-SMA IHCs were also performed with AEC as a chromogen, to avoid misinterpretation of haemosiderin deposits (table 4).

Antibody	Manufacturer	Type	Dilution	Pre-treatment
α-SMA	Dako	Monoclonal, HFF35	1:500	Low pH
TTF-1	Abcam	Monoclonal, 8G7G3/1	1:500	High pH
SP-C	LS-BIO	Polyclonal	1:100	High pH

Table 4: Lung marker IHC – details.

Afterwards, each slide was scanned at 20X via Aperio CS2 Scanscope (Leica Microsystems UK Ltd), and then, any non-lung tissue in the slide was cut out manually. For picrosirius red, haemosiderin, SP-C and α-SMA, Orbit image analysis software (Idorsia Pharmaceuticals Ltd, Allschwil, Switzerland) has been used for image analysis. A training step was done by manually drawing 50 representative annotations per tissue class (e.g. collagen vs background) through multiple slides; positive areas were selected near negative areas to maximise the discrimination between the two categories. Finally, a ratio between positive pixels (e.g. picro-sirius red collagen, Surfactant C) of the total pixels analysed per horse was counted per marker. Overlays are highlighted in figure 11.

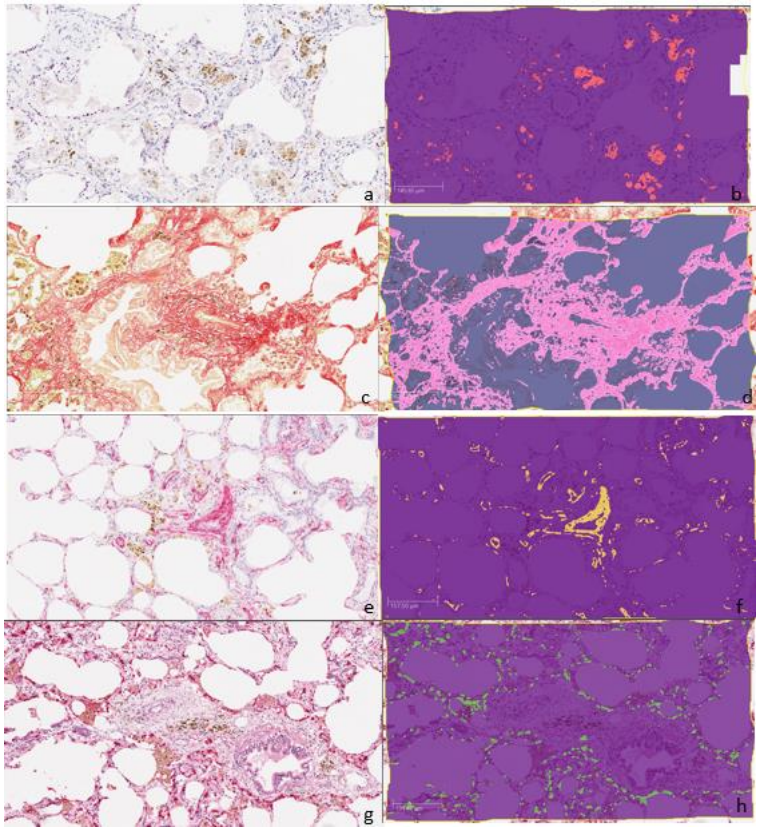

Figure 11. a: EIPH horse, TTF-1 IHC slide used for haemosiderin analysis. b: overlay of the image a after training for haemosiderin. Brown = haemosiderin; violet = background. c: EIPH horse, picrosirius red stain. d: overlay of image c after training for collagen. pink = collagen; dark blue = background. e: EIPH horse, α-SMA IHC. f: overlay of image e after training for α-SMA. yellow = α-SMA; violet = background. g: EIPH horse, SP-C IHC. h: overlay of image g after training for SP-C. Green= SP-C; violet = background.

For haemosiderin evaluation, slides for TTF-1 using AEC as a chromogen (i.e. red stain) were used. For TTF-1 analysis, the software Medical Image Manager (MIM, HeteroGenius Limited 2016) was used according to manufacturer instructions (cell count function). TTF-1 nuclear positivity

ratio was calculated by dividing the number of positive nuclei by the total nuclei detected in the selected slide per horse. SPSS statistics software (IBM, UK) was used for statistical analysis between groups. For all markers, the normality of distributions was verified through Kolmogorov and Smirnov tests, and then a Kruskal Wallis test was applied to identify if the distribution was significantly different among groups; when significant differences were present, Bonferroni correction pairwise comparison was conducted as a *post-hoc* test. A similar assessment was made comparing the lung location (i.e. caudal and dorsal), irrespective of the grouping, using Mann Whitney U test.

Results

Forty-five horses were included in the study, with 10, 7, 11 and 17 horses from the non-TB control group, TB control group, EIPH group and EAFPH group, respectively. Results are summarised in table 5 and images 12 and 13.

ID	Breed	Group	Location	Collagen %	Haemosiderin %	α-SMA %	SP-C %	TTF-1 %*
8810	Cob	Non-TB control	Caudal	20.899	N/A	3.653	3.3494	N/A
8877	Shetland Pony	Non-TB control	Caudal	26.4702	0.01	1.0515	1.0951	2.8309
10974	WB	Non-TB control	Dorsal	18.8303	0.0054	0.7877	2.1373	0.762
11353	Arab	Non-TB control	Caudal	15.7394	0.0048	N/A	0.746	6.1802
11402	WB	Non-TB control	Dorsal	12.5503	0.0006	0.4827	0.478	3.7567
16218	Irish DX	Non-TB control	Caudal	23.7761	0.0167	1.8969	0.4804	0.4665
16276	Welsh	Non-TB control	Caudal	15.5651	0.0239	0.6794	0.7738	0.677
18759	Arab x	Non-TB control	Caudal	20.4445	0.0107	1.0097	1.2636	13.2811
17L-2281	Cob	Non-TB control	Dorsal	15.9196	0.0079	N/A	0.3911	0.2125
5415	Andalusian	Non-TB control	Dorsal	14.9849	0.0633	1.7809	1.3308	5.0993
7899	TB	TB control	Caudal	16.5354	0.0122	2.6536	0.9729	1.5132
9177	TB	TB control	Caudal	19.4906	0.0097	2.2465	2.4903	4.9397
523	TB	TB control	Dorsal	9.6149	0.0233	1.34	1.2275	0.8139
14603	TB	TB control	Dorsal	13.5912	0.0006	0.8931	2.055	9.1664
15826	TB	TB control	Caudal	24.8367	0.0088	2.7554	1.2427	0.2867
15880	TB	TB control	Dorsal	8.9777	0.2346	2.0026	3.563	0.2772
14234	TB	TB control	Dorsal	11.9004	0.0941	1.7759	0.5477	0.9726
4773	TB	EIPH	Caudal	22.0501	0.1679	1.3338	2.3128	0.3451
6645	TB	EIPH	Caudal	39.1849	1.0542	1.7973	3.9359	3.3075
7435	TB	EIPH	Caudal	25.0901	7.7047	3.387	5.4674	3.3003
9593	TB	EIPH	Caudal	31.7816	3.196	1.641	5.4219	0.9205
9711	TB	EIPH	Caudal	34.8182	1.3429	2.3536	5.0873	9.3923
10209	TB	EIPH	Caudal	21.5323	0.5285	1.1698	1.2141	4.2756
10210	TB	EIPH	Caudal	19.5274	3.5939	3.801	2.1539	4.8405
10211	TB	EIPH	Caudal	13.6257	0.1902	2.1346	1.331	1.3615
14542	TB	EIPH	Caudal	16.9031	0.0052	1.2493	1.5107	0.8758
14734	TB	EIPH	Caudal	14.8241	0.774	0.633	0.5664	4.2
3852	TB	EAFPH	Dorsal	2.3577	N/A	0.0546	0.2032	7.0835
4850	TB	EAFPH	Dorsal	7.7569	0.1812	0.3753	0.4117	0.7343
4997	TB	EAFPH	Dorsal	10.0546	0.3087	0.1919	0.3077	2.8905
5272	TB	EAFPH	Dorsal	8.815	0.399	1.0463	0.2554	3.4312
5642	TB	EAFPH	Dorsal	8.5362	N/A	0.6896	0.4197	4.6765
7491	TB	EAFPH	Dorsal	9.618	0.1395	1.4528	N/A	0.6046
8121	TB	EAFPH	Caudal	20.401	0.0155	3.1801	0.9764	0.7554
8173	TB	EAFPH	Caudal	9.472	0.3423	0.7796	1.9725	1.0734
8541	TB	EAFPH	Caudal	26.5578	1.2134	1.9628	4.2689	1.0246
9447	TB	EAFPH	Caudal	22.9192	0.0848	1.6919	3.2999	0.6536
9694	TB	EAFPH	Caudal	28.426	0.098	0.7934	0.9014	4.2269
10815	TB	EAFPH	Caudal	27.7292	0.5698	N/A	2.7886	4.6989
11637	TB	EAFPH	Caudal	23.4628	0.5936	0.7414	1.4945	6.9593
12545	TB	EAFPH	Caudal	16.6431	0.0714	1.2627	0.1788	1.6693
14298	TB	EAFPH	Caudal	16.2062	0.1215	0.6671	2.5281	1.382
15166	TB	EAFPH	Caudal	23.3541	0.018	0.459	3.1346	2.0192
15278	TB	EAFPH	Caudal	18.2971	1.5575	1.9174	1.1616	5.8822

Table 5. Results of image analysis of pulmonary features and immunohistochemical markers. The value is expressed in percentage of tissue analysed. *: for this marker, the percentage expressed is nuclei positive / total nuclei analysed.

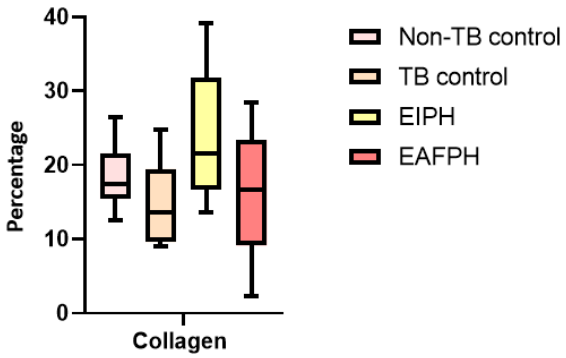

Figure 12 . Comparison of pulmonary collagen percentage between groups.

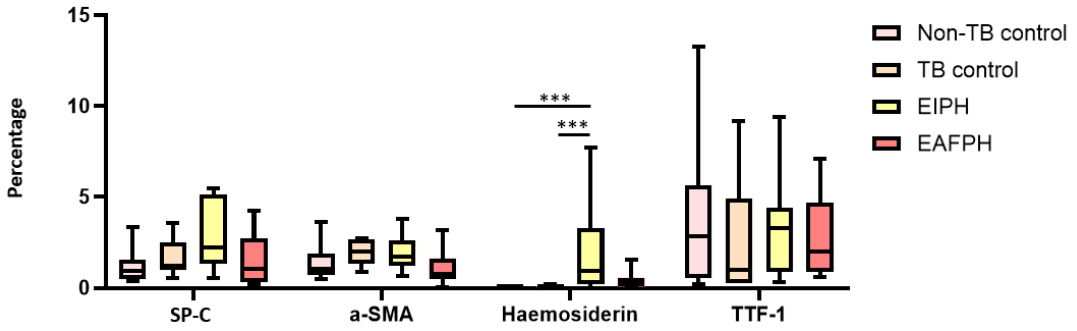

Figure 13. Comparison of pulmonary haemosiderin and IHC markers percentage between groups. *** = P < 0.001. SP-C = Surfactant protein C; a-SMA = Alpha smooth muscle actin. TTF-1 = Thyroid transcription factor 1.

No significant differences between the four groups were observed regarding collagen, SP-C, and TTF-1. Both control groups exhibited significant (P = < 0.001) lower haemosiderin ratio when compared to the EIPH group; EAFPH showed less haemosiderin compared to EIPH horses but not significantly. α-SMA ratio was significantly different between groups (P = 0.031) but when *post-hoc* test was performed no significant differences were observed between groups: the unique comparison approaching statistical significance was the EAFPH group showing lower α-SMA ratio compared to the control non-runner TB group (P = 0.76). Comparison between anatomical location revealed no significant changes for haemosiderin and TTF-1; collagen (P < 0.001), SP-C (P = 0.003) and α-SMA (P = 0.023) ratios were significantly higher in the caudal lung compared to the dorsal one.

Discussion

The present findings don't highlight significant differences between the EAFPH and the other groups, able to explain the cause of the severe pulmonary haemorrhage, except for haemosiderin and, almost, for α-SMA.

The higher haemosiderin ratio in both EIPH and EAFPH groups compared to both control groups seems to reinforce that recurrent pulmonary bleeding (i.e. haemosiderin accumulation) is associated with the running activity: pulmonary haemosiderin accumulation was indeed reported in almost all TB in training horses using BAL cytology (McKane et al., 1993).

Furthermore, the horses included in the control TB group were not in training or running and showed scant to none haemosiderin as the non-TB control group. All these data suggest that in the absence of inflammatory diseases, the presence of pulmonary haemosiderin is not a breed-related finding but is most likely associated with exercising. As expected, the EIPH group showed the highest amount of haemosiderin compared to all groups, , but not significantly more than the EAFPH. A possible explanation is that the low amount of brown to black formolic pigment observed within the haemorrhagic lungs of the EAFPH group was partially considered as haemosiderin by Orbit, raising the score of positivity of the EAFPH mildly.

The α-SMA results also revealed interesting findings: the presence of significant difference on the Kruskal-Wallis test but not by the *post-hoc* tests might be due to differences close to being significant. α-SMA is expressed in all smooth muscle cells, including the vascular smooth muscle cells and the airway ones; a possible explanation for such difference is the smaller amount and thickness of larger blood vessels. It is possible that increasing the number of horses included in the study might generated more statistically relevant results.

The comparison between anatomical locations revealed more significant relevant findings.

First of all, the amount of collagen was significantly more abundant in the caudal lung compared to the dorsal. The collagen might come from the pleura, from the vessel adventitia, from the septa or all these micro-compartments combined. Such findings might reflect that the caudal lung, being a peripheral location, contains more collagen fibrotic and, is less elastic compared to the dorsal area: indeed anaesthetised horses show reduced compliance in the caudal location compared to the dorsal area (Ambrisko, Schramel, Hopster, Kastner, & Moens, 2017). In light of these findings, the larger amount of adventitial collagen of the intra-lobular small venules (a.k.a. vascular remodelling) (Williams et al., 2008) might be reflecting an “anatomical” feature rather than an actual “pathological” change. This hypothesis seems supported by a recent study (Rocchigiani et al., 2022) and it is worth considering that the control group used in one of Williams’ papers was composed of only

eight horses (Williams et al., 2013). This hypothesis needs a larger cohort of horses to be confirmed or rejected.

The caudal lung also exhibited larger amount of surfactant C protein compared to the dorsal region. It is already known, that lower calibre airways and reduced alveolar surface (as it occurs in the caudal location compared to the dorsal one) require a large amount of surfactant proteins to prevent alveolar collapse (Klein, 2013). What was surprising is that the surfactant C wasmore abundant, the TTF-1 cells (i.e. pneumocytes type II) were not. Such a finding might suggest that even though TTF-1 cells were not more numerous, they might produce more surfactant C.

The results rejected the main hypothesis of the present chapter. However, anatomical differences between dorsal and caudal lobes were observed.

Image analysis of potential cardiac immunohistochemical markers in runner and non-runner horses: a pilot study

Introduction

After extensively analysing all pulmonary changes in EAFPH and EIPH, the heart was the last organ to be analysed more in detail. Even though no evident gross and histological changes were observed in any horse used in the present study, we couldn't exclude the possibility of conduction system disturbances of sudden structural damage. It is indeed reported that arrythmias and peracute myocardial necrosis (< than 6 hour of duration) (Robinsons & Robinson, 2015) are commonly inapparent histologically, in all animal species. Numerous clinical studies conducted in racehorses reveal the common occurrence of cardiac arrhythmias (Nath et al., 2021); furthermore, cardiac arrhythmias have also been hypothesised to be a common cause of sudden death in horses (Lyle et al., 2011) and possibly also to pulmonary bleeding (Decloedt, Van Steenkiste, Vera, Buhl, & van Loon, 2020). Cardiac arrhythmia and other functional cardiac disturbances in horses have been investigated in numerous studies (Lyle et al., 2011), (Kiryu et al., 1999) (Gelberg et al., 1985) (Molesan et al., 2019), but no specific correlation between cardiac histological changes and cardiac function disturbance has been

undisputedly proven: a plausible explanation is also because arrhythmogenesis may reflect a functional rather than a structural cell abnormality (Lyle et al., 2011). Since numerous studies focused on equine athletes' sudden death found little to absent cardiac changes at light microscopy (Rocchigiani et al., 2022) (Molesan et al., 2019), we decided to focus on immunohistochemical markers of cardiac damage.

In equine medicine, there are few fully approved biomarkers of cardiac damage, including cardiac troponin I, troponin T (Van Der Vekens, Decloedt, Ven, De Clercq, & van Loon, 2015) and asymmetric dimethylarginine (Ertelt et al., 2021) but not a single one of those have been evaluated via immunohistochemistry. Despite this lack of knowledge, we explored the expression of cardiac troponin T, connexin 43 and cleaved caspase 3. Cardiac troponins are globular proteins that, along with tropomyosin and actin, form part of the thin filament of the contractile apparatus in striated muscle and heart (Rossi et al., 2018); due to their structural presence within cardiomyocytes, they are valuable markers of cardiac damage also used in human medicine (Van Der Vekens et al., 2015). Connexin 43 is the most frequent gap junction protein in the ventricular myocardium in humans; altered expression of these molecules has been induced by multiple pathological cardiac conditions; there is also evidence that altered expression facilitates arrhythmogenesis in mice (Delmar et al., 2018). Due to relatively frequent arrhythmias in exercising horses, connexin 43 appeared as a valid candidate to study, trying to link cardiac functional disturbances to histological changes. Cleaved caspase 3 is a widely used marker for apoptosis, considered anessential component of ageing-induced cardiac damage (Kwak, 2013).

The main hypothesis evaluated in this chapter is that EAFPH and EIPH horses could express significantly different amounts of cardiac troponin T, connexin 43 and cleaved caspase 3.

Materials and methods

Equine *post-mortem* examinations submitted for diagnostic purposes to the Laboratory of Veterinary Pathology, University of Liverpool, between 2017 and 2022, were considered for this study. Four groups of horses were issued as previously (see previous chapter). For all horses included, a single fragment of cardiac tissue (tentatively interventricular septum)

extending from the endocardium to the opposite endocardium/epicardium was used for each horse; when not possible, a section of the left ventricle was used instead. All cardiac samples were fixed by immersion in 10% formalin, pH 7.4 for at least 48 hours, paraffin-embedded, and cut to produce 4-µm-thick sections before staining them with haematoxylin & eosin (H&E) as per standard protocol; furthermore, cardiac troponin T, connexin 43 and cleaved caspase 3 IHCs were also performed (table 6).

Antibody	Manufacturer	Type	Dilution	Pre-treatment
Cardiac troponin T	Santa Cruz	Monoclonal, TC3	1:100	Low pH
Connexin 43	Abcam	Polyclonal	1:1000	High pH
Cleaved caspase 3	Cell signaling	Polyclonal	1:500	Low pH

Table 6. Cardiac marker IHC – details.

Afterwards, each slide (apart from cleaved caspase 3, see Results paragraph) was scanned at 20X via Aperio CS2 Scanscope (Leica Microsystems UK Ltd) and then, any non-heart tissue in the slide was cut out manually. Orbit image analysis software (Idorsia Pharmaceuticals Ltd, Allschwil, Switzerland) has been used for image analysis for each marker, as in the previous chapter (see previous chapter for details). Overlays are highlighted in figure 14.

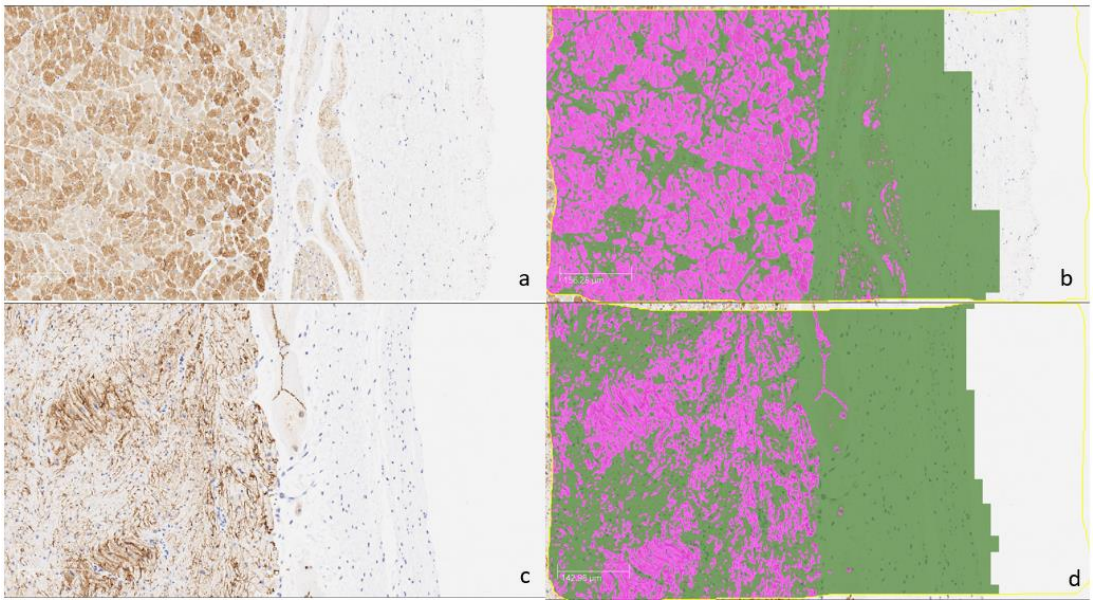

Figure 14. a: EIPH horse, cardiac troponin T IHC. b: overlay of the image a after training for cardiac troponin T. Pink = cardiac troponin T; green = background. c: EIPH horse, connexin 43 IHC. d: overlay of the image c after training for connexin 43. Pink = connexin 43; green = background.

SPSS statistics software (IBM, UK) was used for statistical analysis between groups: for all markers, the normality of distributions was verified using Kolmogorov and Smirnov tests, and then a Kruskal Wallis test was applied to identify if the distribution was significantly different among groups; when significant differences were present, Bonferroni correction pairwise comparison was conducted as a *post-hoc* test.

Results

Forty-five horses were included in the study, with 10, 7, 11 and 17 horses, from the control non-TB group, control non-runner TB group, EIPH group and EAFPH group, respectively. Results are summarised in table 7 and figure 15.

Original ID	Breed	Group	C. troponin	Connexin 43
8810	Cob	Non-TB control	N/A	41.0285
8877	ıetland Por	Non-TB control	61.8354	22.166
10974	WB	Non-TB control	61.0754	14.5408
11353	Arab	Non-TB control	67.242	19.4606
11402	WB	Non-TB control	25.6704	5.157
16218	Irish DX	Non-TB control	2.1462	1.1565
16276	Welsh	Non-TB control	52.0571	13.139
18759	Arab x	Non-TB control	59.8406	22.7161
17L-2281	Cob	Non-TB control	64.5289	12.6442
5415	Andalusian	Non-TB control	N/A	N/A
7899	TB	TB control	39.8114	5.3832
9177	TB	TB control	56.7227	25.0025
523	TB	TB control	54.9359	49.7273
14603	TB	TB control	77.0601	80.5624
15826	TB	TB control	31.0881	2.4763
15880	TB	TB control	64.9125	41.3233
14234	TB	TB control	52.9988	4.8116
4773	TB	EIPH	40.3343	8.4793
6645	TB	EIPH	2.2203	1.4358
7435	TB	EIPH	50.8353	4.0485
7995	TB	EIPH	36.8399	9.5105
9593	TB	EIPH	N/A	N/A
9711	TB	EIPH	43.471	26.6297
10209	TB	EIPH	58.5198	19.2105
10210	TB	EIPH	58.1748	3.8582
10211	TB	EIPH	25.7062	3.9414
14542	TB	EIPH	32.752	12.3987
14734	TB	EIPH	3.2417	3.704
3852	TB	EAFPH	1.7963	0.5644
4850	TB	EAFPH	N/A	N/A
4997	TB	EAFPH	0.49	3.5574
5272	TB	EAFPH	1.0984	1.1766
5642	TB	EAFPH	0.086	1.4106
7491	TB	EAFPH	0.0902	0.0954
8121	TB	EAFPH	1.185	0.1465
8173	TB	EAFPH	7.0442	2.195
8541	TB	EAFPH	0.3854	0.497
9447	TB	EAFPH	62.3805	14.7894
9694	TB	EAFPH	22.3249	2.0103
10815	TB	EAFPH	30.6975	N/A
11637	TB	EAFPH	47.3257	5.6096
12545	TB	EAFPH	6.6819	0.681
14298	TB	EAFPH	63.42	13.2871
15166	TB	EAFPH	71.7959	42.5726
15278	TB	EAFPH	N/A	0.4818

Table 7. Results of image analysis of immunohistochemical cardiac markers. The value is expressed in percentage of tissue analysed. N/A= non assessable.

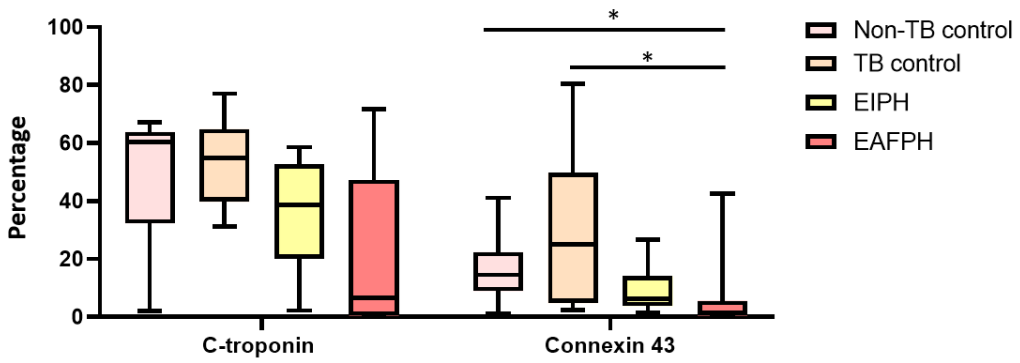

Figure 15. Comparison of IHC myocardial markers percentage between groups. * = P < 0.05. SP-C = Surfactant protein C; a-SMA = Alpha smooth muscle actin. TTF-1 = Thyroid transcription factor 1.

Cleaved caspase 3 IHC revealed scattered and scarce signal observed within cardiomyocytes sarcoplasm; since it was so scant and rare, it was not considered useful to be scanned and included in the following analytic steps. Cardiac troponin T was commonly present within cardiomyocytic sarcoplasm, showing diffuse to granular pattern, commonly highlighting the sarcoplasmic banding pattern; Purkinje's fibres exhibited lower positivity with scattered sarcoplasmic labelling, occasionally exhibiting a filamentous pattern. No significant difference between the four groups was observed for cardiac troponin T. Connexin 43 IHC revealed standard, moderate labelling of the cellular membrane, scattered rarer round accumulation within the sarcoplasm and an intense labelling was observed at the junction between cardiomyocytes (i.e. intercalated disks); a similar pattern was observed within the Purkinje fibres' cells. Connexin 43 ratio was significantly lower in the EAFPH group compared to both controls.

Discussion

This study constitutes the first attempt to characterise the distribution of biomarkers of cardiac damage using immunohistochemistry in the horse.

Connexin 43 revealed the most significant results: the distribution of connexin 43 seemed consistent with the distribution of the same protein in other animals where they are primarily present along the membrane and the intercalating disc (Boengler & Schulz, 2017); the distribution was present in most of cardiomyocytes and even with stronger signal within

the Purkinje's fibres, suggesting a plausible higher expression within the conduction system. The presence of scattered round clusters of connexin 43 was not fully understood. It might represent a *post-mortem* or not relevant change rather than an *intra-vitam* change, as such a pattern was observed in all groups. The reduced amount of connexin 43 in the EAFPH group compared to both control groups might be interpreted differently. One possibility is that in EAFPH horses, the disruption of connexins (and therefore of gap junctions) could be secondary to widespread myocardial damage. In support of this theory, there is evidence in multiple animal species that connexin 43 expression decreases in conditions such as myocardial infarction or arrhythmia (Boengler & Schulz, 2017). Another possibility is that the reduction of connexin 43 is due to incipient autolysis: EAFPH horses showed the highest degree of autolysis, and possibly the autolysis hampered the antigen detection via immunohistochemistry. Therefore, further studies focusing on connexin 43 expression in healthy and autolytic equine heart are warranted to solve this doubt.

Cardiac troponin T was commonly and largely present within cardiomyocytes, in smaller amounts within the Purkinje's fibres. Such finding seems to withfollowing the literature, where these cells have fewer fibrillary components than ventricular cardiomyocytes (Boyden, Hirose, & Dun, 2010). The EAFPH horses showed a reduced amount of this protein, but the differences between the other groups was not significant.

While the cleaved caspases 3 was mostly absent within the cardiomyocytes of all groups, the control tissue (i.e lymph node) showed evident positivity within lymphoid follicles. These results suggest that the immunohistochemistry was performed correctly. Finally, the lack of cleaved caspase 3 indicates that apoptosis is not involved in any process occurring within the heart of normal controls, EIPH and EAFPH horses.

Conclusions

The present study document how EIPH and EAFPH manifest. EIPH is usually macroscopically inapparent and shows up with the classic triad of lesions encompassing fibrosis, vascular remodelling and haemosiderin accumulation; in addition, we suggest including iron encrustation among

the characteristic lesion of EIPH. On the other hand, EAFPH shows up more commonly with interstitial oedema and haemorrhage of the cranial lobes, while histologically exhibits severe and widespread haemorrhages with usually few to absent chronic changes (e.g. haemosiderin accumulation, iron encrustation). Electron microscopy and immunohistochemistry of pulmonary antigens didn't spot - clear cut differences that distinguish EIPH from EAFPH, except for mild changes in the perivascular collagen. Finally, analysis of myocardial damage markers revealed a low amount of connexin 43 in EAFPH horses compared to controls but not compared to the EIPH horses. While the hunt for the pathogenetic mechanism of EAFPH is still on, we made progresses in distinguishing EIPH and EAFPH and shedding light on their pathological phenotypes.

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