

# Regional distribution of collagen and haemosiderin in the lungs of horses with exercise-induced pulmonary haemorrhage

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## Summary

**Reasons for performing study:** Regional veno-occlusive remodelling of pulmonary veins in EIPH-affected horses, suggests that pulmonary veins may be central to pathogenesis. The current study quantified site-specific changes in vein walls, collagen and haemosiderin accumulation, and pleural vascular profiles in the lungs of horses suffering EIPH.

**Hypothesis:** In the caudodorsal lung regions of EIPH-affected horses, there is veno-occlusive remodelling with haemosiderosis, angiogenesis and fibrosis of the interstitium, interlobular septa and pleura.

**Methods:** Morphometric methods were used to analyse the distribution and accumulation of pulmonary collagen and haemosiderin, and to count pleural vascular profiles in the lungs of 5 EIPH-affected and 2 control horses.

**Results:** Vein wall thickness was greatest in the dorsocaudal lung and significantly correlated with haemosiderin accumulation. Increased venous, interstitial, pleural and septal collagen; lung haemosiderin; and pleural vascular profiles occurred together and changes were most pronounced in the dorsocaudal lung. Further, haemosiderin accumulation colocalised with decreased pulmonary vein lumen size. Vein wall thickening, haemosiderin accumulation and histological score were highly correlated and these changes occurred only in the caudodorsal part of the lung.

**Conclusion:** The colocalisation of these changes suggests that regional (caudodorsal) venous remodelling plays an important role in the pathogenesis of EIPH.

**Potential relevance:** The results support the hypothesis that repeated bouts of venous hypertension during strenuous exercise cause regional vein wall remodelling and collagen accumulation, venous occlusion and pulmonary capillary hypertension. Subjected to these high pressures, there is capillary stress failure, bleeding, haemosiderin accumulation and, subsequently, lung fibrosis.

## Introduction

Equine exercise-induced pulmonary haemorrhage (EIPH) occurs during strenuous exercise (Pascoe *et al.* 1981; Raphel and Soma

1982) and, in racehorses, the incidence is up to 75% (Birks *et al.* 2002). EIPH severity varies from a few erythrocytes in bronchoalveolar lavage fluid to visible intratracheal blood and even epistaxis (Kindig *et al.* 2001; Hinchcliff *et al.* 2005a). Performance is compromised by more than just a few drops of blood in the trachea (Hinchcliff *et al.* 2005b).

The most widely hypothesised mechanism of EIPH is alveolar capillary wall stress failure caused by exercise-induced pulmonary hypertension (West *et al.* 1993; West and Mathieu-Costello 1994). Lesions in lungs of EIPH-affected horses include bronchiolitis/distorted bronchioles, the presence of haemosiderophages and an increased amount of connective tissue (O'Callaghan *et al.* 1987; Oikawa 1999). Individual histological findings had not been placed in spatial context with each other and changes to the pulmonary vasculature had not been emphasised until Williams *et al.* (2008) reported colocalisation of veno-occlusive remodelling with haemosiderosis, angiogenesis and fibrosis of the interstitium, interlobular septa, pleura and the bronchovascular bundle, primarily in the caudodorsal lung regions, and not in cranioventral lung regions. These changes are similar to those observed in human pulmonary veno-occlusive disease (Wagenvoort *et al.* 1985). The current study used morphometric methods to quantify site-specific changes in the amounts of collagen and haemosiderin, and the number of pleural vascular profiles in the lungs of EIPH affected horses. The results confirm the caudo-dorsal predilection of EIPH lesions, and establish veno-occlusive remodelling as a component of the characteristic lesions. The results suggest that venous obstruction in the dorsal parts of the lung may underlie the development of EIPH and that the most extensive veno-occlusive remodelling is associated with regions of haemorrhage, haemosiderin accumulation and lung fibrosis.

## Materials and methods

### Horses

Lung tissue was obtained from 5 EIPH-affected Thoroughbred racehorses aged 5–7 years (Horses 1–5 in Williams *et al.* 2008) with approval of the MSU All-University Committee on Animal Use and Care. Horses had at least 3 bouts of racing-associated

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epistaxis. Average time from last race to necropsy was 130 days. Two 2-year-old unraced Thoroughbred horses were used as controls.

#### *Tissue collection and processing*

Twelve samples collected from 6 regions of the right and left lungs of each horse (Fig 1) (Williams *et al.* 2008) were paraffin embedded, and stained with haematoxylin and eosin (H&E), Prussian blue, Verhoeff von Gieson, and the collagen-specific stain Picrosirius red (PR) (Pick *et al.* 1989). Immunohistochemistry for  $\alpha$  smooth muscle actin ( $\alpha$ -SMA) was used as described (Williams *et al.* 2008). All slides were evaluated blindly using brightfield microscopy, except for the PR-stained slides, which were evaluated using polarised light.

#### *Lung histology and scoring*

On each H&E slide, each of 5 lesions: 1) interstitial fibrosis; 2) haemosiderin; 3) pleural/interlobular septal thickness; 4) evidence of neovascularisation; and 5) venous collagen accumulation and wall thickness were scored 0 (not present) to 3 (severe) and a cumulative histological score (HS) was calculated (maximum = 15). Slides were placed into 3 categories based upon HS: 0 = histologically normal; 1–9 = mild to moderate lung changes; 10–15 = marked to severe lung lesions.

#### *Morphometric analysis*

Except for measurements of haemosiderin, and vascular profiles, all morphometric measurements were performed on images of PR-stained sections obtained with an Olympus CX41 microscope, a polarising filter set, and either a 2x, 4x or 10x objective. The microscope had a QColor-3 camera<sup>1</sup>. Morphometric analysis was performed using MicroSuite5 Biological Software<sup>1</sup>.

#### *Imaging*

For evaluation of intralobular veins, each section was divided in 4 quadrants. Starting at the upper left, using a 10x objective, a photograph was taken of every third field within each quadrant, providing vessels fit within the field of view. All photographed veins were analysed, on average 13 veins in each slide (780 total).

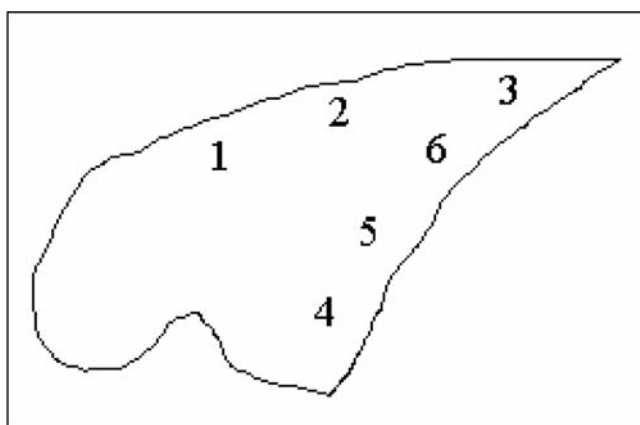


Fig 1: Diagrammatic representation of a horse's left lung in its normal position with respect to gravity. The locations of the sampled regions are shown.

Lung parenchyma was similarly selected. Fields that contained large airways, veins, arteries or septa were not photographed. A maximum of 4 images per quadrant and 16 images per slide were captured.

Each septum attached to the pleura was imaged in every section using a 4x objective. Two adjacent photographs were taken of each septum, starting at the junction of pleura and septum. With 4 exceptions, all sections contained pleura. In PR-stained sections, 4 images were taken of the pleura using a 4x objective. For the  $\alpha$ -SMA sections, the entire length of the pleura was imaged using a 4x objective.

For measurement of haemosiderin, Prussian blue-stained slides were imaged using a 2x objective. One image was taken per quadrant.

#### *Vein wall measurements*

Intralobular veins were traced using an annotation tool. The inner and outer lumen boundaries were the endothelial lining and the interface between the collagen-rich adventitia and the surrounding parenchyma, respectively. MicroSuite5 Biological Software was used to calculate luminal and outer perimeter lengths. Average vein wall thickness was determined by the difference between outer and inner perimeter, divided by  $2\pi$ . This formula assumes a circular vein wall cross-section. Vein wall collagen content in the bronchovascular bundle was not quantified because airway, artery and vein wall collagen could not be separated.

#### *Collagen quantification*

Camera settings were optimised for each objective to eliminate intensity saturation. MicroSuite5 Biological Software converted images to greyscale for intensity measurements. For intralobular veins, wall collagen content and collagen concentration (content/area) were measured. Interstitial collagen concentration was measured over the entire image. The annotation tool delineated the septa and pleura, and thickness and collagen content and concentration were measured. Seven measurements of pleural thickness were made in each image.

#### *Haemosiderin quantification*

Camera settings were optimised to eliminate intensity saturation. We measured the percentage of image area stained with Prussian blue and the number of blue particles.

#### *Number of vessels in the pleura*

Alpha-SMA immunohistochemistry was used to identify and count the number of vascular profiles in the pleura per length of pleural surface.

#### *Statistical analysis*

For each slide, an average value was calculated for each variable. The data from EIPH-affected horses were then analysed by 2 methods. First, the regional variation in lesions was examined using a 2-way repeated measures ANOVA with lung (right or left) and region (1–6) as main effects. When significant main effects or interactions were present, each region was compared to Region 3 by Dunnett's test with significance of  $P < 0.05$ . Second, slides were

**TABLE 1: Regional values for histological score, pulmonary vein wall thickness, haemosiderin particle count, and percentage of lung parenchyma occupied by haemosiderin in 5 EIPH-affected horses**

	Region 3	Region 2	Region 6	Region 1	Region 4	Region 5	P value L>R
Histological score	7.2 ± 8.1	5.8 ± 8.8	3.6 ± 7.9	3.3 ± 8.5	0.2 ± 3.8*	1.0 ± 4.5*	0.075
Vein wall thickness (microns)	52.9 ± 6.5	47.5 ± 4.3	49.2 ± 4.6	50.6 ± 5.3	37.2 ± 2.7*	36.3 ± 2.7*	0.287
Haemosiderin particle count	718 ± 233	363 ± 200	138.9 ± 168.3	364 ± 239	1.7 ± 0.1*	3.3 ± 1.4*	0.029
Haemosiderin % area	3.43 ± 1.33	4.96 ± 3.26	0.38 ± 0.25	3.49 ± 2.85	0.00 ± 0.00*	0.00 ± 0.00*	0.071

Values are mean ± s.e. \* = Significantly different from Region 3 (Dunnett's test). The P value (2-way ANOVA) for right left comparisons also is shown.

grouped by HS, and compared using a repeated measure analysis of variance. When statistical significance was found (P<0.05), means were compared by Student-Newman-Keuls test. For graphic presentation, data are expressed as a percentage of the HS 0 group except for haemosiderin data in which the HS 0 score was zero.

**Results**

*Histopathology*

The histopathology of these EIPH-affected lungs has been described (Williams *et al.* 2008). In HS 10–15, there was considerable remodelling of the intralobular veins. A prominent collar of mature collagen surrounded most intralobular veins, and the Verhoeff von Gieson-stained sections revealed collagen interposed between the external elastic lamina and lumen, often resulting in a reduced venous lumen. Colocalised with these changes was extensive haemosiderosis and severe fibrosis, involving the alveolar interstitium, bronchovascular bundle, pleura and septa. There was a considerable increase in the number of arteriolar profiles within the fibrotic alveolar interstitium, pleura/sub-pleura, and within the thickened interlobular septa. No such lesions were observed in the control horses.

*Histopathological scoring*

Of sections from the EIPH-affected horses, 22/60 (37%) were in HS 0, 30/60 (50%) in HS 1–9, and 8/60 (13%) in HS 10–15. The average HS was highest (7.2) in Region 3 but not significantly different from Regions 1 (HS = 3.3), 2 (HS = 5.8) and 6 (HS = 3.6).

**TABLE 2: Regional values (mean) for histological score, pulmonary vein wall thickness, particle count of haemosiderin, and percentage of lung parenchyma occupied by haemosiderin in two 2-year-old unraced control horses**

	Region					
	3	2	6	1	4	5
Histological score	0	0	0	0	0	0
Vein wall thickness (microns)	21.5	24.5	26.1	21.5	22.1	21.3
Haemosiderin particle count	0	0	0	0	0	0
Haemosiderin % area	0	0	0	0	0	0

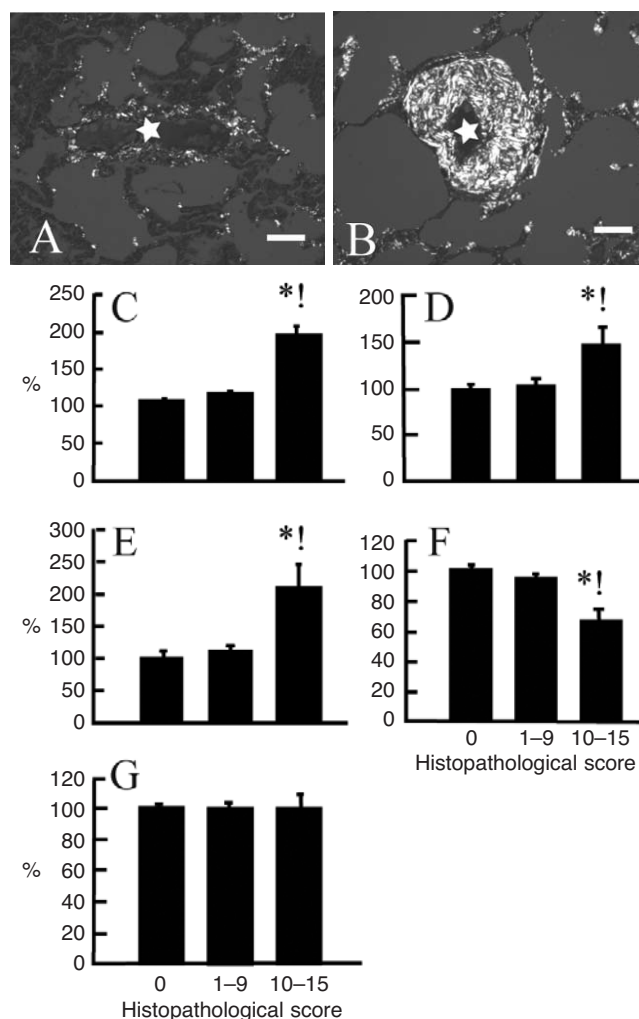
**TABLE 3: Correlations between vein wall thickness, haemosiderin accumulation and histopathological score in lungs of 5 EIPH-affected horses. Numbers are: correlation coefficient (P value)**

	Haemosiderin particle count	Haemosiderin % area	Histopathological score
Vein wall thickness	0.694 (<0.00001)	0.497 (<0.00001)	0.696 (<0.00001)
Haemosiderin particle count		0.830 (<0.00001)	0.816 (<0.00001)
Haemosiderin % area			0.745 (<0.00001)

Scores for Regions 4 (HS = 0.2) and 5 (HS = 1.0) were significantly less than those of Region 3 (Table 1). All sections from control horses were scored HS = 0.

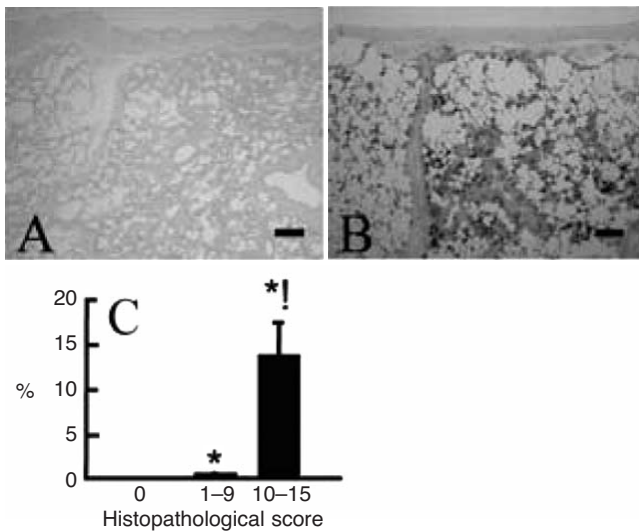
*Morphometric analysis by lung region*

In the EIPH-affected horses, vein wall thickness was greater in the dorsal than in the ventral regions (Table 1) and greatest in

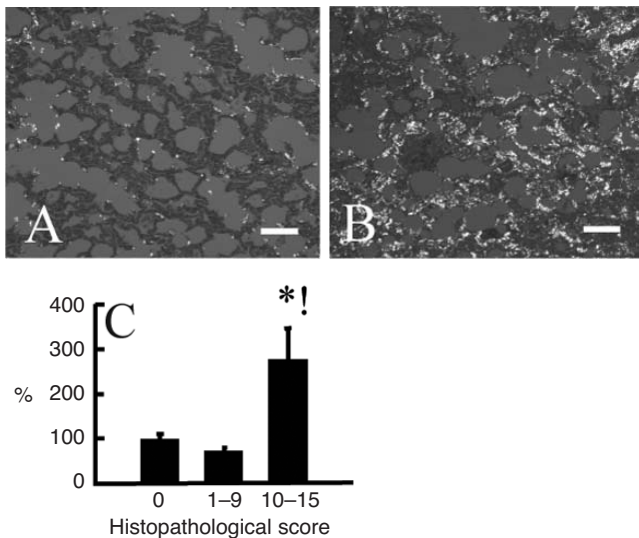


**Fig 2: Intralobular vein collagen, EIPH lung; HS = 0 (A), a small amount of collagen (white) is restricted to the adventitia of the vein wall, and the lumen is evident (\*); HS = 10–15 (B), the collagen is greatly increased around the vein, and the lumen diameter is reduced (\*). Picrosirius red stain. Bar = 50 µm. Vein wall thickness (C), collagen concentration (D), total collagen (E), inner wall (F) and outer wall perimeter (G) in lung regions with a histopathological score (HS) of 0, 1–9 and 10–15. Data (mean ± s.e.) are expressed as percent of HS 0. \* = Significantly different from HS = 0; ! = Significantly different from HS = 1–9.**

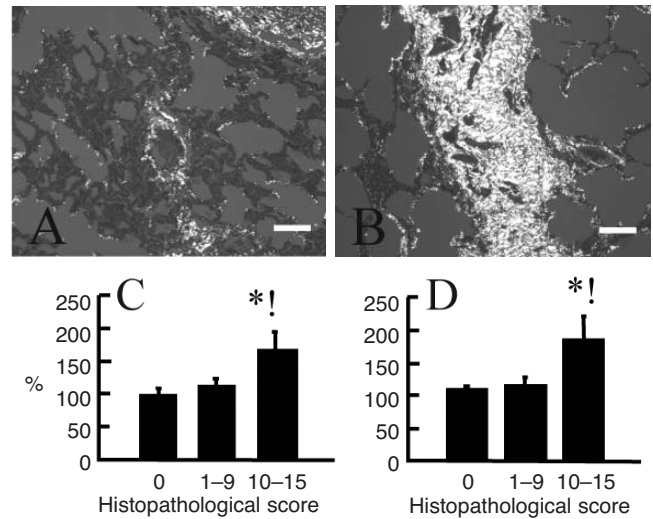
Region 3. Thickness of Regions 1, 2, 3 and 6 did not differ but thickness was significantly less in Regions 4 and 5 than in Region 3. There were no regional differences in venous wall collagen concentration or total collagen content or in inner vein perimeter. Regional differences in haemosiderin particle number and percent of lung parenchyma occupied by haemosiderin were similar to vein wall thickness. The number of pleural blood vessels was higher in Region 3 than all other regions. The amount of haemosiderin was significantly greater in left than right lung, as were the number of pleural blood vessels (data not shown).



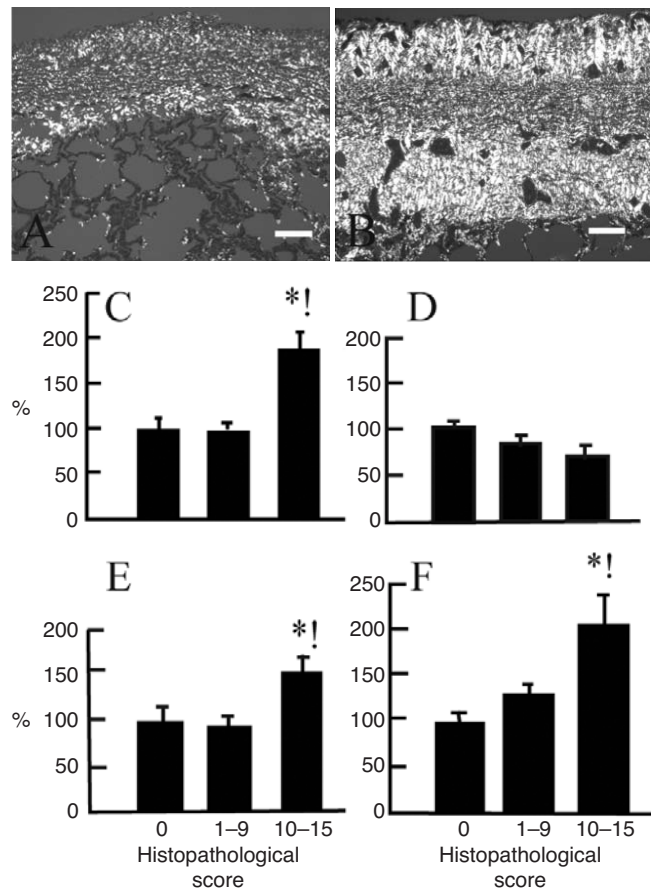
**Fig 3:** Haemosiderin accumulation, EIPH lung; HS = 0 (A), there is no significant detectable haemosiderin; HS = 10-15 (B), haemosiderin (dark pigment) is abundant throughout the lung region. Prussian blue stain. Bar = 400  $\mu$ m. Haemosiderin (% area staining with Prussian blue) (C) in lung regions with a histopathological score (HS) of 0, 1-9 and 10-15. \* = Significantly different from HS = 0; ! = significantly different from HS = 1-9.



**Fig 4:** Interstitial collagen, EIPH lung; HS = 0 (A), small foci of collagen (white) are restricted to the junction of alveolar walls; HS = 10-15 (B), the collagen is greatly increased within the alveolar interstitium. Picrosirius red stain. Bar = 100  $\mu$ m. Interstitial collagen concentration (C) in lung regions with a histopathological score (HS) of 0, 1-9 and 10-15. Data (mean  $\pm$  s.e.) are expressed as percent of HS 0. \* = Significantly different from HS = 0; ! = significantly different from HS = 1-9.



**Fig 5:** Septal collagen, EIPH lung; HS = 0 (A), a small amount of collagen (white) is evident within the septum, and surrounding the septal vasculature; HS = 10-15 (B), the septa are greatly expanded by collagen. Picrosirius red stain. Bar = 100  $\mu$ m. Septum collagen concentration (C) and total collagen (D) in lung regions with a histopathological score (HS) of 0, 1-9 and 10-15. Data (mean  $\pm$  s.e.) are expressed as percent of HS 0. \* = significantly different from HS = 0; ! = significantly different from HS = 1-9.



**Fig 6:** Pleural collagen, EIPH lung; HS = 0 (A); HS = 10-15 (B), the collagen (white) is greatly increased within the pleura. Picrosirius red stain. Bar = 200  $\mu$ m. Pleural thickness (C), collagen concentration (D), total collagen (E), and number of pleural vascular structures (F) in lung regions with a histopathological score (HS) of 0, 1-9 and 10-15. Data (mean  $\pm$  s.e.) are expressed as percent of HS 0. \* = Significantly different from HS = 0; ! = significantly different from HS = 1-9.

No regional differences in vein wall thickness or haemosiderin content were observed in the lungs of control horses (Table 2). On average, vein wall thickness was less in these horses, and in caudodorsal regions, was half that in EIPH-affected animals.

In EIPH-affected horses, vein wall thickness was significantly correlated with both haemosiderin particle number and the percent of parenchyma occupied by haemosiderin. All of these measures were significantly correlated with HS (Table 3).

#### Morphometric analysis based on HS

Intralobular vein wall thickness, collagen concentration, and total collagen content were significantly greater in HS 10–15 than in HS 0 and HS 1–9, which did not differ. Outer venous wall perimeter did not differ between the groups but luminal perimeter was significantly smaller in the HS 10–15 than HS 0 and HS 1–9 (Fig 2). The percentage of each section staining with the Prussian blue was 0 in HS 0 and was increased significantly in the HS 1–9, and further in HS 10–15 (Fig 3). In the lung parenchyma, collagen concentration was not different between HS 0 and HS 1–9, but increased significantly in HS 10–15 (Fig 4). Interlobular septa in HS 10–15 had significantly greater collagen concentration and total collagen content than in HS 0 and HS 1–9, which did not differ (Fig 5). Pleural thickness, total collagen content, and the number of pleural vessels were increased significantly in HS 10–15 compared to HS 0 and HS 1–9, but pleural collagen concentration was not different between the groups (Fig 6).

#### Discussion

The prevailing hypothesis about EIPH pathogenesis is capillary stress failure in response pulmonary hypertension during exercise (West *et al.* 1993; West and Mathieu-Costello 1994). The observations in the current paper suggest that stress failure may be exacerbated by venous remodelling. Inward venous remodelling, and the resultant increased resistance downstream from alveolar capillaries, result in elevated pulmonary capillary pressures, especially during exercise. We suggest that the resultant bleeding leads to colocalisation of haemosiderin, fibrosis and angiogenesis.

The colocalisation of the venous remodelling, haemosiderin, fibrosis and angiogenesis was apparent in lung regions sorted by HS. Throughout HS 10–15, most intralobular veins had excess collagen in the adventitia, often interposed between the elastic lamina and the lumen (Williams *et al.* 2008). Compared to HS 0, vein collagen concentration in the HS 10–15 was increased by 48%, while the total collagen content more than doubled. Consequently, mean intralobular vein wall thickness in HS 10–15 was 83% greater than in HS 0. Interestingly, venous outer wall perimeter did not differ between groups but, compared to HS 0, lumen perimeter was reduced by 33% in HS 10–15. This inward remodelling results in increased vascular resistance (Herity *et al.* 1999) that leads to increased upstream pulmonary capillary and arterial pressure, and diverts blood flow to other regions.

When the distribution of lesions was examined by region, the highest HS was in Region 3, but HS in other dorsal regions, although lower, did not differ significantly from Region 3. In contrast, HS in the 2 most ventral parts of the lung (Regions 4 and 5) was significantly less than in Region 3. Vein wall thickness and haemosiderin had the same regional distribution and were highly correlated giving further support to their colocalisation and to the presence of veno-occlusion as the underlying cause of

haemosiderosis. The strikingly thinner vein wall and the lack of regional variability in the young, unraced control horses increases our confidence that regional changes in EIPH-affected horses are related to the disease and not to regional variability in normal Thoroughbred lungs. The control horses were younger than the EIPH-affected horses; therefore we cannot rule out that the difference in vein wall thickness between these 2 groups of horses is a function of age rather than EIPH.

One possible cause of venous remodelling in EIPH-affected horses is venous hypertension and, in exercising horses, pulmonary wedge pressure averages 70 mmHg (Manohar and Goetz 1996; Manohar *et al.* 1996). Even brief venous hypertension elicits vessel wall remodelling. For example, 300 mmHg for 2 min in a jugular vein graft results in up-regulation of matrix-degrading metalloproteinases (MMP-2, MMP-9) and formation of new intima (Chung *et al.* 2005). Also, in sheep, 4 days after induction of pulmonary hypertension, there is remodelling of pulmonary veins, an increased number of muscular veins, thickened vein walls and reduced vein lumen (Johnson *et al.* 1997). During training, racehorses are exercised vigorously several times weekly, subjecting the pulmonary circulation to repeated bouts of hypertension that may cause the venous wall thickening.

In human pulmonary veno-occlusive disease, haemosiderosis and fibrosis are colocalised (Holcomb *et al.* 2000; Mandel *et al.* 2000). Therefore, it seems likely that in horses, venous obstruction causes local capillary hypertension, bleeding and accumulation of haemosiderin, which leads to interstitial fibrosis because of oxygen radical injury exacerbated by the high iron content of haemosiderin (O'Connell *et al.* 1986; Grady *et al.* 1989). Fibrosis apparently requires interstitial haemosiderin because autologous blood infused into bronchi results in haemosiderin in the airways only, but no accompanying fibrosis (Derksen *et al.* 2007). Interestingly, the amount of haemosiderin was significantly greater in left than right lung, as were the number of pleural blood vessels. A previous endoscopic study indicated a right lung predisposition for EIPH (Hillidge 1986) and we have no explanation for the discrepancy between the 2 investigations. Even if venous hypertension causes venous wall thickening, why is the change restricted to the dorso-caudal region of the lung? This might occur if caudodorsal veins experience a higher intraluminal pressure as a consequence of the preferential caudodorsal distribution of blood flow during exercise (Bernard *et al.* 1996). Furthermore, even within an isogravitric

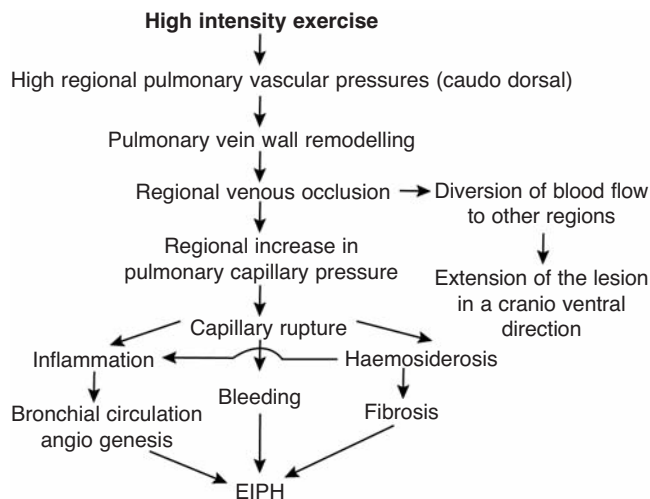


Fig 7: Hypothesis for the pathogenesis of EIPH.

plane, pulmonary blood flow can vary considerably (Bernard *et al.* 1996; Hlastala *et al.* 1996; Galvin *et al.* 2007) suggesting that, within the caudodorsal region, lesions of EIPH may well be focal.

In summary, our data support the following hypothesis for the pathogenesis of EIPH (Fig 7). Repeated bouts of pulmonary venous hypertension during strenuous exercise result in regional vein wall remodelling. The resultant venous occlusion leads to capillary hypertension, capillary stress failure, bleeding, haemosiderin accumulation and lung fibrosis.

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### Manufacturer's address

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