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Differences in extracellular matrix proteins between Friesian horses with aortic rupture, unaffected Friesians and Warmblood horses

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Summary

Background: Unlike in Warmblood horses, aortic rupture is quite common in Friesian horses, in which a hereditary trait is suspected. The aortic connective tissue in affected Friesians shows histological changes such as medial necrosis, elastic fibre fragmentation, mucoid material accumulation and fibrosis with aberrant collagen morphology. However, ultrastructural examination of the collagen fibres of the mid-thoracic aorta has been inconclusive in further elucidating the pathogenesis of the disease.
Objectives: To assess several extracellular matrix (ECM) components biochemically in order to explore a possible underlying breed-related systemic ECM defect in Friesians with aortic rupture.
Study design: Cadaver study.
Methods: Tissues from affected Friesians (n = 18), unaffected Friesians (n = 10) and Warmblood horses (n = 30) were compared. Samples were taken from the thoracic aorta at the level of the rupture site, from two locations caudal to the rupture and from the deep digital flexor tendon. Total collagen content, post-translational modifications of collagen formation including lysine hydroxylation, and hydroxylysylpyridinoline (HP), lysylpyridinoline (LP) and pyrrole cross-links were analysed. Additionally, elastin cross-links, glycosaminoglycan content and matrix metalloproteinase (MMP) activity were assessed.
Results: Significantly increased MMP activity and increased LP and HP cross-linking, lysine hydroxylation and elastin cross-linking were found at the site of rupture in affected Friesians. These changes may reflect processes involved in healing and aneurysm formation. Unaffected Friesians had less lysine hydroxylation and pyrrole cross-linking within the tendons compared with Warmblood horses. No differences in the matrix of the aorta were found between normal Warmbloods and Friesian horses.
Main limitations: Small sample size.
Conclusions: The differences in collagen parameters in tendon tissue may reflect differences in connective tissue metabolism between Friesians and Warmblood horses.

Keywords: horse; aorta; Friesian horse; extracellular matrix; collagen; glycosaminoglycans; elastin

Introduction

Aortic rupture is quite rare in Warmblood horses [1,2]. In most cases, the horse presents with an aneurysm or tear of the aortic root [3,4]. The event can occur in older stallions shortly after breeding and often presents as sudden death [5]. In Friesian horses, aortic arch rupture is much more common and frequently presents as a chronic disease characterised by pseudoaneurysm formation [6]. Histopathological examination of the aortic tissues in Friesians showed accumulation of mucoid material, elastic fibre fragmentation and medial fibrosis with aberrant collagen morphology, suggesting a possible connective tissue disorder [7]. In human subjects, nontraumatic aortic rupture often occurs as a consequence of Ehlers–Danlos syndrome or Marfan's syndrome [8,9]. In Marfan's syndrome, there is abnormal transforming growth factor-β (TGF-β) signalling with changes in matrix composition and elastin and collagen content or structure [10].

Collagen determines the tensile strength of the aortic wall [11] and elastic fibres are necessary to the stretching and recoil of arteries. In human abdominal aortic aneurysm, elastin degradation has been implicated in the dilation and aberrant collagen structure that predispose to aneurysmal rupture [12]. Matrix metalloproteinases (MMPs) have proteolytic activities and degrade various extracellular matrix (ECM) proteinaceous components [13]. In man, aberrant MMP activity is associated with increased risk for abdominal aortic aneurysm [14] and aortic rupture [15].

Collagen cross-linking is fundamental to tensile strength and also increases the resistance of collagen fibres to proteolytic activity [16]. Enzymatic cross-linking of (hydroxy)lysine residues in collagen produces immature cross-links [17], which subsequently mature into trivalent pyridinoline (hydroxylysylpyridinoline and lysylpyridinoline) and pyrrole cross-links [18]. Defects in collagen or elastic cross-linking lead to an abnormal arterial structure and weakening of the aortic wall. Microstructural alterations of collagen occur in human aortic disease. For instance, an increase in collagen (pyridinoline) cross-linking is present in the aneurysms of patients with Marfan's syndrome and abdominal aortic aneurysm [19]. Patients with Marfan's syndrome are also deficient in desmosin (elastin) cross-linking and have decreased elastin content [20].

The purpose of this study was to investigate the possible existence of an underlying systemic, breed-related alteration in the biochemical composition of the ECM in aortic rupture in Friesian horses. The study focused on the assessment of collagen content, post-translational modifications of collagen, collagen cross-links, elastin cross-links, MMP activity and glycosaminoglycan (GAG) content of the aortic wall distant

from the rupture site and in another mesenchymal tissue site (deep digital flexor tendon [DDFT]), and compared affected Friesians with nonaffected Friesians and nonaffected Friesians with a control population of Warmblood horses.

Materials and methods

Animals

Eighteen affected Friesian horses (mean age: 5.7 years; age range: 3–10 years; 11 females, seven males) were included in the study. All had been diagnosed with aortic rupture by post-mortem examination at the Faculty of Veterinary Medicine, Ghent University, Belgium (n = 9) or the Faculty of Veterinary Medicine, Utrecht University, the Netherlands (n = 9). Ten unaffected Friesian horses (mean age: 4.2 years; age range: 0–10 years; five females, five males) without a history of cardiovascular or orthopaedic disease were used as a control group. Additionally, 30 Belgian Warmblood horses (mean age: 6.2 years; age range: 1–9 years; 10 females, 20 males) were used as a separate control group. Aortic and tendon tissue were collected from four unaffected Warmblood horses during post-mortem examination at Ghent University and from 11 unaffected Warmblood horses at a slaughterhouse. Tendon tissue was collected from a further 15 unaffected Warmblood horses at a slaughterhouse. Horses sampled at the slaughterhouse were apparently healthy, but their disease history was unknown.

Sampling procedure

In each horse the aorta was divided into five equal sections from the base of the heart to the diaphragm (site AO5). Full-thickness aortic wall samples of approximately 2 × 2 cm were taken at the level of aortic rupture (AO1) and from two more caudal sections (AO2 and AO3). A tissue sample was taken from the mid-metacarpal DDFT. Samples were frozen and stored at −20°C until further analysis.

Sample digestion

Aorta samples were digested for approximately 20 h by use of 50 μL elastase solution composed of 1 U/ml elastase[a] at 37°C in 200 μL of 50 mmol/L TRIS-HCl buffer (pH 8.5). Tendon samples were digested for approximately 20 h by use of 50 μL papain solution composed of 1 U/ml papain[b] at 56°C in 200 μL of 50 mmol/L phosphate buffer (pH 6.5) containing 2 mmol/L Na_2EDTA and 2 mmol/L cysteine.

Pyridinoline-, (iso)desmosine cross-links and amino acid analysis

Samples were prepared for high-performance liquid chromatography (HPLC) as described previously [21]. Briefly, after lyophilising for 24 h, approximately 5 mg of aorta or tendon sample was hydrolysed in 600 μL 6 mol/L HCL at 110°C for 16 h before 100 μL 2.4 mmol/L homo-arginine (internal standard) was added. The samples were dried in a speedVac and dissolved in 600 μL sample buffer (1.2 mmol/L heptafluorobuteric acid and 2.5 mmol/L ammonium acetate, pH 5.6, in 20% acetonitril). The amino acids proline, hydroxyproline and hydroxylysine, and the collagen crosslinks lysylpyridinoline (LP) and hydroxylysylpyridinoline (HP) were quantified by HPLC/mass spectrometry analysis as described previously [21]. This method was modified for the analysis of elastin-specific cross-links by adding the MRM-transitions 526.4/481.3 and 526.4/397.2 for desmosine and isodesmosine, respectively. The parameters were expressed as g collagen/g dry weight for hydroxyproline (as a measure for total collagen) and mol hydroxylysine/mol collagen (as a measure for collagen hydroxylation). LP and HP collagen cross-links were expressed as mol/mol collagen and elastin cross-links were expressed as desmine and isodesmosine mol/g dry weight.

Pyrrole cross-links

Pyrrole cross-links were analysed in the enzyme digests of the samples using a modified version of the method described by Thorpe *et al.*[22] using digestion by papain instead of trypsin. Briefly, 20 μL of the digest was diluted with 180 μL H_2O, after which 40 μL of Ehrlich's reagent (500 mg 4-dimethylbenzalhyde in 4.4 ml 60% perchloric acid completed to 10 ml with H_2O) was added. The absorbance of the samples was measured at 650 nm as a background reference. Then, 1-methyl-pyrrole was used to create a standard curve. Results were expressed as mol pyrrole per g Lowry protein.

Metalloproteinase activity

General MMP activity was measured using a fluorimetric assay based on the cleavage of a fluorogenic peptide substrate FS-6[c] [23]. Briefly, 0.1 g tissue was added to 500 μL of cooled MMP buffer (0.1 mol/L Tris, 0.1 mol/L NaCl, 10 mmol/L $CaCl_2$, 0.05% [w/v] triton X-100, 0.1% [w/v] PEG6000, pH 7.5) and homogenised (MagNA Lyser homogeniser[d]). Subsequently, 100 μL of this mixture was added to 100 μL of 10 μmol/L FS-6 solution and the fluorescent signal was monitored for 30 min using a Clariostar® fluorimeter[e] . The slope of the resulting linear curve (relative fluorescence units/s [RFU/s]) was calculated as a measure of general MMP activity. Measurements were corrected with the sample protein concentration, determined by a modified Lowry assay [24].

Glycosaminoglycans

The GAG content in the digests of the samples was assessed spectrophotometrically with the modified 1,9-dimethylmethylene blue assay [25,26]. Amounts of GAGs were expressed as mg per g Lowry protein.

Data analysis

Comparisons were made between affected and unaffected Friesians and between unaffected Friesians and Warmbloods. Statistical analysis (SAS Version 6.4[f]) was performed using generalised linear mixed models with the horse as random effect, and sample location and type of horse as categorical effects. Normality was tested using the Shapiro–Wilks test. *F*-tests were used for testing at the 5% significance level. The significance level for multiple comparisons was adjusted by Bonferroni's technique, leading, with eight comparisons, to a P≥0.006.

Results

Horse group had a significant effect on LP cross-links (P<0.001), desmosine content (P<0.001) and isodesmosine content (P<0.001). Location had a significant effect on total collagen content (P<0.001), lysine hydroxylation (P = 0.002), LP cross-links (P<0.001), desmosine content (P<0.001), isodesmosine content (P<0.001), pyrrole cross-links (P<0.001) and MMP activity (P<0.001). There were significant interactions between group and location for lysine hydroxylation (P<0.001), LP cross-links (P<0.001), desmosine content (P<0.001), isodesmosine content (P<0.001) and MMP activity (P<0.001).

Total collagen, pyridinoline cross-links and amino acid analysis

There were no significant differences between the horse groups in total collagen content. In affected Friesians, samples from the proximal segment of the aorta (i.e. AO1) had a significantly higher degree of lysyl hydroxylation (P = 0.003) (Fig 1) and a significantly higher number of HP cross-links compared with samples from unaffected Friesians (P = 0.002) (Fig 2a). Unaffected Friesian horses had a significantly lower degree of lysyl hydroxylation (P<0.001) (Fig 1) and a significantly lower number of HP cross-links in the DDFT (P = 0.004) compared with Warmblood horses (Fig 2a).

The affected Friesians had a significantly higher number of LP cross-links in AO1 compared with unaffected Friesians (P<0.001) (Fig 2b). There were no significant differences in the number of LP cross-links between Warmblood horses and unaffected Friesians in any of the samples. Affected Friesians had significantly higher desmosine content and isodesmosine content in AO1 compared with unaffected Friesians (P<0.001) (Fig 3).

Pyrrole cross-links

Location had a general effect on pyrrole cross-links; there were more pyrrole cross-links in DDFT compared with aortic wall samples in both breeds (Fig 4). Pyrrole cross-links were significantly lower in the DDFT of unaffected Friesians compared with that of Warmblood horses ($P = 0.003$) (Fig 4).

Metalloproteinase activity

Metalloproteinase activity in AO1 was significantly higher in affected Friesians compared with unaffected Friesians ($P = 0.004$) (Fig 5). Warmblood horses had significantly higher MMP activity in AO2 compared with unaffected Friesians ($P<0.001$) (Fig 5).

Glycosaminoglycans

Horse group had an effect on GAG content. GAG content was lower in unaffected Friesians and affected Friesians than in Warmblood horses (Fig 6).

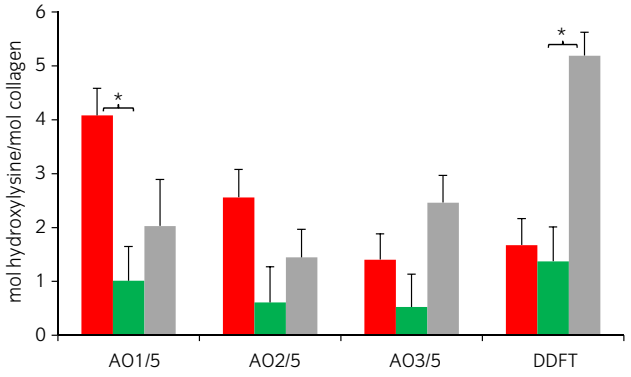

Fig 1: Mean ± s.d. lysyl hydroxylation in affected Friesians (red), unaffected Friesians (green) and Warmblood horses (grey) for locations AO1, AO2 and AO3 and deep digital flexor tendon (DDFT) samples. *Significant difference ($P<0.01$).

Discussion

The ECM of the aorta plays an important role in maintaining the normal structure of the arterial wall [27]. Abnormalities of ECM components, their structure or their interactions can alter matrix turnover and have consequences for aortic wall strength and subsequent vascular disease.

Collagen hydroxylation and levels of LP and HP collagen cross-linking were significantly higher at the aortic rupture site in affected horses in comparison with the same location in unaffected Friesians. These substantial differences in the biochemical characteristics of the aortic wall are consistent with damage and subsequent remodelling at the rupture site in affected Friesian horses.

Affected Friesian horses had significantly higher MMP activity at the site of rupture than did unaffected Friesians, a difference that is likely to reflect greater MMP activity in the context of increased repair and remodelling activity as a sequel to rupture. A considerable degree of remodelling of the aortic wall can be expected when horses with rupture or aortopulmonary fistulation remain alive for weeks to months after the onset of the condition. In these circumstances, a new equilibrium is established and is characterised by higher tissue turnover and increased MMP activity. Greater MMP activity at the rupture site concords with the well-recognised role of MMPs in the pathogenesis of degenerative abdominal and thoracic aortic aneurysm formation seen in human aortic disease [28,29]. The present study found no significant differences in the composition of the ECM of the aorta between Warmblood horses and Friesians and no evidence that an underlying systemic disorder relating to aberrant MMP activity exists in Friesian horses with aortic rupture.

Elastin cross-link levels in proximal aortic tissue were found to be significantly increased in affected Friesians compared with unaffected Friesian horses. This is likely to reflect the result of healing and remodelling. It may be an attempt to strengthen the rupture area as compensation for the loss of structural integrity of the aortic tissue and increased turbulence of blood flow within the pseudoaneurysms. In human subjects with aneurysms, TGF-β stimulation of chronically activated smooth muscle cells results in increased elastin formation and elastin cross-linking [30]. A similar mechanism may explain the increased elastin cross-linking found at the rupture site in affected Friesian horses in the current study.

There were significant differences in DDFT ECM components between Friesian horses and Warmbloods, providing evidence for inherent differences in connective tissue ECM between the breeds. Collagen lysine hydroxylation was lower in the flexor tendons of unaffected Friesian horses than in those of Warmbloods, suggesting a significant difference between the breeds with respect to the metabolism of collagen, the most abundant

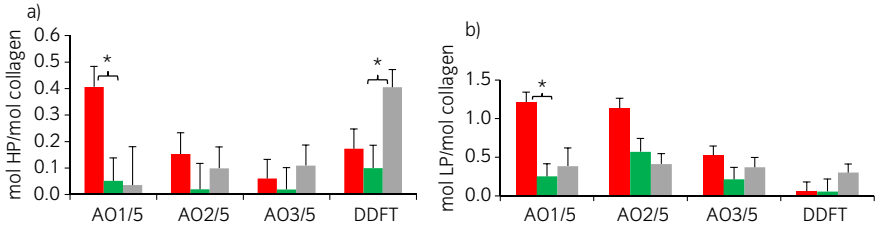

Fig 2: Mean ± s.d. numbers of a) hydroxylysylpyridinoline (HP) cross-links and b) lysylpyridinoline (LP) cross-links in affected Friesians (red), unaffected Friesians (green) and Warmblood horses (grey) for locations AO1, AO2 and AO3 and deep digital flexor tendon (DDFT) samples. *Significant difference ($P<0.002$).

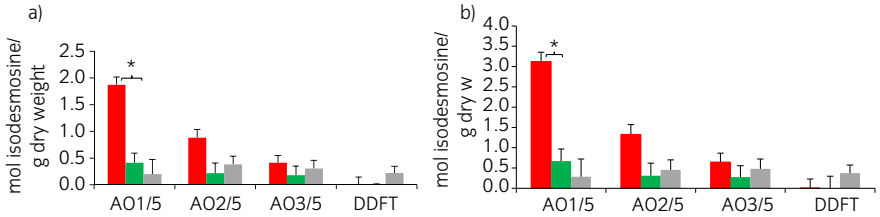

Fig 3: Mean ± s.d. a) desmosine content and b) isodesmosine content, measured by the number of desmosine and isodesmosine cross-links per amount of tissue in affected Friesians (red), unaffected Friesians (green) and Warmblood horses (grey) for locations AO1, AO2 and AO3 and deep digital flexor tendon (DDFT) samples. Dry w, dry weight. *Significant difference $P<0.0001$.

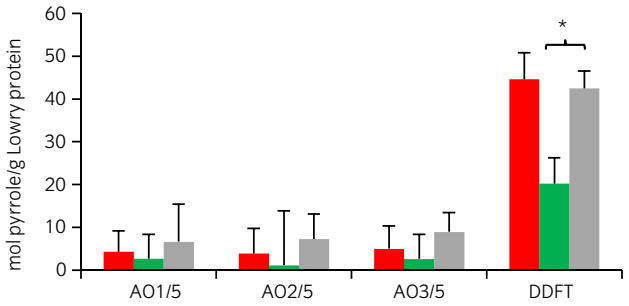

Fig 4: Mean ± s.d. pyrrole cross-links in affected Friesians (red), unaffected Friesians (green) and Warmblood horses (grey) for locations AO1, AO2 and AO3 and deep digital flexor tendon (DDFT) samples. *Significant difference (P<0.01).

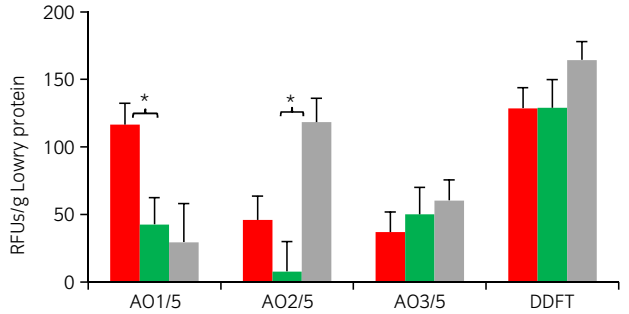

Fig 5: Mean ± s.d. matrix metalloproteinase activity (relative fluorescence units [RFUs]/g Lowry protein) in affected Friesians (red), unaffected Friesians (green) and Warmblood horses (grey) for locations AO1, AO2 and AO3 and deep digital flexor tendon (DDFT) samples. *Significant difference (P<0.01).

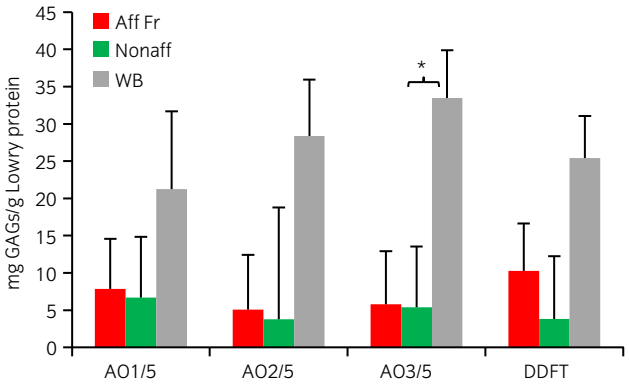

Fig 6: Mean ± s.d. glycosaminoglycan content in affected Friesians (red), unaffected Friesians (green) and Warmblood horses (grey) for locations AO1, AO2 and AO3 and deep digital flexor tendon (DDFT) samples. *Significant difference (P<0.01).

structural protein in the mammalian body. The exact role of collagen lysine hydroxylation (and possible subsequent glycosylation) is still unknown [31,32]. An increase in this post-translational modification is linked with a decrease in fibril diameter [31,32] and decreased tensile strength. HP cross-linking and pyrrole cross-linking also differed in flexor tendon tissue between Friesian and Warmblood horses. These ECM elements were significantly lower in Friesian horses than in Warmbloods, a finding that may reflect fundamental differences in collagen turnover between the breeds. These findings support previous suggestions of breed-specific variation and may partly explain the less stiff tendons of Friesians [33].

Affected Friesians are thought to have a phenotype that differs from that of unaffected Friesians. However, the present study shows significant differences between these Friesian horse groups only at the site of aortic rupture. Other significant changes may be masked by the fact that the different horse groups in this study included only small numbers of horses. It is possible that smaller mutations superimposed upon breed-related aberrant connective tissue metabolism in Friesians explain several diseases of the Friesian horse breed.

Levels of HP collagen cross-links reported in the current study are very low compared with previously published results in which HP levels of 0.7 mol/mol collagen were determined in 60 adult slaughter horses [34], whereas approximately 0.2–0.4 mol/mol collagen were observed here. However, the present study not only included horses in a younger age range, but also investigated findings in a different tendon with different properties. The DDFT was sampled at a more proximal site in comparison with that in the previous study and tendon composition can differ greatly between regions within the same tendon [34]. The LP cross-links reported in DDFT in the current study are high (1–5 mol/mol collagen) compared with the 0.02–0.04 mol/mol collagen previously documented in superficial digital flexor tendon [34].

The present study is limited by its small sample size, which precluded any examination of sex and age effects. No sex predisposition was found in Friesians with aortic rupture in a previous study [6]. The age of 4–5 years at which Friesians present with aortic rupture coincides with the age at which most Friesian horse owners start to prepare horses for active competition. Therefore, it may be more likely that an increase in exercise is an important triggering factor, rather than age per se.

Conclusions

This study shows that there is extensive ECM remodelling at the site of rupture in affected Friesian horses, demonstrated by increases in LP cross-links, elastin cross-linking and MMP levels at the rupture sites compared with matched sites in unaffected Friesians. No differences in the ECM of the aorta were found between normal Warmbloods and Friesian horses. The present study shows differences in collagen lysine hydroxylation and pyrrole cross-links in flexor tendon tissue between Friesian horses and Warmbloods, which suggests the existence of differences in connective tissue metabolism and homeostasis between the breeds. These findings warrant further research.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

The study included abattoir material and material from client-owned horses collected during post-mortem examination. Explicit owner informed consent for participation in this study was not stated, but general permission for post-mortem examination was given and owners were able to opt out of research studies.

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Authorship

M. Ploeg, A. Gröne, C.H.A. van de Lest and C. Delesalle contributed to the study design. M. Ploeg, C.H.A. van de Lest and C. Delesalle contributed to the study execution. All authors contributed to data analysis and interpretation, and to the preparation of the manuscript.

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References

1. Ploeg, M., Saey, V., van Loon, G. and Delesalle, C. (2016) Thoracic aortic rupture in horses. *Equine Vet. J.* **49**, 269-274.
2. Rooney, J.R. (1979) Rupture of the aorta. *Mod. Vet. Pract.* **60**, 391-392.
3. Sleeper, M.M., Durando, M.M., Miller, M., Habecker, P.L. and Reef, V.B. (2001) Aortic root disease in four horses. *J. Am. Vet. Med. Assoc.* **219**, 491-496.
4. Marr, C.M., Reef, V.B., Brazil, T.J., Thomas, W.P., Knottenbelt, D.C., Kelly, D.F., Baker, J.R., Reimer, J.M., Maxson, A.D. and Crowhurst, J.S. (1998) Aorto-cardiac fistulas in seven horses. *Vet. Radiol. Ultrasound.* **39**, 22-31.
5. Rooney, J.R., Prickett, M.E. and Crowe, M.W. (1967) Aortic rupture in stallions. *Pathol. Vet.* **4**, 268-274.
6. Ploeg, M., Saey, V., de Bruijn, C.M., Gröne, A., Chiers, K., van Loon, G., Ducatelle, R., van Weeren, P.R., Back, W. and Delesalle, C. (2013) Aortic rupture and aorto-pulmonary fistulation in the Friesian horse: characterisation of the clinical and gross post mortem findings in 24 cases. *Equine Vet. J.* **45**, 101-106.
7. Ploeg, M., Saey, V., Delesalle, C., Gröne, A., Ducatelle, R., de Bruijn, M., Back, W., van Weeren, P.R., van Loon, G. and Chiers, K. (2015) Thoracic aortic rupture and aortopulmonary fistulation in the Friesian horse: histomorphologic characterization. *Vet. Pathol.* **52**, 152-159.
8. Eder, J., Laccone, F., Rohrbach, M., Giunta, C., Aumayr, K., Reichel, C. and Trautinger, F. (2013) A new COL3A1 mutation in Ehlers-Danlos syndrome type IV. *Exp. Dermatol.* **22**, 231-234.
9. Scheck, M., Siegel, R.C., Parker, J., Chang, Y.H. and Fu, J.C. (1979) Aortic aneurysm in Marfan's syndrome: changes in the ultrastructure and composition of collagen. *J. Anat.* **129**, 645-657.
10. Tsamis, A., Krawiec, J.T. and Vorp, D.A. (2013) Elastin and collagen fibre microstructure of the human aorta in ageing and disease: a review. *J. R. Soc. Interface* **27**, 1-21.
11. Burton, A.C. (1954) Relation of structure to function of the tissues of the wall of blood vessels. *Physiol. Rev.* **34**, 619-642.
12. Dobrin, P.B., Baker, W.H. and Gley, W.C. (1984) Elastolytic and collagenolytic studies of the arteries: implications for the mechanical properties of arteries. *Arch. Surg.* **119**, 405-409.
13. Hellenthal, F.A., Buurman, W.A., Wodzig, W.K. and Schurink, G.W. (2009) Biomarkers of abdominal aortic aneurysm progression. Part 2: inflammation. *Nature Rev. Cardiol.* **6**, 543-552.
14. Rizas, K.D., Ippagunta, N. and Tilson, M.D. (2009) Immune cells and molecular mediators in the pathogenesis of the abdominal aortic aneurysm. *Cardiol. Rev.* **17**, 201-210.
15. Wilson, W.R., Anderton, M., Schwalbe, E.C., Jones, J.L., Furness, P.N., Bell, P.R. and Thompson, M.M. (2006) Matrix metalloproteinases-8 and -9 are increased at the site of abdominal aortic aneurysm rupture. *Circulation* **113**, 438-445.
16. Vater, C.A., Harris, E.D. and Siegel, R.C. (1979) Native cross-links in collagen fibrils induce resistance to human synovial collagenase. *Biochem. J.* **181**, 639-645.
17. Bailey, A.J., Paul, R.G. and Knott, L. (1998) Mechanisms of maturation and ageing of collagen. *Mech. Ageing Dev.* **106**, 1-56.
18. Hanson, D.A. and Eyre, D.R. (1996) Molecular site specificity of pyridinoline and pyrrole cross-linking in type I collagen of human bone. *J. Biol. Chem.* **271**, 26508-26516.
19. Lindeman, J.H.N., Ashcroft, B.A., Beenakker, J.M., van Es, M., Koekkoek, N.B.R., Prins, F.A., Tielemans, J.F., Abdul-Hussien, H., Bank, R.A. and Oosterkamp, T.H. (2010) Distinct defects in collagen microarchitecture underlie vessel-wall failure in advanced abdominal aneurysms and aneurysms in Marfan syndrome. *Proc. Natl Acad. Sci. USA* **107**, 862-865.
20. Abraham, P.A., Perejda, A.J., Carnes, W.H. and Uitto, J. (1982) Marfan syndrome. Demonstration of abnormal elastin in aorta. *J. Clin. Invest.* **70**, 1245-1252.
21. Souza, M.V., van Weeren, P.R., van Schie, H.T.M. and van de Lest, C.H.A. (2010) Regional differences in biochemical, biomechanical and histomorphological characteristics of the equine suspensory ligament. *Equine Vet. J.* **42**, 611-620.
22. Thorpe, C.T., Stark, R.J.F., Goodship, A.E. and Birch, H.L. (2010) Mechanical properties of the equine superficial digital flexor tendon relate to specific collagen cross-link levels. *Equine Vet. J.* **42**, 538-543.
23. de Grauw, J.C., van de Lest, C.H., Brama, P.A., Rambags, B.P. and van Weeren, P.R. (2009) In vivo effects of meloxicam on inflammatory mediators, MMP activity and cartilage biomarkers in equine joints with acute synovitis. *Equine Vet. J.* **41**, 693-699.
24. Dulley, J.R. and Grieve, P.A. (1975) A simple technique for eliminating interference by detergents in the Lowry method of protein determination. *Anal. Biochem.* **64**, 136-141.
25. Farndale, R.W., Buttle, D.J. and Barret, A.J. (1986) Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylene blue. *Biochim. Biophys. Acta* **883**, 173-177.
26. van der Harst, M.R., de Groot, J., Kiers, G.H., Brama, P.A., van de Lest, C.H. and van Weeren, P.R. (2005) Biochemical analysis of the articular cartilage and subchondral and trabecular bone of the metacarpophalangeal joint of horses with early osteoarthritis. *Am. J. Vet. Res.* **66**, 1238-1246.
27. Berillis, P. (2013) The role of collagen in the aorta's structure. *Open Circ. Vasc. J.* **6**, 1-8.
28. Hobeika, M.J., Thompson, R.W., Muhs, B.E., Brooks, P.C. and Gagne, P.J. (2007) Matrix metalloproteinases in peripheral vascular disease. *J. Vasc. Surg.* **45**, 849-857.
29. Pearce, W.H. and Shively, V.P. (2006) Abdominal aortic aneurysm as a complex multifactorial disease: interactions of polymorphisms of inflammatory genes, features of autoimmunity, and current status of MMPs. *Ann. N. Y. Acad. Sci.* **1085**, 117-132.
30. Kothapalli, C.R. and Ramamurthi, A. (2010) Induced elastin regeneration by chronically activated smooth muscle cells for targeted aneurysm repair. *Acta Biomater.* **6**, 170-178.
31. Brinckmann, J., Notbohm, H., Tronnier, M., Acil, Y., Fietzek, P.P., Schmeller, W., Müller, P.K. and Batge, B. (1999) Overhydroxylation of lysyl residues is the initial step for altered collagen cross-links and fibril architecture in fibrotic skin. *J. Invest. Dermatol.* **113**, 617-621.
32. Notbohm, H., Nokelainen, M., Myllyharju, J., Fietzek, P.P., Müller, P.K. and Kivirikko, K.I. (1999) Recombinant human type II collagens with low and high levels of hydroxylysine and its glycosylated forms show marked differences in fibrillogenesis *in vitro*. *J. Biol. Chem.* **274**, 8988-8992.
33. Gussekloo, W.S., Lankester, J., Kersten, W. and Back, W. (2011) Effect of differences in tendon properties on functionality of the passive stay apparatus in horses. *Am. J. Vet. Res.* **72**, 474-843.
34. Lin, Y.L., Brama, P.A.J., Kiers, G.H., van Weeren, P.R. and DeGroot, J. (2005) Extracellular matrix composition of the equine superficial digital flexor tendon: relationship with age and anatomical site. *J. Vet. Med.* **52**, 333-338.