Challenging Friesian horse diseases: aortic rupture and megaesophagus

Uitdagen van Friese paarden ziekten: aortaruptuur en megaoesofagus (met een samenvatting in het Nederlands)

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Proefschrift

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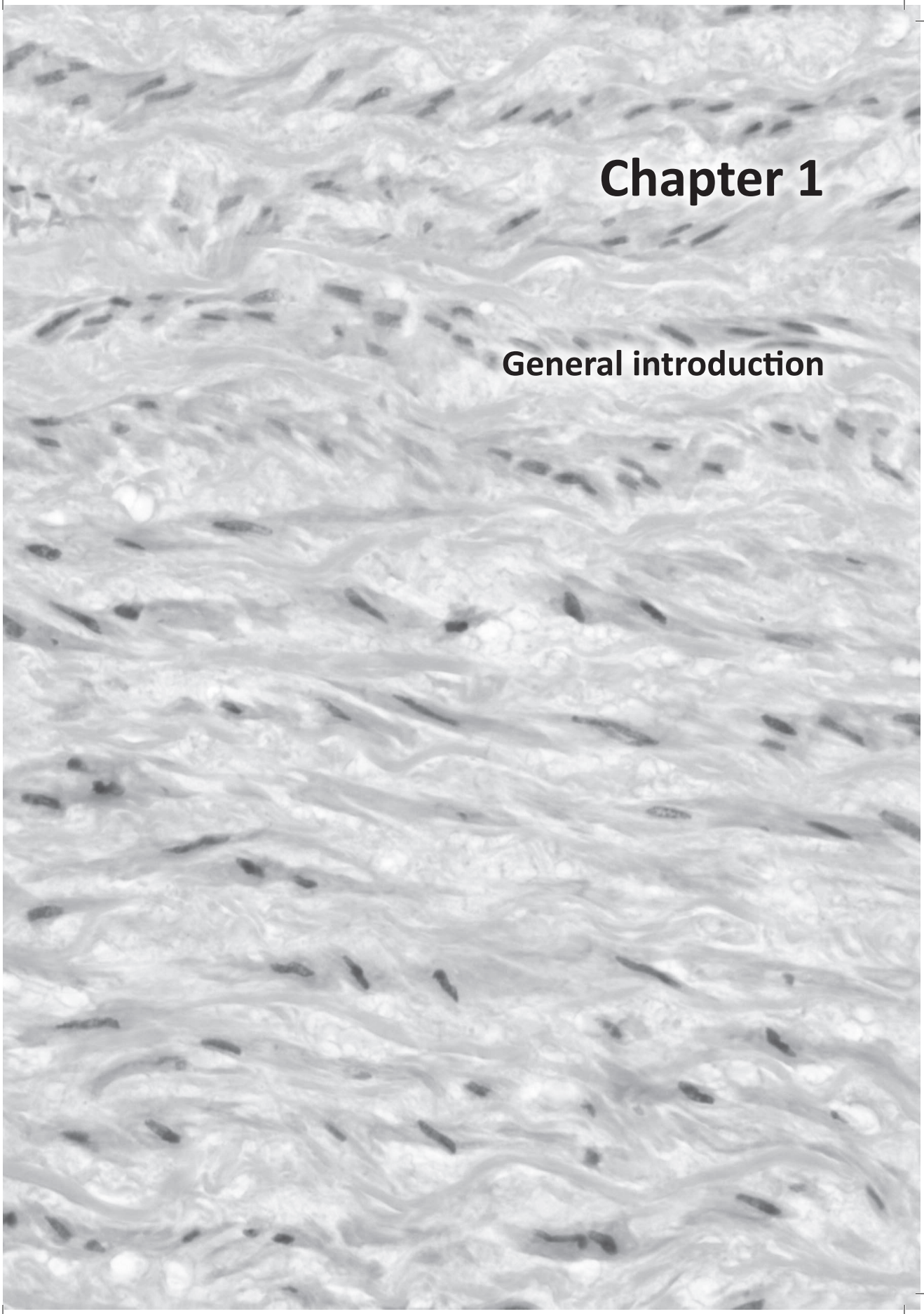

Chapter 1

General introduction

Equine aortic rupture

Aortic rupture is a rare and usually fatal disease in horses.[21] In November 2011 the famous show jumper Hickstead collapsed shortly after finishing a round and died of an aortic rupture in the ring during an important jumping event, thereby putting this disease prominently in the public spotlight.

The classic picture of aortic rupture in horses is the older stallion that develops extreme hypertension during breeding, causing rupture of a weakened aortic wall.[4]

Only a few reports are available in the scientific literature that describe aortic rupture in horses. In most cases rupture of the aortic sinus is described and frequently an aneurysm of the aorta was present as well.[26] Additionally, other forms of aortic disease include dissecting aneurysm of the aorta; dissecting tracts in the septal myocardium; aorto-cardiac fistulas into the right ventricle, right atrium or left ventricle; and the presence of a fistula between the aortic arch and the pulmonary trunk.[15,19,23,24,31] Suggested causes of aortic rupture in horses include congenital aneurysm; degenerative disease processes that weaken the aorta predisposing it to rupture[22] or migrating *Strongylus vulgaris* larvae, causing structural weakness of the aortic wall.[27]

Until the 1990s only one report had been published about three Friesian horses diagnosed with aortic rupture during post-mortem investigation. These Friesians presented clinically with severe and progressive symptoms of cardiac distress. Necropsy of these horses showed a transverse rupture of the aorta near the insertion of the ligamentum arteriosum. The authors suggested that probably the ligamentum arteriosum represented a weak spot in the aortic wall, which, together with hemodynamic and mechanical factors, would create the perfect setting for rupture. The specific area of the aortic tear was thought to be weak due to the transition between the fibrous tissue of the ligamentum arteriosum and the walls of the aorta and pulmonary artery. Rupture of the pulmonary artery was considered to be caused by necrosis provoked by pressure from hematomas surrounding the aorta. There were no new cases or additional information published about aortic rupture in Friesians following this publication in 1985.[31] However, in the last decade increasing numbers of Friesian horses suffering from aortic rupture, all with fatal outcome, were presented to the equine clinics of Ghent and Utrecht University and to Wolvega Equine Clinic. As a breed-specific cardiovascular disease was suspected, it was decided that further study was warranted to get more insight into the pathogenesis of aortic rupture in Friesian horses.

The population of Friesian horses has passed through a so-called bottle neck several times in the past 100 years. This happened for the last time in the 1970s. The rapid mechanization in agriculture triggered a temporary decline in the demand for the horse

and in that particular period less than 100 Friesian foals were born each year. In fact, the breed was threatened with extinction.[18] Although demand has increased since and the population has grown considerably, these bottlenecks led to a very narrow genetic basis and a concomitant strong increase in inbreeding.

Human aortic disease

In abdominal aortic aneurysms (AAA), there is usually first aneurysm formation before the aorta ruptures. Abdominal aortic aneurysms (AAA's) are the most common causes of aortic rupture and associated with atherosclerosis, transmural degenerative processes, neovascularization, degeneration of vascular smooth muscle cells, and chronic inflammation mainly located in the outer aortic wall.[17] Risk factors involved in abdominal aneurysms are female sex, increased blood pressure, chronic obstructive pulmonary disease and smoking.[6] The four most well-known pathogenic factors and hence areas of research are proteolytic degradation of aortic connective tissue, inflammation and immune response, molecular genetics and biomechanical wall stress.[34]

Thoracic aortic aneurysms and aortic dissections (TAAD) in older patients are mainly caused by degenerative aortic lesions associated with smoking, arterial hypertension and hyperlipidemia. In younger persons without the above mentioned risk factors, genetic disease is frequently the base of TAAD. Genetically, aneurysmal disease can be divided into inherited syndromes such as Marfan syndrome, familial forms of TAAD and sporadic forms of TAAD.[8]

The predominant histological finding in thoracic aortic aneurysms (TAA) is cystic medial degeneration, pointing towards a non-inflammatory loss of medial vascular smooth muscle cells (VSCMs), fragmentation of elastic lamellae, and mucoid degeneration.[10] Furthermore, small extracellular matrix (ECM) elements including cytokines and growth factors also can have a role in the pathogenesis of connective tissue disorders that are associated with aortic aneurysm formation. For example, the growth factor TGF-β can stimulate VSCMs and fibroblasts to increase collagen synthesis[29] and has been associated with Marfan syndrome.[8] In Marfan syndrome, increased TGF-β1 activity and fibrillin-1 degradation products lead to aortic elastin fragmentation and VSMC apoptosis with subsequent aneurysm formation.[12]

Structure of the aorta

The aorta is a large elastic artery and the wall consists of three primary layers: intima (innermost layer), media (middle layer), adventitia (outermost layer).[13] The intima

consists of a single layer of endothelial cells lining the arterial wall and resting on a thin basement membrane.[28]
The media is characterized by a three-dimensional network of bundles of collagen fibrils, elastin and smooth muscle cells.[7] All intercellular fibers and ground substance, including a large amount of glycosaminoglycans, are synthesized by smooth muscle cells[9] The adventitia consists mainly of elastic and collagen fibers.[20]

Aortic connective tissue
Connective tissues provide structural and metabolic support for tissues and organs. All connective tissues contain two major components, the cells and the ECM. The cells are all derived from the mesenchyme and include fibroblasts, myofibroblasts, adipocytes and leukocytes. The ECM determines the physical properties and is composed of a mixture of fibers within a matrix of organic material called ground substance.[35]
Ground substance, which is present in the media of equine elastic arteries such as the aorta,[20] consists of a mixture of unbranched polysaccharide chains, each containing repeating disaccharide units such as glycosaminoglycans. GAG molecules are very hydrophilic because of their negatively charged sulfate groups, thereby attracting a large volume of water and positive ions, which is necessary for the characteristic turgor of connective tissue. The mechanical strength of ground substance is increased by the fibrous proteins of the ECM, mainly collagen and elastin.[35]

Of these two important fibrous components of connective tissue, collagen is relatively inextensible and functions as a stiff reinforcing structural component. Collagens are synthesized as propeptides, which undergo intracellular posttranslational modifications including hydroxylation, glycosylation and triple helix formation to form procollagen.[1,16] Subsequently, procollagens are secreted into the ECM where the N- and C-terminal ends are enzymatically cleaved to form tropocollagen. Lastly, with help of the extracellular enzyme lysyl oxidase, which acts on lysines and hydroxylysines and produces aldehyde groups, covalent bonding by cross-link formation between tropocollagen molecules will ensue and polymers are formed that are the final collagen fibrils.[1,16] In humans, the aorta contains approximately 30% type I and 70% type III collagen,[25,33] which are both fibrillar collagens.[1] Elastin, the other structural protein is necessary for stretching and elastic recoil. Elastin is essential for the uniform load absorbing capacity in both circumferential and longitudinal directions, thereby attenuating oscillating arterial shock waves.[11] Elastin is synthesized by fibroblasts as tropoelastin, which polymerizes in the ECM.[35]

Outline of this thesis

Thus far, a single article has reported the existence of aortic rupture in the Friesian horse breed, but recently increasing numbers of Friesians suffering from this disorder have been presented to several equine clinics, prompting further research in this area.
A first step in unraveling the pathogenesis of this Friesian disease is the in-depth investigation of the clinical histories and necropsy reports of the Friesian horses that were presented to these equine clinics, *i.e.* detailed phenotyping. A comprehensive clinical characterization is important for equine clinicians so that they know which clinical signs are associated with aortic rupture. Additionally, this will help clinicians to recognize this disease in an earlier stage, which can be valuable for possible future therapeutic interventions. Further, this macroscopic inventory and phenotyping will help further future (genetic) research by making an accurate selection of affected horses (Chapter 2).

The histomorphological abnormalities within the aortic wall of aortic rupture patients have been characterized extensively in humans. As a result, the wide range of hypothetically possible underlying causes, including infectious agents, genetic abnormalities or degenerative processes could be narrowed down considerably, enabling focusing on specific elements in follow-up research. A similar approach has been followed in the research on equine aortic rupture as presented in this thesis, with, after the macroscopic description in Chapter 2, an in-depth histomorphological study on the aortic walls of the equine patients in Chapter 3.

Friesian horses are known to suffer from several connective tissue-related clinical entities, which include, apart from aortic rupture, megaesophagus,[2] tendon and ligament laxity[14] and aseptic desmitis of the intersesamoidean ligament in the hind limb.[32] For this reason, it was decided to include in the thesis megaesophagus, which has, like aortic rupture, an increased prevalence in the Friesian breed, as an additional focus area. For this disorder a similar approach as for aortic rupture was chosen and Chapter 4 presents the gross and histological features of the esophagus of Friesians with clinically diagnosed megaesophagus.

The principal component of connective tissues in the mammalian body is collagen and interestingly, morphological examination (Chapters 3 and 4) revealed aberrant collagen depositions within the aortic wall of Friesians with aortic rupture, but also within the esophagus of Friesians presented with megaesophagus. These observations support the contention that a general collagen-based systemic disorder might underlie

several, if not all connective tissue-related disorders mentioned above.[3] Further, in humans displaying aortic disease several connective tissue abnormalities, including increased collagen cross-linking, have been demonstrated to be related to aortic disease.[5,30]
These known pathogenetic sequences, together with the observations as made in Chapters 3 and 4, led to the next step of the project, which was the biochemical analysis of the ECM components of the aortic wall in Friesians with a focus on collagen (Chapter 5).

Finally, Chapter 6 presents a general discussion of the main findings in the preceding chapters against the background of the existing literature in the horse and, there where this is useful for comparative reasons, of relevant literature from the human field.

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Chapter 2

Aortic rupture and aorto-pulmonary fistulation in the Friesian horse: Characterisation of the clinical and gross post mortem findings in 24 cases

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Abstract

In horses, aortic sinus of Valsalva aneurysms or tears in the aortic root are well-recognized conditions in breeding stallions, often leading to sudden death. A more uncommon form of aortic rupture, located proximal to the ligamentum arteriosum has been reported in 3 Friesian horses.

The purpose of this study was to phenotypically characterize aortic rupture and aorto-pulmonary fistulation in Friesian horses in terms of clinical and post-mortem data based on 24 cases.

Friesian horses that were diagnosed with aortic rupture and aorto-pulmonary fistulation over a period of 13 years (1997-2010) at the Department of Equine sciences of Utrecht University (n=15) and Wolvega Equine Hospital (n=9), were included in this study. Case history, results of clinical examination and gross post-mortem findings were screened and analysed.

Some cases were found dead without prior symptoms, but in several cases signs such as recurrent colic, peripheral oedema and sustained tachycardia were present for several weeks prior to cardiac failure, indicating a non-acute condition. Clinical examination during hospitalisation revealed increased rectal temperature, peripheral oedema and increased jugular pulse with a bounding arterial pulse. In the majority of horses an aortic rupture of the aortic arch near the ligamentum arteriosum, concurrent with a circumferential cuff of perivascular haemorrhage and aorto-pulmonary fistulation, was found at post-mortem examination.

Aorto-pulmonary fistulation in conjunction with aortic rupture is more common in Friesians than previously estimated. In some cases findings demonstrate a progressive pathology rather than acute cardiac failure and sudden death. An appropriate cardiac approach is necessary during autopsy in order not to overlook the diagnosis.

Equine practitioners should realize that in Friesian horses presented with a history of recurrent false colic, coughing, sustained tachycardia and/or peripheral oedema, aortic rupture and aorto-pulmonary fistulation should be included in the differential diagnosis.

Introduction

In western countries, abdominal aortic aneurysm formation and rupture is the 13th leading cause of death in man.[4] This is a multifactorial disease in which both genetic risk factors and inflammation play an important role.[1] Approximately 74% of human aortic aneurysms are in the abdominal part and approximately 23 % are in the thoracic part of the aorta.[2,22] In horses, several cases of aortic root disease have been reported, which are characterized by an aneurysm of the aortic sinus of Valsalva or a tear in the aortic root. This type of aortic rupture usually occurs very close to the junction of the aorta with the heart.[16,17] Aortic root rupture is well-known in breeding stallions during coitus or shortly thereafter.[3] The rupture usually results in acute haemorrhage into the pericardial sac, leading to signs of acute cardiac failure and often death within seconds. Sometimes the aorta ruptures into the right atrium, right ventricle or interventricular septum.[11,14,15,17]

Van der Linde-Sipman et al.[19] reported on three Friesian horses manifesting with aortic rupture not at the level of the aortic root, but at a more distal location, at the level of the aortic arch near the ligamentum arteriosum.
Since then, many similar cases in Friesian horses have been presented to Utrecht University Equine hospital and Wolvega Equine hospital in The Netherlands, making this specific form of aortic rupture a quite commonly encountered pathology in Friesian horses.

Thus far there is no study describing the characteristic phenotypical appearance of aortic rupture and aorto-pulmonary fistulation in the Friesian breed in a large case series, which makes this type of pathology difficult to recognize, especially in countries where the Friesian breed is less common. The purpose of this article is to describe the phenotype of this condition in terms of case history, clinical and post-mortem data from 24 Friesian horses diagnosed with aortic rupture and/or aorto-pulmonary fistulation. This phenotypical characterization is a first step in an attempt to unravel the pathophysiology and possibly the genetic background of this almost invariably fatal cardiac pathology in the Friesian horse breed.

Material and methods

Animals

Twenty four Friesian horses that were diagnosed with aortic rupture or aorto-pulmonary fistulation over a period of 13 years (1997-2010) at the Department of Equine Sciences of Utrecht University (n=15) and at Wolvega Equine Hospital (n=9) were included in this study. Case logs were screened for case history, clinical signs at admission, haematological variables, findings at diagnostic imaging and post-mortem examinations.

Case history
Clinical history data included age, gender, previous episodes of fever (ºC) or tachycardia (pulse >50 beats/min), duration of illness (days) from the onset of overt clinical complaints, and time point of manifestation of one of the following clinical signs in the past: epistaxis, recurrent colic, coughing and (intermittent) presence of peripheral oedema (brisket, abdomen and/or prepuce).

Clinical examination at arrival
Clinical parameters included: presence of peripheral oedema, rectal temperature (ºC), assessment of mucous membranes, respiratory rate (breaths/minute), arterial pulse (beats/min, type), presence of carotid hammer pulse and/or jugular pulse , findings at thoracic auscultation such as pronounced vesicular breathing and shallow breathing sounds, findings at cardiac auscultation such as presence of specific murmurs, presence of cardiac arrhythmias and sustained sinus tachycardia at rest.

Haematological parameters
Haematological parameters included measurement of total white blood cell count and differentiation, haematocrit and thrombocytes.

Diagnostic imaging
A standard echocardiographic (Pie Medical Esaote Mylab 50[a], Philips HD 11 XE (NZE 439)[b] evaluation was performed by visualization of the right parasternal long-axis views: 4 chamber view, the left ventricular outflow tract and the right ventricular inflow and outflow tract view during which the aortic and pulmonary artery diameter were evaluated, the area of the left ventricle in systole and diastole and fractional shortening were assessed. Right parasternal short-axis views of the left ventricle, the mitral valve and the aortic valve were obtained. Measurement of the diameter of the left ventricle in systole and diastole and evaluation of left ventricular systolic function using M-mode was performed. Finally, a left parasternal long-axis view was obtained. The dilation of the PA, RA and RV were based on subjective assessment.
Thoracic radiographs (Philips Super-100CP[b], Philips ZA91[c]) were used to assess the presence of pleural effusion or dilatation of the aorta.

Gross post mortem findings
Post-mortem examination was performed according to standard necropsy procedures.[12] Attention was paid to the possible presence of the following morphological features during gross examination of the organs: lung oedema, pleural effusions, peripheral (brisket, abdomen or prepuce) oedema, aorto-pulmonary fistulation, aortic rupture near the ligamentum arteriosum, pulmonary artery rupture and macroscopic liver congestion (as illustrated by swollen borders and bloody appearance on cut surface).

Results

Initial case history

The primary attending practitioners reported several relevant clinical data in the study population. The reports included 14 females, 5 intact males and 4 geldings and the gender of one horse was not registered. Mean ± s.d. age at which the aortic disease was diagnosed was 4.9 ± 3.9 years (range 1-20, median 4 years). More than one-third of all cases (n = 10) showed recurrent signs such as colic, anorexia, repeated recumbency, depression, poor performance or coughing in the days or weeks prior to the fatal cardiac failure. Epistaxis was reported in 4 horses. In all cases suffering from recurrent colic, rectal examination revealed no abnormalities such as impaction or displacement of the intestine with the exception of one case, in which caecal impaction was found. Other distinctive features often reported were (intermittent) peripheral oedema (n = 8), fever (≥38.5 ºC; n = 5) and sustained sinus tachycardia at rest (n = 8). These latter signs were often only reported at a later stage in the disease process, one to two weeks before cardiac failure. Duration of overt illness was 4-9 days in 7 and ≥ 10 days in 9 horses. Three horses (Nos. 5, 18 and 21) were reported as found dead without prior manifestation of any clinical symptoms. The report of one horse (No. 2) was incomplete and did not contain data on all features mentioned.

Clinical examination at presentation in referral clinic

Characteristic features at presentation were increased rectal temperature (>38.5 ºC; n = 12), peripheral oedema (n = 11) and increased respiratory rate (n = 7; >14 breaths/min) (Table 1). The arterial pulse rate was frequently increased (>40 beats/min; n = 17) and judged to be hyperkinetic in 10 cases. A carotid hammer pulse was palpated in 3 horses and a pronounced jugular pulsation was detected in the same 3 horses (Supporting item S1) was palpated in 3 cases. Pale mucous membranes were reported in 7 cases. Cardiac auscultation revealed cardiac arrhythmias (n = 3), sustained sinus tachycardia (n = 6) and presence of murmurs (n = 11), which were further characterized in 8 cases. Of these, 6 horses presented a systolic murmur and 2 a diastolic murmur on the left side.

Haematology

Haematological parameters were recorded in 13 horses. White blood cell count was measured in 10 horses of which 2 showed an increased count (15.3 $x10^9$ cells/l [reference range 5.5-12.1] and 11.9 x 10^9 cells/l [5-10]). The PVC was frequently decreased (11/13; mean ± s.d. 0.25 ± 0.046 l/l, median 0.27 l/l). Thrombocytes were within reference limits for all horses.

TABLE 1: Overview of clinical signs at presentation

							Thoracic auscultation			Cardiac auscultation	
Horse	Oedema	Rectal Temperature (ºC)	Repiratory rate (breath/min)	Pulse rate (beats/min)	Steep jugular pulse	Bounding arterial pulse	Pronounced vesicular breathing	Shallow breathing sounds	Arrythmias	Sustained tachycardia	Murmur
1	+	38.1	40	80	-	-	+	-	-	-	3/6, Systolic, L
2	+	39.1	NA	90	NA	-	NA	NA	NA	+	Systolic
3	-	NA	NA	80	+	+	-	+	-	-	NA
4	-	37.8	NA	90	+	-	NA	NA	-	+	-
5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
6	+	NA	NA	NA	-	-	NA	NA	NA	NA	NA
7	+	NA	NA	85	+	+	-	-	-	-	NA
8	-	38.7	NA	75	+	-	-	-	-	-	Systolic + Diastolic, L
9	+	NA	NA	100	+	+	NA	NA	NA	NA	NA
10	-	NA	NA	NA	-	-	NA	NA	NA	NA	NA
11	-	NA	NA	120	-	-	-	-	+	-	Diastolic, R
12	+	38.5	40	72	-	-	+	-	-	-	-
13	-	NA	NA	NA	-	-	NA	NA	NA	NA	NA
14	-	38.1	NA	90	+	-	-	-	-	+	Systolic, L
15	+	38.1	40	88		-	-	-	-	+	+
16	-	37.9	22	72	+	-	-	-	-	+	4/6, Systolic, L
17	+	38.4	24	80	+	-	+	+	+	+	-
18	NA	NA	NA	NA	NA	-	NA	NA	NA	NA	NA
19	+	39.0	NA	70	-	-	-	-	+	+	+
20	-	39.0	NA	80	+	-	-	-	-	-	4/6, Systolic, L
21	-	NA	NA	NA	-	-	NA	NA	NA	NA	NA
22	+	38.8	24	100	+	-	-	-	-	-	Systolic, R
23	+	38.8	12	120	-	-	+	-	-	-	4/6, Systolic + 2/6, Diastolic, L
24	+	38.9	40	80	-	-	+	+	-	-	+

+: present, -: absent, NA: data not available.

Echocardiographic and radiographic examination
Echocardiography was performed in 16 cases and revealed a dilated pulmonary artery in 5. Three horses showed a wider diameter of the pulmonary artery compared to the aortic diameter. In 6 cases a dilated right atrium was detected and 5 horses in this group also showed a dilated right ventricle. Presence of a pleural effusion was reported in 8 horses and in 3 cases a pericardial effusion was detected. In the 11 horses presented with murmurs only 3 also showed valvular insufficiencies. All 3 horses showed a tricuspid insufficiency and 2 also had mitral valve regurgitation. In one other horse there were no murmurs detected although echocardiography showed both tricuspid and mitral valve regurgitation.

Radiographic examination was performed in 5 horses (3 of which also had echocardiography). Thoracic pleural effusion was seen in 4 cases and in one horse a dilated aorta was identified.

Gross post-mortem examination
In all cases, there was a transverse aortic rupture located at the level of the aortic arch, in proximity (1-2 cm caudal) of the *ligamentum arteriosum*. A circumferential cuff of blood around the aorta, covering the aortic rupture and extending up to 40 centimetres in length, was present in 8 cases (Figs. 1-3). A few cases (n = 4) also had a cuff of perivascular haemorrhage around the pulmonary artery. Closer inspection revealed that these circumferential blood cuffs were not due to obvious aneurysmal dilatations, but were formed by leakage of blood out of the ruptured site into the connective tissue surrounding the arteries. None of the horses showed concurrent aneurysmal dilatation of the arteries. The majority of the horses suffering from an aortic rupture also had an aorto-pulmonary fistulation (n = 13) with transverse pulmonary artery rupture (Supporting item S2), which was located opposite the aortic rupture and approximately 8 cm above the pulmonic semilunar valves. In 5 cases the post-mortem records clearly described a pronounced frayed rim of the ruptured aorta (Fig. 4). In contrast the ruptures of the pulmonary arteries were in some cases described as less irregular. The route of fistulation was described as a connection between aorta and pulmonary artery with multiple pockets of 0,5-7 cm in diameter expanding the lumen in many cases (n = 10).

Presence of pleural effusion was reported in 9 horses. In 4 of these horses there was haemothorax and mild to moderate amount of hemorrhagic fluid was also present at the nares of these horses. Pulmonary oedema was marked and diffuse in 9 cases and in 7 of these horses a cuff of perivascular haemorrhage around the aorta with aorto-pulmonary fistulation was detected. Moderate to severe peripheral oedema (located around the ventral thorax, ventral abdominal wall and preputial sheath) was found in 7 animals and in 5 of them also concurrent pulmonary oedema was described. A

congested liver was reported in 10 cases and confirmed histopathologically by periacinar congestion (n = 10) with necrosis (n = 2) of hepatocytes and fibrosis (n = 1). Other findings were pale mucous membranes (n = 5), a dilated right ventricle (n = 5) and oedema of the intestinal mesentery (n = 1).

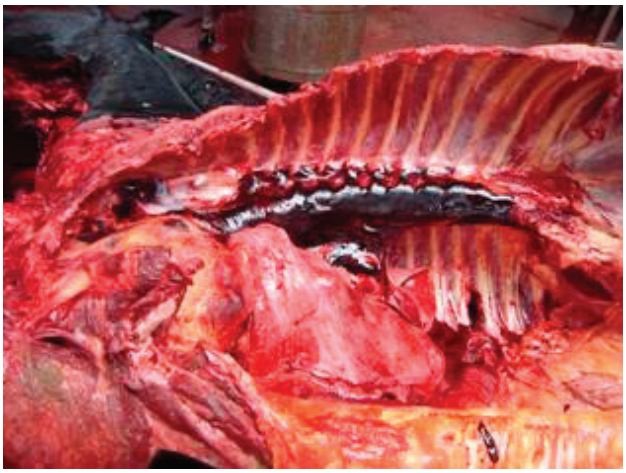

Figure 1. A marked circumferential cuff of blood around the aorta at necropsy, covering the aortic rupture and extending up to 40 cm in length.

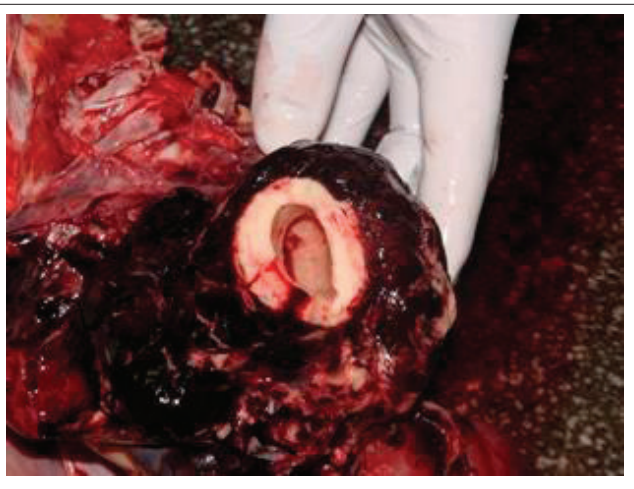

Figure 2. Cross-section of a marked cuff of perivascular haemorrhage around the aorta.

Discussion

This demonstrates that aortic rupture in conjunction with aorto-pulmonary fistulation at the level of the aortic arch near the ligamentum arteriosum is much more common in Friesians than previously estimated. Van der Linde-Sipman et al.[19] reported on three Friesian horses manifesting a similar transverse aortic rupture proximal to the scar of the former outlet of the ductus arteriosus of Botalli. These horses also showed a transverse rupture in the pulmonary trunk opposite the aortic rupture, forming a fistulation into the pulmonary artery functioning as a left to right shunt. Only one other case, referred to as dissecting aortic lesions connecting with the pulmonary artery, has been described in a horse from a different breed (Dutch crossbred).[19]

A feature already described by Van der Linde-Sipman et al.[19] is the presence of haemorrhage, mainly found around the aorta, but also around the pulmonary trunk. These haemorrhages were previously regarded as dissecting aneurysms. However, it has now become clear that these were not aneurysms but circumferential cuffs of perivascular haemorrhage (Figs. 1-3) formed by leakage of blood out of the ruptured site into the connective tissue surrounding the arteries. This observation has consequences for the possible aethiopathogenesis and also for the nomenclature of this pathology.

Hepatic fibrosis found in one of the horses, which was considered to be a sign of chronic heart failure, indicates that aortic rupture and aorto-pulmonary fistulation in Friesian horses can manifest not only as an acute event, but also as less acute pathology, eventually culminating in acute fatal cardiac failure over the course of a few days to several weeks. It appears that some Friesian horses developed an acute aortic tear with haemothorax and subsequent death within minutes, whereas others formed an aortic tear and/or aorto-pulmonary fistulation with cuff a of perivascular haemorrhage around the aorta and/or the pulmonary artery allowing for stabilization during several weeks in some cases. Finally, there was a group of horses that formed a quite stable aorto-pulmonary fistulation that led to right-sided heart failure, after weeks to months. In these non-acute cases complaints like recurrent colic, epistaxis, coughing, and intermittent manifestation of peripheral oedema in the weeks prior to the final stage of overt cardiac failure were reported. Experience with these patients in the two equine hospitals has demonstrated that unless overt cardiac failure or haemothorax are present, some of these horses can be kept in a stable condition for several weeks. They show intermittent manifestation of peripheral oedema, however always with the continuous presence of tachycardia (pulse >50 beats/min) at rest and sometimes with a bounding arterial pulse. The finding of signs of chronic liver congestion in several of these horses supports a chronic course of the disease. This slow progression makes early recognition of these patients very important and

potentially offers possibilities for treatments, such as application of intravascular occlusion devices.[20]

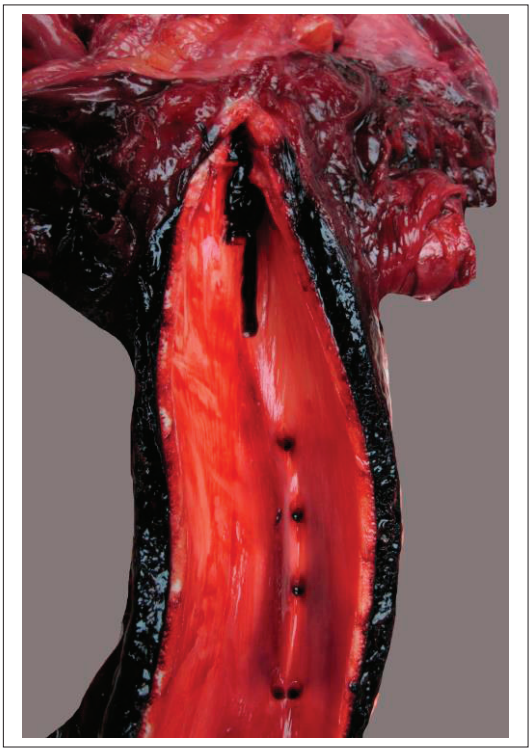

Figure 3. Longitudinal section of a marked cuff of perivascular haemorrhage around the aorta.

This study demonstrates that accurate ante mortem diagnosis of this condition is quite a challenge at present. Case history and specific clinical signs may give helpful indications. Equine clinicians should realize that aortic rupture and aorto-pulmonary fistulation should be included in the differential diagnosis when confronted with a Friesian horse that presents with a case history of recurrent nongastrointestinal related colic and/or coughing, exercise intolerance, fever, epistaxis, sustained tachycardia and possibly a bounding arterial pulse. However, these signs are not conclusive and it is clear that there is a need for the development of additional techniques to diagnose this condition in the Friesian breed an earlier stage. It should also be realised that this aortic disease requires a specific approach of the post-mortem examination as well, especially with respect to the cardiac incisions. These should be made in such a manner that the diagnosis cannot be missed (Supporting item S3). It is therefore

advised to slightly modify the standard procedure of opening the heart [12]: after *in situ* examination and opening of the pericardial sac, the thoracic aorta at the diaphragm should be dissected. The heart, thoracic aorta, some lung tissue that is left attached and the undamaged pulmonary artery are removed from the thoracic cavity. The left ventricle (up to the aortic valves) and atrium are opened by cutting from the apex following the caudal border of the left ventricle. Then the right ventricle and atrium are opened with a V-shaped incision with the apex of the heart forming the tip of the V. The truncus pulmonalis is then cut open and, when present, the edges of a pulmonary rupture can be observed. Next the dorsal side of the aorta is incised from the thoracic side towards the heart base. By cutting through the cuff of blood surrounding the aorta and transecting the aortic tear, the scar of the ligamentum arteriosum will be transected. In this area a small probe is needed to visualize the aorto-pulmonary fistula, if present.
There are several explanations possible for the typical localization and presentation of aortic rupture and aorto-pulmonary fistulation in the Friesian breed.

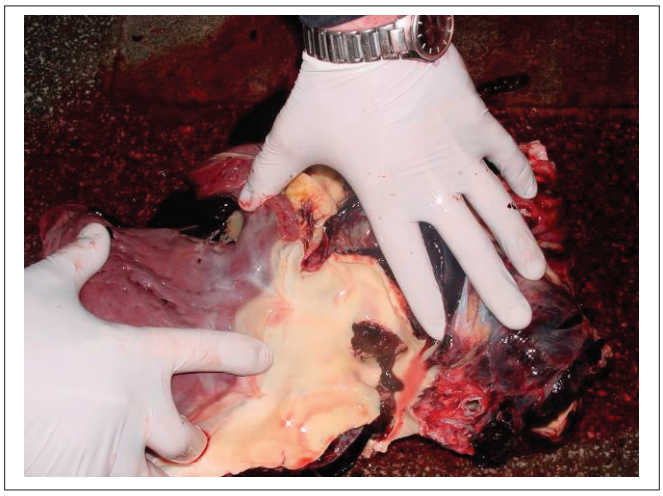

Figure 4. Aortic rupture. Note the frayed rim at the site of rupture.

Van der Linde-Sipman et al.[19] proposed that the tension of the ligamentum arteriosum on the previously damaged walls of the aorta and pulmonary trunk might facilitate rupture. In the affected area the fibrous tissue of the ligamentum arteriosum connects the aorta and pulmonary trunk and this merging of different tissues may constitute a weak area in the vessel wall. Van der Linde-Sipman (1985) also described the histological features, which consisted of media necrosis and intimal thickening and/or medial fibrosis in many vasa vasorum in the media and adventitia of aorta and pulmonary trunk.[19] Histopathological examination of samples of the cases presented

will be performed in more depth to further unravel the aetiopathogenesis as proposed by van der Linde-Sipman et al.[19] Holmes et al.[6] proposed that the rupture of the pulmonary trunk was due to pressure necrosis induced by the haematomas around the aorta.[6]

In man, the precise mechanism of spontaneous nonsyndromal aortic rupture without prior formation of an aneurysm is also not clear. The major pathological changes in these cases are atherosclerotic plaques together with longstanding hypertension.[5] Atherosclerotic disease is not recognized in horses, although Teeter et al.[18] have demonstrated the frequent presence of arterial calcifications (arteriosclerosis) in the pulmonary artery of racehorses. However, no calcifications were detected during autopsy of the Friesian horses in this study.

In humans, many cardiac outflow tract defects appear to have an embryonic pathogenesis involving the neural crest cell lineage or vascular smooth muscle cell defects.[7,10] Similarly, an embryologic defect originating from the fusion of the dorsal aorta and the developing heart could be a possible reason for the rupture seen in the Friesian breed. Finally, besides embryological developmental aortic malformations, there are also other syndromes that could result in cardiac disease and in particular in aortic rupture. For example, Marfan syndrome is an elastin connective tissue disorder caused by fibrillin-1 gene mutation; Ehlers-Danlos is known as a collagen connective tissue defect and Loeys-Dietz syndrome is known to be caused by heterozygous mutations in the genes encoding for *type I* or *II* transforming growth factor-beta (TGF-beta) receptor.[8]

Van der Linde-Sipman et al.[19] already suggested that a genetic susceptibility is likely, as the aortic and pulmonary trunk ruptures occurred in 3 Friesian horses that were descendants from the same sire. Van Vliet and Back[21] demonstrated that 75% of horses admitted to the Utrecht Teaching Hospital with 'aortic rupture' were of the Friesian breed, whereas Friesians comprise only 7% of the general hospital population. The Friesian breed is notorious for having low levels of variation in protein and microsatellite markers, possibly due to a sharp reduction in the number of breeding stallions after World War II.[9] Today's whole genome scan techniques already have proven to be successful in studying simple genetic diseases in the Friesian breed.[13] For comple*x* genetic diseases it is essential to do in-depth phenotypic research, of which this study is a first step with respect to vascular pathology. The improved phenotypic description of aortic rupture in Friesians may also prove helpful to find more accurate, ante mortem diagnostic tools as 'early warning signs' and possibly in the development of treatment modalities that may prevent fatal rupture.

Manufacturers' details
[a]Pie Medical Esaote Mylab 50, Esaote Benelux B.V., Maastricht, The Netherlands.
[b]Philips HD 11 XE (NZE 439), Philips Medical systems, Eindhoven, The Netherlands.
[c]Philips ZA91, Philips Healthcare Nederland, Eindhoven, The Netherlands.
[d]Philips Super-100CP, Philips Medical systems, Eindhoven, The Netherlands.

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Chapter 3

Thoracic aortic rupture and aortopulmonary fistulation in the Friesian horse: histomorphologic characterization

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Abstract

Aortic rupture in horses is a rare condition. Although it is relatively common in the Friesian breed, only limited histopathological information is available.

Twenty Friesian horses (1-10 years old) were diagnosed with aortic rupture by postmortem examination. Ruptured aortic walls were analyzed using histology and immunohistochemistry.

Based on the histological and immunohistochemical findings these cases were divided into acute (4 cases, 20%), subacute (8 cases, 40%) and chronic (8 cases, 40%) groups. Features common to samples from horses in all groups included: 1) accumulation of mucoid material; 2) disorganization and fragmentation of the elastic laminae; 3) aortic medial smooth muscle hypertrophy; and 4) medial necrosis of varying degrees, ranging from mild and patchy in the acute cases to severe midzonal necrosis in the chronic cases.

Inflammation, most likely secondary to medial necrosis, varied from predominantly neutrophilic infiltrates in the media and periadventitial tissue in the acute group, to the presence of mainly hemosiderophages in the periadventitial tissue in the chronic group. Medial fibrosis with aberrant collagen morphology was seen in the subacute, and more commonly in the chronic, groups. Only minimal changes were seen in the aortic vasa vasorum. Smooth muscle hypertrophy and accumulation of mucoid material were not related to the age of the lesions.

The findings of this study suggest that a connective tissue disorder affecting elastin or collagen in the aortic media is potentially the underlying cause of aortic rupture in Friesian horses.

Introduction

More than 40 cases of aortic rupture in Friesian horses have been reported to date.[38,51] These aortic ruptures occurred spontaneously, in the absence of trauma, infectious disease or iatrogenic injury. There was no sex predilection and the median age of affected horses was 4 years and ranged from 1-20 years. Ruptures occurred consistently in the thoracic aorta near the ligamentum arteriosum. Frequent findings at postmortem examination included a concurrent circumferential cuff of perivascular hemorrhage (periaortic hematoma) and aorto-pulmonary fistulation, which is well described in the Friesian breed.[38]

Aortic rupture in non-Friesian horse breeds is rare and mainly affects breeding stallions in which it manifests as acute death.[42] In these cases, the rupture appears near the sinuses of Valsalva and the macroscopic lesions and clinical signs differ from the those seen in Friesian horses with aortic rupture.

An aneurysm is a localized abnormal dilation of any vessel. True aneurysms are composed of all, or most, of the layers of the intact vessel wall. False aneurysms, also called pseudoaneurysms, result from rupture of an artery or aneurysm with disruption of all 3 layers of the arterial wall and communication with the arterial lumen.[27,52] Aortic aneurysms are rare in horses and occur mainly near the aortic arch.[40] In some cases *Strongylus vulgaris* arteritis can cause aneurysm formation in the abdominal or thoracic aorta.[45]

Spontaneous aortic rupture in humans is a multifactorial disease[1] and a relatively uncommon cause of death.[13,19,29] Histopathological findings include abnormalities of the cellular and matrix constituents of the aortic media. Aortic rupture in humans occurs mostly in the abdominal portion of the vessel[10] and is frequently associated with atherosclerosis[12]. Thoracic aortic rupture is uncommon and predominantly linked to hereditary diseases.[16,24,30] In abdominal aortic aneurysms (AAA) the structure and the amount of the matrix proteins are abnormal, there being decreased elastin and increased collagen.[5,31] Both the vasa vasorum and the increased synthesis of matrix metalloproteinases by smooth muscle cells have been implicated in the progression of AAAs.[18,37] Additionally, inflammatory processes have been shown to play an important role in the development of this disease. These can be divided into noninfectious etiologies such as atherosclerosis [1,49] and infectious causes such as mycotic aneurysms of the aorta.[36] In thoracic aortic rupture, alterations in elastin (Marfan syndrome), fibrillin (Ehlers-Danlos syndrome type IV) and proteoglycans (biglycan gene deficiency) have been determined.[16,24,30] In rare cases, a functional connection occurs between the ascending aorta and the pulmonary artery. Such an aorto-pulmonary fistula is a diagnostically challenging condition.[20] It occurs most

frequently as a result of erosion and/or rupture of a chronic process or pseudoaneurysm of the aorta.[26,46]

Histological examination of the ruptured aortic wall has proved to be a very useful tool for investigation of the underlying causes of aortic rupture in humans.[3,44] To date, only 3 cases of aortic rupture with aorto-pulmonary fistulation in Friesians have been histologically examined and described in the literature.[51] In 2 horses the lesion was described as scattered medial necrosis throughout the wall surrounded by neutrophils and granulation tissue, and in the third horse no inflammatory reaction was observed. Many vasa vasorum with intimal thickening and/or medial fibrosis were seen in the aortic adventitia, and this may predispose to aortic medial necrosis and rupture.[51] The purpose of the present study was to describe the histological lesions in a larger series of Friesian horses with aortic rupture, in order to gain better insight in to the underlying mechanisms of this unique disease.

Material and methods

Animals

Twenty Friesian horses (1-10 years old; 14 mares, 3 geldings, 2 stallions and 1 animal of unknown gender) were included in this study; gross lesions in 7 of these animals have been described previously.[38] All horses were diagnosed with aortic rupture by means of postmortem examination over a period of 14 years (1997-2011) at Wolvega Equine Hospital (n = 6), the Faculty of Veterinary Medicine, Ghent University, Belgium (n = 5) or the Faculty of Veterinary Medicine of Utrecht University, the Netherlands (n = 9). Six horses were found dead or showed acute clinical signs in the few hours preceding death. However, the majority (n = 14) showed signs such as fever, tachycardia and colic in the days, weeks or even months prior to euthanasia. The animals, clinical signs and gross lesions are presented in Table 1.

Histopathology

Tissue samples were taken at the level of the aortic rupture, fixed in phosphate-buffered formalin, routinely embedded in paraffin wax and cut in 4 μm thick sections. Periaortic hematomas, pseudoaneurysms and arterial dissections were also analyzed. Sections were stained with hematoxylin and eosin (HE), Von Gieson's stain (Klinipath 64089, Duiven, the Netherlands) to visualize possible collagen deposition of the media, Alcian Blue (Sigma A4045-25G, Zwijndrecht, the Netherlands) for detection of accumulation of mucoid material and Von Kossa stain for demonstration of mineralization.

Immunohistochemistry
The immunohistochemical antibodies were used according to manufacturers' instructions. Presence of smooth muscle was demonstrated using mouse anti-smooth muscle actin (SMA) (Biogenex, Fremont, USA) as the primary antibody and horse anti-mouse/biotin (Vector Laboratories, Peterborough, UK) as the secondary antibody. Vasa vasorum were identified by visualization of factor VIII-related antigen using rabbit anti-factor VIII (Dak, Glostrup, Denmark) as primary antibody and goat anti-rabbit IgG (Vector Laboratories) as secondary antibody. Additionally, factor VIII-related antigen was used to determine the nature of the lining of the pseudoaneurysms and dissections. Lymphocytes were analyzed with a polyclonal rabbit anti-CD3 antibody (Dako, Glostrup, Denmark) that labels human T-lymphocytes (both helper and cytotoxic T-lymphocytes), a polyclonal rabbit anti-CD20 antibody that labels human B-lymphocytes (Thermo Fisher Scientific, Erembodegem, België), and a monoclonal mouse antibody that labels reactive and tissue macrophages (MAC387, Abcam, Cambridge, UK). Aortic elastin was visualized using a monoclonal anti-elastin antibody BA-4 (Leica Biosystems, Diegem, Belgium). A standard avidin biotin complex method with diaminobenzidine as chromogen was used for visualization (Envision, Dako). Negative controls were prepared from serial sections in which the primary antibody was omitted and replaced by dilution buffer. The specificity of the primary antibodies was validated with recognized positive control tissues.

Scoring of lesions
Histological scoring of necrosis of the media, accumulation of mucoid material, altered smooth muscle orientation, elastin fragmentation and fibrosis was based upon protocols described previously.[43] This semi-quantitative grading system uses a scale from 0 for no changes to 3 for severe changes. The smooth muscle cells in the media were also assessed for the presence of hypertrophy.

The presence or absence of fibrin and mineralization was assessed. The degree of medial and adventitial inflammation (density of inflammatory cells, grade 0 to 3) was scored with grade 0 representing no inflammation and grade 3 for severe inflammation.[23] Finally, the numbers of vessels in the media and adventitia were assessed by counting the number of vasa vasorum in 5 randomly chosen fields irrespective of the size of the vessel**.**

Results

Major findings are summarized in supplementary Table 1.

Macroscopic findings

Macroscopic lesions in the present series (including those in the horses included in the previously mentioned study) were similar to those previously described. In summary, all Friesians had an aortic rupture just proximal to the ligamentum arteriosum and 6 horses showed a hemothorax. A periaortic hematoma was present in 4 cases. Aorto-pulmonary fistulation was present in 13 animals and was, in most cases, associated with one or more pseudoaneurysms (extensive encapsulated perivascular hematoma, retaining communication with the aortic lumen) (Fig.1). Pulmonary artery ruptures were transverse, located at the level of the ligamentum arteriosum and varied from 2 to 7 centimeters in length. In 4 cases a dissection of the aorta was present and in 1 case there was an additional dissection of the pulmonary artery wall. Dissection took the form of a longitudinal split in the mid media creating a "false" lumen running distally over a length of up to 15 centimeters.

Histological findings of ruptured aortas

In 4 animals (acute group, Nos. 1-4), aortic lesions were histologically classified as acute based on the presence of fibrin clots in the hematoma and neutrophils as the dominant infiltrative cell type (Supplementary Table 1). Two of these horses had hemothorax. The other 2 horses had periaortic hematomas and 1 of these had an aorto-pulmonary fistula. All horses in the acute group had mild (n = 3) to moderate (n = 1) (grade 1-2) necrosis of the media, which was mainly patchy. Small numbers (grade 1) of B-and T-lymphocytes, plasma cells and macrophages were seen both in both the media and in the periadventitial tissue, but neutrophils were the predominant inflammatory cell type. Accumulation of mucoid material (grade 1-3) (Fig. 2) and disorganization and fragmentation (grade 1-2) of the elastic laminae (Fig. 3) were seen in the aortic media of all horses in the acute group. Medial fibrosis was not present. Mild mineralization of the midzone of the media was present in a single case and occurred in association with mild medial necrosis. Smooth muscle hypertrophy was present in all affected Friesians, but no animals in this group showed altered smooth muscle orientation. The number of vasa vasora in the media ranged from 26 to 78 (mean 45) and two of these showed moderate intimal thickening by subendothelial smooth muscle cells without medial fibrosis. The number of vasa vasorum in the adventitia was only determined in a single case (17 vasa vasorum), but could not be detected in others due to the extensive hemorrhage or the absence of adventitia.

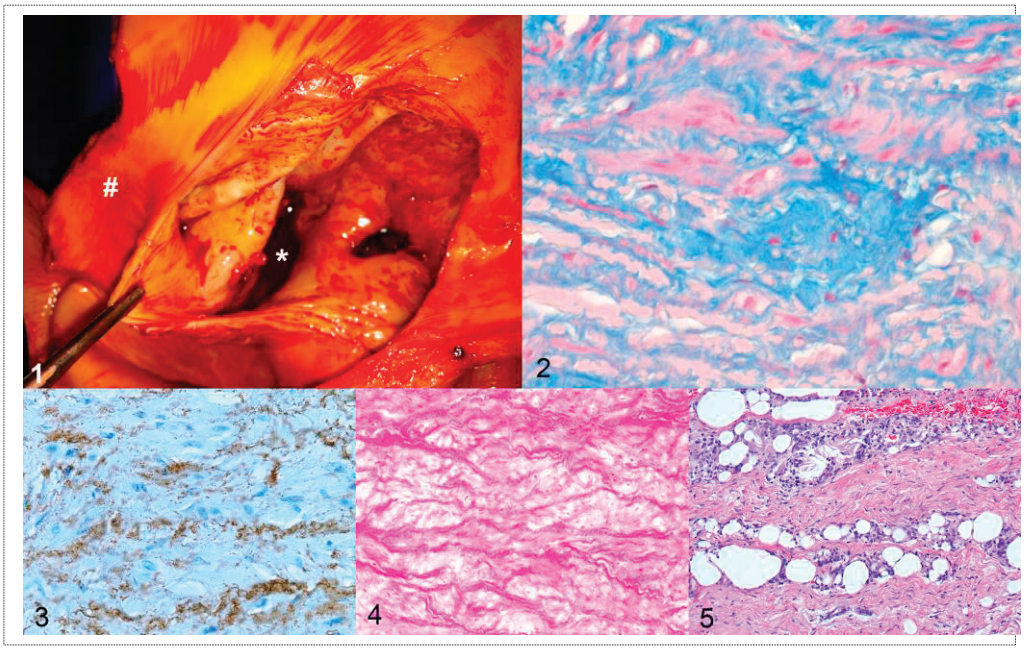

Figure 1. Friesian horse, case No. 19. Aortic rupture with the aortic lumen (#) on the left and a typical pseudoaneurysm (*) in the center characterized by finger-like sacculations in its wall.
Figure 2. Friesian horse, case No. 8. Accumulation of mucoid material in the aortic mid media adjacent to the rupture site. Alcian Blue.
Figure 3. Friesian horse, case No. 14. Note marked disorganization and fragmentation in the elastin within the aortic media. Immunohistochemistry for elastin; hematoxylin counterstain.
Figure 4. Friesian horse, case No. 13. Midregion of the aortic media demonstrating marked disorganization of collagen fibers. Von Gieson.
Figure 5. Friesian horse, case No. 14. Wall of pseudoaneurysm demonstrating fibrosis, fat tissue and inflammatory infiltrate consisting of mainly macrophages. HE.

Subacute stages of the lesions were identified in 8 horses (subacute group, Nos. 5-12) based on the presence of immature fibroblasts in the adventitia and periadventitial tissue and infiltration of mononuclear cells and hemosiderin-laden macrophages (Supplementary Table 1). Five of these horses demonstrated a periaortic hematoma, which was associated with hemothorax in 4 cases, and with an aorto-pulmonary fistula in 1 case. The other 3 horses showed an aorto-pulmonary fistula combined with an aortic dissection. Fibrin clots were observed in the hematoma in 6 cases. Medial

necrosis was seen in all cases with subacute lesions and varied from mild in 3 cases to severe (grade 1-3) in 5 cases. In all cases there was infiltration of the adventitia and periadventitial tissue predominantly by hemosiderin laden macrophages (grade 1-3) was present in all cases. In 4 cases these macrophages were accompanied by neutrophils and in 2 cases they were admixed with several eosinophils. Accumulation of mucoid material (grade 1-3) and disorganization and fragmentation of the elastic laminae (grade 1-3) were seen in the aortic media of all horses in the subacute group. In 2 cases mild to moderate (grade 1-2) medial fibrosis was observed. Increased fibrosis was typically characterized by disorganization, fragmentation or clumping of fibers (Fig. 4). In 1 horse there was multifocal mineralization of well-defined areas of medial smooth muscle cell necrosis and extensive elastin fragmentation. Smooth muscle hypertrophy was present in all affected Friesians, but no animals in the subacute group showed changes in smooth muscle orientation. The number of vasa vasorum in the media ranged from 21 to159 (mean 70) and a single case showed medial fibrosis of vasa vasorum in the aortic media. In the adventitia, the number of vasa vasorum ranged from 37 to 276 (mean 149) and a single case also showed moderate intimal thickening with subendothelial smooth muscle cells and proteoglycans.

The remaining 8 animals (chronic group, Nos. 13-20) showed chronic lesions in the aorta. These were similar to the lesions seen in the subacute cases but additionally showed fibrosis in the adventitia and periadventitial tissue (supplementary Table 1). All of the cases with chronic lesions showed aorto-pulmonary fistulation combined with a pseudoaneurysm and all cases had moderate (n = 3) to severe (n = 5) necrosis (grade 2-3) of the midzone of the media. In all cases, a mild infiltration (grade 1) of B- and T-lymphocytes and plasma cells was found in the media and/or adventitia. In 5 cases there was infiltration of the periadventitial tissue mainly by hemosiderin laden macrophages, sometimes admixed with neutrophils. Accumulation of mucoid material (grade 2-3) and disorganization and fragmentation (grade 1-3) of the elastic laminae were seen in the aortic media of all horses in the chronic group. Mild to moderate (grade 1-2) medial fibrosis was observed in 7 cases and was similar to the morphologically abnormal fibrosis described in the subacute cases. In 5 cases there was multifocal mineralization of well-defined areas of medial smooth muscle cell necrosis with extensive elastin fragmentation. Smooth muscle hypertrophy was present in all affected Friesians, but no animals in the chronic group showed altered smooth muscle orientation. Moderate to marked intimal thickening of the vasa vasorum of 2-5 vessels was seen in 3 of the chronic cases and 2 of these were located in the adventitia. Mild medial fibrosis of the vasa vasorum was present in the media (n = 2) and/or adventitia (n = 3). The number of vasa vasorum in the media ranged from 26 to 133 (mean 59) and in the adventitia from 63 to 434 (mean 163).

Pseudoaneurysms

Pseudoaneurysms were present in 1 of the acute, 4 of the subacute and all of the chronic cases and in all Friesians with chronic histologic lesions. The adventitial side of the wall of the pseudoaneurysm was characterized by a thick layer of spindle shaped cells (fibroblasts) that formed streams and bundles in various directions in a myxomatous matrix. Frequently whorls were formed and multifocal small to medium sized blood vessels were present. The center of the wall was composed of a layer of smooth muscle cells that were mainly longitudinally oriented. The smooth muscle cells were multifocally mixed with a moderate amount of collagen fibers that showed various orientations. Close to the intimal side of the wall of the pseudoaneurysm these smooth muscle cells were replaced in large areas by moderate amounts of collagen, high numbers of small to large sized blood vessels with occasionally marked intimal proliferation, high numbers of B- and T-lymphocytes, multinucleated giant cells, a moderate amount of fat, low numbers of viable and degenerate neutrophils, plasma cells and a few cholesterol clefts (Fig. 5). The luminal side was lined by well-differentiated endothelial cells, confirmed by staining with factor VIII-related antigen. Pseudoaneurysms contained large blood clots with high numbers of degenerated and viable neutrophils, and low numbers of eosinophils and multinucleated giant cells.

Periaortic hematomas

Periaortic hematomas were observed in 2, 5 and 1 animals demonstrating acute, subacute and chronic histologic lesions, respectively. The periphery was bordered by a moderately thick layer of longitudinally arranged thick collagen fibers. In some cases the adventitia showed large areas of fragmented (degenerate) collagen, hemorrhage and an inflammatory infiltrate consisting of large numbers of lymphocytes and plasma cells. In a few cases, the adventitia showed multifocal proliferation of randomly aligned bundles of fibroblasts admixed with fat tissue. Multifocal areas with large numbers of neutrophils and a moderate amount of necrotic debris admixed with small deposits of hematoidin were also present.

Aortic dissections

In 4 cases (3 subacute and 1 chronic) the aortic wall was dissected within the media close to the adventitia with the formation of a pseudolumen. This pseudolumen was walled off by large numbers of smooth muscle cells, a large amount of collagen and moderate numbers of medium to large sized vessels. The smooth muscle cells were longitudinally aligned parallel to the lumen, but this alignment became less organized further from the lumen. The variable arrangement of collagen fibers became increasingly loose and disorganized towards the periphery. Both sides of the dissection were lined by endothelial cells (positive factor VIII-related antigen immunostaining).

Discussion

The histological lesions described in the present study correlated well with the gross lesions and clinical signs. All horses in the acute group suffered from acute death or presented with acute signs, whereas all horses of the chronic group had shown clinical signs over a prolonged period due to aorto-pulmonary fistulation. To date, there is only a single report in the literature describing the histological features in 3 cases of Friesian horses with aortic rupture and aorto-pulmonary fistulation. These animals had severe progressive symptoms of cardiac distress, died within a few hours to 8 days after referral and are comparable to the chronic group in the present study. These authors reported the histologically detected alterations in the vasa vasorum as a possible cause of aortic medial necrosis. Furthermore, the scar of the former site of the ductus arteriosus was considered to be predisposed to rupture and fistulation.[51]

In this study, aortic medial necrosis was observed in all horses. By weakening the aortic wall this may predispose affected vessels to dissection and spontaneous rupture.[33] Medial necrosis has also been suggested as a predisposing lesion in non-Friesian horses dying from acute rupture of the aorta at the sinuses of Valsalva.[42] Chronic histologic lesions as seen in the Friesian horses such as fibrosis of the adventitia and peri-adventitial tissue with infiltration of hemosiderin-laden macrophages however have not been described.

Nearly all affected Friesian horses (85%) showed laminar medial necrosis (LMN) as a histologically evident laminar pink band with loss of nuclei in the mid media. The middle part of the media is situated between the area supplied by the vasa vasorum and the area nourished by the intraluminal blood, and is therefore, most prone to ischemic damage.[53] For the same reason, aortic dissection usually occurs at the junction of the middle and the outer third of the media, as seen in the Friesian horses in this study.[54]

The laminar medial necrosis of the aorta in the Friesian horse could be attributed to ischemia. A high number of vasa vasorum with intimal thickening and/or medial fibrosis in the media and adventitia of the aorta was described in 3 Friesian horses with aortic rupture.[51] The lumen of the vasa vasorum was completely obliterated and this was suggested to cause hypoxia or anoxia of the aortic wall resulting in local circulatory compromise, necrosis and finally, wall rupture. In the present study only 5 of the 20 cases with mild to moderate intimal thickening of the vasa vasorum without complete occlusion. Medial fibrosis in 2-5 vessels of the vasa vasorum was infrequently present in subacute and chronic cases. There is a high discrepancy between our findings and those previously reported, therefore making ischemic damage as an underlying cause, unlikely. The alterations observed in the vasa vasorum in the present study are believed to be a secondary phenomenon related to the blood

flow changes such as the severe circulatory disturbances caused by ruptured aortas or pseudoaneurysms.

Another cause of medial necrosis is connective tissue abnormalities that, in humans, are often related to gene dysfunctions. In such cases, cystic medial necrosis (degeneration of elastic fibers and collagen in the media of the aorta and subsequent accumulation of mucoid material) is often observed.[55] Accumulation of mucoid material in the tunica media resembling cystic medial necrosis was present in all cases reported here, suggesting that a primary connective tissue disorder may contribute to aortic rupture in Friesian horses. There is controversy about the significance of cystic medial necrosis in human aortic rupture. It has been suggested by some authors that it is primarily an aging process[43], but other studies have indicated that cystic medial necrosis is an expression of metabolic activity rather than the result of a degenerative process.[9,43] In this study, the role of aging is questionable as the majority of animals were less than 7 years old. Furthermore, cystic medial necrosis has been observed in non-diseased horses[41]. Therefore, it is possible that cystic medial necrosis is not a primary feature of the disease.

Some degree of medial necrosis is related to aging and is typically observed as small focal defects in the human aorta.[7] In a small minority of affected Friesian horses a patchy distribution of necrotic medial foci was observed and could be attributed to age. However, 1 of these horses was only 4 years old, suggesting that age may not be a factor in the development of this lesion. In addition, this patchy medial necrosis was seen only in acute and subacute cases, indicating that this lesion may represent an early stage in the disease.

Finally, medial necrosis can be initiated by mediators from structural elements of the media. This mechanism is seen in abdominal aortic aneurysms in humans, where vascular smooth muscle cells promote matrix metalloproteinase release resulting in medial damage.[22] In such cases, inflammation is a prominent and consistent secondary finding characterized by extensive infiltrates of B- and T-lymphocytes, plasma cells and dispersed macrophages in the adventitia.[17] Since smooth muscle hypertrophy, fibrosis and the infiltration of lymphocytes and macrophages were observed in the affected Friesian horses, involvement of proteinases cannot be excluded.

Smooth muscle cell hypertrophy of the human aorta, often accompanied with protein deposition, is associated with hypertension and aberrant blood flow creating cyclic stretch and shear stresses.[4,15,34] In bovine Marfan syndrome smooth muscle hypertrophy is considered to be a secondary reaction, replacing damaged elastic fibers of the aortic wall.[39] The extracellular matrix can play a pivotal role in the regulation of arterial vascular smooth muscle cell differentiation and proliferation.[4,25,28] In the

present study, all Friesians showed aortic medial smooth muscle cell hypertrophy, accumulation of mucoid material and disorganization of the elastin. It is, therefore, possible that primary changes in the extracellular matrix with subsequent aberrant stretching may have caused smooth muscle hypertrophy in these cases.

In about half of the chronically affected Friesian horses, abnormal collagen deposition was seen predominantly in the midzone of the media. As is seen in humans, this collagen deposition may reflect a reactive strengthening of the vessel wall as a reaction to the abnormal hemodynamic changes caused by the pseudoaneurysm and/or aorto-pulmonary fistula.[1,8] Additionally, as fibrosis was most obvious in the chronic group, the collagen deposition may be a secondary reaction to chronic injury. Possible triggers for repair in vascular tissues are necrosis of smooth muscle fibers, disruption of elastic laminae, or loss of endothelial continuity.[35] However, the collagen deposition observed in the affected Friesian horses was clumped and disorganized which is not a common characteristic of fibrosis.

Disorganization and fragmentation of elastic laminae in the media was seen in all horses and could be due to proteolytic activity caused by the damaged extracellular matrix at the site of the rupture. In the past, elastin fragmentation was interpreted as being secondary to smooth muscle cell necrosis.[14] However, elastin degeneration was present in both chronic and acute cases. This may suggest an underlying primary connective tissue disorder in the Friesians. Well-known causes of elastin defects include Williams syndrome [11], "cutix laxa" [48], and human and bovine Marfan syndromes.[2,39] In these syndromes, vascular anomalies are accompanied by alterations in other organs. Since no other abnormalities were observed, a syndromal elastin defect seems unlikely in the Friesian horses.

In humans, aorto-pulmonary fistulation is very rare and occurs most frequently as a complication of aortic pseudoaneurysm. The latter has been associated with a true aneurysm, aortitis, atherosclerosis, arteriosclerosis, aortic dissections, traumatic aortic tear, or as a post-surgical event.[6] Occasionally, a foreign body has been the inciting factor.[20] In the present study, there were no indications for infectious agents, atherosclerosis or trauma as being the primary cause. In one human case, a small aortic aneurysm extended into the pulmonary artery and this was believed to be due to pressure and the close proximity of the pulmonary artery and the aorta.[21] In Friesian horses the location of the aorta-pulmonary fistulation was previously assumed to be due to tension created by the ligamentum arteriosum on the previously damaged walls of aorta and pulmonary trunk.[51] However in this study, an ongoing chronic process with weakening of the aortic wall and pseudoaneurysm formation towards the pulmonary artery is believed to be the primary cause in at least some of the cases. Nevertheless, as there are several cases with a fistula in the absence of a

pseudoaneurysm, rupture of the pulmonary artery may be caused by an intrinsic defect.

Histological lesions seen in affected Friesian horses differ from non-Friesian horses suffering from abdominal aneurysms. In 45% of the *Strongylus vulgaris*-mediated equine arteritis one or more larvae can be found and this finding is consistently associated with thrombosis.[32] The current study showed no true aneurysms and there were no macroscopic or histologic indications for parasite migration in the affected Friesian horses. Rupture of the uterine artery is a common condition in predominantly aged, multiparous mares and copper deficiency is presumed to be a predisposing factor.[47] Peripartum hemorrhage in these mares is characterized by atrophy of smooth muscle cells with fibrosis of the *tunica media* and disruption and/or calcification of the internal elastic lamina.[50] In this study, the median age of the affected Friesians was only 4 years old, suggesting a different pathogenesis.
The finding of a shared set of clinical signs and gross and histological findings in all cases of aortic rupture reported here supports the recognition of this syndrome as a distinct clinical entity unique to this breed. It is conceivable that an underlying genetic defect of the connective tissue in the aortic media predisposes these animals to aortic rupture, dissection and aorto-pulmonary fistulation at an anatomically and hemodynamically predisposed site. The Friesian horse population appears to be the only animal species in which aorto-pulmonary fistulation is regularly encountered. In light of these findings, biochemical and ultrastructural examination of the extracellular matrix in the aorta of affected Friesians may be useful in furthering our understanding of the pathogenesis of this syndrome.

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Chapter 4

Esophageal dysfunction in Friesian horses: morphologic features

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Abstract
Megaesophagus appears to be more common in Friesian horses compared to other breeds. A prevalence of approximately 2% was observed amongst Friesian horses presented to the Wolvega Equine Clinic and the Utrecht University Equine Clinic.
In this study, morphological changes in the esophagus of Friesian horses with a megaesophagus were compared to those of six control horses.
Of 18 horses with a clinically observed megaesophagus, only 12 animals presented with esophageal dilation at necropsy, usually involving the thoracic part. Muscular hypertrophy of the distal esophagus was present in only a third of the affected horses, indicating that this change is not the most relevant cause of megaesophagus in Friesian horses. Histopathologically, an increased deposition of collagen, that was clumped and disorganized, was present in these clinically affected horses mainly in the non-dilated part of the esophagus. Furthermore, there was a decrease in neural elements and elastin in horses clinically diagnosed with megaesophagus. Similarly, this change was more evident in horses with megaesophagus at necropsy. Mild degeneration and necrosis along the whole length of the esophagus was present in clinically affected horses and encountered only rarely in control horses. There were no significant differences between the affected and control horses with respect to inflammation, mineralization or the number of cells of Cajal.
The increased occurrence of megaesophagus in the Friesian breed compared to other horse breeds, together with the presence of aberrant collagen in very young foals supports the hypothesis that megaesophagus is a hereditary trait in Friesian horses.

Introduction

Megaesophagus is a chronic dilation of the esophagus. Over the years, several different underlying pathophysiological processes for the development of megaesophagus have been proposed including the loss of neurogenic input at the level of the esophageal interstitial cells of Cajal (ICC) and idiopathic muscular hypertrophy of the distal esophagus (IMHO). [9,10]

Megaesophagus has been reported with higher frequency in Friesians when compared to other breeds.[3,10] The percentage of Friesian horses with megaesophagus presented to Utrecht University equine hospital is high (2.2%) in comparison to esophageal disease in other breeds (0.5%).[10] This suggests that it could be a hereditary trait such as dwarfism and hydrocephalus that are known to affect the Friesian breed.[12,15]
In addition, it was observed that in some cases, in which a megaesophagus was diagnosed clinically, no dilation was present during postmortem investigation.[10] In order to better understand these features, it is important to characterize the histopathological elements of this disease in far more detail than has been done so far. Therefore the morphological characteristics of megaesophagus in Friesian horses, with emphasis on esophageal elements that are important for normal esophageal function, were analyzed.

Material and methods

Case selection

Eighteen Friesian horses, presented to the Faculty of Veterinary Medicine, Utrecht University and to Wolvega Equine Clinic, Oldeholtpade, The Netherlands between 2009 and 2012 were diagnosed clinically with megaesophagus (supported by endoscopy and contrast radiography). They were euthanized because of therapeutic unresponsiveness, and necropsied. Three non-affected Friesian horses (Supplemental Table 1) were selected to serve as control horses. These were selected based on a preliminary screening of pedigrees and chosen because they were not closely related to affected horses. Additionally, three warmblood horses (Supplemental Table 1) presented for autopsy for orthopedic reasons were selected to serve as non-Friesian controls.

Histopathology

For every horse at least three samples were collected. In horses with a dilation the first sample was taken at the widest point of esophageal dilation (Fig. 1, location B). The second and third samples were taken at the oral and aboral sides of the dilation and at a distance of at least 10 cm away from the dilation (Fig. 1, location A and C, respectively). In horses without a dilation, samples were taken at three anatomically similar locations.

Masseter and semitendinosus muscles were collected from 13 of the 18 megaesophagus horses and all control horses to assess possible signs of primary systemic muscular disease potentially causing the megaesophagus. Additionally, the vagal nerve and brain stem (nucleus dorsalis and nucleus ambiguus) of the same Friesians were sampled and assessed for signs of primary neural disorders.

Tissues were fixed in 10% buffered formalin, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (HE) for routine evaluation. Furthermore, HE was used to assess the amount of degeneration and necrosis of the tunica muscularis, inflammation and mineralization within all layers of the esophagus, and the number of neurons in the submucosa (plexus of Meissner) and tunica muscularis (plexus of Auerbach). Von Gieson's stain (Klinipath 64089, Duiven, The Netherlands) was used to visualize the amount of collagen in the mucosa and tunica muscularis.
Histological findings were analyzed using a semi-quantitative method.
Presence of degeneration and/or necrosis, inflammation, mineralization and collagen deposition were scored as absent (0), mild (grade 1), moderate (grade 2) and severe (grade 3). The number of neurons was assessed and graded as no neurons (0), presence of small numbers (1), moderate numbers (2) or marked numbers of neurons (grade 3). Results from affected Friesians horses were compared to the control horses.

The non-dilated parts only were assessed with regard to caudal muscular hypertrophy. The striated muscle cells located on the oral side of the dilation (Fig 1, location A), and the smooth muscle cells located on the aboral side of the dilation (Fig. 1, location C) in the tunica muscularis of the esophagus were analyzed for the presence of hypertrophy and hyperplasia. Two randomly chosen fields were photographed at a magnification of 40x and scored for the number of cells and the width of 20 randomly chosen cells. Results from affected Friesian horses were compared to control horses.

Immunohistochemistry
The immunohistochemical stains were performed according to the manufacturer's instructions. Elastin was visualized using a monoclonal anti-elastin antibody BA-4 (Leica Biosystems, Diegem, Belgium). Smooth muscle abundance was examined using mouse anti-SMA (1:1200 Lot MU1281008, Biogenex, Duiven, The Netherlands) as primary antibody and horse anti-mouse/biotin (1:125, Lot W1123, Vector Laboratories, Amsterdam, The Netherlands) as secondary antibody. Cells of Cajal were evaluated using polyclonal rabbit anti-human CD117 (1:100, Lot W2206, DAKO, Eindhoven, The Netherlands) as primary and biotinylated anti-rabbit IgG (1:250, Vector Laboratories, Amsterdam, The Netherlands) as secondary antibody.
Nervous tissue was examined using monoclonal mouse anti-human neurofilament protein (1: 160, DAKO, Lot 00006119, Eindhoven, The Netherlands) as primary and biotinylated anti-mouse IgG (1:125, Vector laboratories, Lot W1123, Amsterdam, The

Netherlands) as secondary antibody. Nervous tissue and the cells of Cajal were evaluated by using a grid (hundred squared, Carl Zeiss, E-PI 10x/20) that was placed at six different, randomly chosen locations of the sections of the esophagus excluding the epithelial layer. The number of squares that contained immunohistochemically positive-labeled tissue, using a 10x objective were counted.

Negative controls were prepared from serial sections in which the primary antibody was omitted and dilution buffer was applied in its place. Small intestine was used to ensure the specificity of the primary antibodies.

Retrospective clinical phenotyping
Phenotypical characterization may help to unravel the pathophysiology and the genetic background of this esophageal pathology in Friesians. Patient records of Friesian horses that were presented to the Faculty of Veterinary Medicine, Utrecht University and to Wolvega Equine Clinic, Oldeholtpade, The Netherlands between 1999 and 2012 were analyzed for the occurrence of megaesophagus. In total 89 Friesian horses were identified based on clinical signs, endoscopic findings and/or contrast radiography as having a megaesophagus. Specific data including gender, age at onset of problems and clinical signs were recorded for all 89 cases. Additionally, the total number of non-Friesians that were presented to the Utrecht University equine clinics was compared with the prevalence of Friesians presented with megaesophagus.

Statistics
Clinically affected horses (Nos. 1-18) were compared to control horses (Nos. 19-24). Additionally, Friesians with macroscopically visible dilation (Nos. 1-12) were compared to Friesians without a grossly visible dilation (Nos. 13-18) and control horses. Comparisons were made for either all sampled locations of the esophagus (A+B+C), or at (A+C) or at (B) alone.
Hypertrophy and hyperplasia were assessed for the smooth musculature at location C and striated musculature at location A only.

Results were analyzed statistically using the Kruskal-Wallis test (nonparametric ANOVA) SigmaPlot 12.5. A difference of $P < 0.05$ was considered significant.

Results

Major findings are depicted in Supplemental Tables 1 and 2.

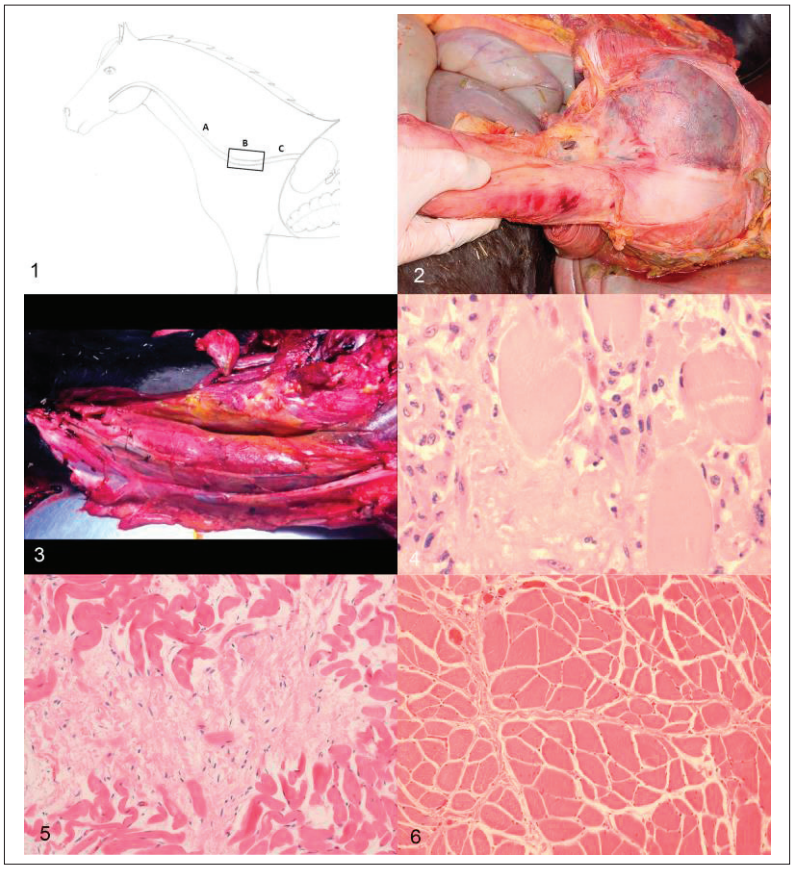

Figure. 1. Esophagus with the three locations sampled: A. Oral side of dilatation. B. Site of esophagus where dilatations usually occur in Friesian horses. C. Aboral site of dilatation.

Figure 2. Focal dilatation of thoracic part of the esophagus, Friesian horse, case number 7.

Figure 3. Friesian horse (No. 10) showing a dilated esophagus over its full length involving the thoracic as well as the cervical parts of the esophagus.

Figure 4. The esophagus of an affected Friesian horse (No. 5) showing marked degeneration and necrosis (grade 3) of the striated muscle fibers, (HE).

Figure 5. Esophagus of an affected Friesian horse (No. 10) showing marked accumulation of fibrous connective tissue in between muscle fibers of the tunica muscularis, (HE).

Fig. 6. Esophageal muscle of a control horse (No. 2) showing a minimal amount of interstitial connective tissue between the muscle fibers of the tunica muscularis.

Necropsy findings
At necropsy not all clinically affected horses (n = 18, Nos. 1-18) had a dilated esophagus. Only in 12 horses (Nos. 1-12) was a megaesophagus present, whereas the remaining six (Nos. 13-18) had a macroscopically normal esophagus.

The esophageal dilation usually presented as a gradual widening towards a centrally located broadest point. Eight horses had a dilation that was mainly located in the thoracic part of the esophagus (Fig. 1, location B) over a distance of 10-150 cm in length (Fig. 2). In most cases (6/8, Nos. 1,2,5,6,8 and 12) the dilation extended caudally to the cardia (aboral side, Fig 1, location C). In four (4/8) of these cases (Nos. 2,4-6), the dilation extended cranially to the cervical part (oral side, see Fig. 1, location A) of the esophagus. Two horses (Nos. 10-11) had a dilation of the esophagus over its full length (Fig. 3) with involvement of not only the thoracic, but also the cervical part. In four of the affected horses (Nos. 6,8,10 and12) with a gross dilation, a concentric hypertrophy of the tunica muscularis was present in the most caudal part of the esophagus over a distance of 10-30 cm.

Histology
There was more degeneration and necrosis present in the esophagus of clinically affected horses (Nos. 1-18) at locations (A+B+C) than in control horses ($P<0.05$). In the majority of these horses changes were mild and mainly encountered in the striated muscle of the tunica muscularis. In control horses, degeneration and necrosis was only present in individual cases.

There was significantly more collagen deposition in between and separating the large muscle fibers (Fig. 5) of the muscularis ($P < 0.05$) in both the entire group of clinically affected horses (Nos. 1-18) and in the subgroup of horses with a megaesophagus at necropsy (Nos. 1-12) for locations (A+C) when compared with the controls. Additionally, in the lamina propria of clinically affected horses (Nos. 1-18) there was more collagen deposition than in the control horses ($P < 0.01$) at locations (A+B+C). Some increased collagen deposition was also encountered in two control horses, but in those two cases this was quite subtle and scored as grade 1, whereas in the Friesians with a megaesophagus at necropsy (Nos. 1-12) changes were scored as grade 2 to 3. In addition, the collagen fibers in affected horses were disorganized, less condensed and presented as small, clumped structures (Fig. 6).
A significant decrease in elastin in the lamina propria ($P < 0.05$) was found when the clinically affected horses (Nos. 1-18) were compared to controls for locations (A+B+C). The horses without a megaesophagus at necropsy had significantly less elastin ($P < 0.05$) for locations (A+B+C) and (A+C) when compared to Friesians with a dilation.

A decrease in neurons was present in the mucosa as well as the tunica muscularis of the horses with a esophageal dilation at necropsy (Nos. 1-12) compared to controls ($P < 0.05$) or to grossly non-affected Friesians (Nos. 13-18) ($P < 0.05$) at locations (A+B+C) and B. Additionally, there were fewer neurons in the mucosa in clinically affected horses (Nos. 1-18) compared to controls for locations (A+B+C).

Neurofilament staining showed a significant decrease of nervous tissue at locations (A+B+C) of clinically affected horses (Nos. 1-18) when compared with controls. This decrease in nervous tissue at locations (A+B+C) was also significant when the subgroup (Nos. 1-12) was compared with grossly non-affected Friesians ($P < 0.05$). There were no significant differences in the number of Cajal cells between the different groups.

An inflammatory component was present in several cases of both clinically affected (Nos. 1-18) and control horses. Inflammation consisted in most cases of low numbers (grade 1) of lymphocytes and plasma cells, macrophages and a few neutrophils. A small amount (grade 1) of mineralization was present within the tunica muscularis of the esophagus in five clinically affected horses (5,9,10,12 and 15). Overall, differences between the groups with respect to inflammation and mineralization in the mucosa, submucosa and muscularis were not significant.

No significant histological abnormalities were encountered in either the vagal nerve, the brain stem (nucleus dorsalis and nucleus ambiguus) or the semitendinosus and masseter musculature.

Assessment of hypertrophy and hyperplasia of the tunica muscularis showed no significant differences between the clinically affected Friesians (Nos. 1-18) and control horses. There were no significant differences in hypertrophy and hyperplasia of the smooth musculature (location C) between the groups. There was significantly less hypertrophy and hyperplasia of the striated muscle component (location A) in the Friesians without a macroscopically visible dilation (Nos. 13-18) when compared to Nos. 1-12.

Retrospective Clinical Phenotyping

Age was documented in 73 of the 89 clinically affected Friesian horses (Supplemental Table 1). Of these, the majority were younger than five years old and the oldest horse diagnosed with megaesophagus was 26 years old (range = one day to 26 years; mean = 3.8 years). The reported onset of clinical signs varied from a few days to several years before the date of admission to the clinic and the final diagnosis. There was no gender predilection (n = 40 ♀; n= 35 ♂ and n = 5 geldings, of the remaining nine horses no sex was recorded) (Supplemental Table 1).

Clinical signs reported included recurrent episodes of esophageal obstruction (n = 17), coughing (n = 12), bilateral (alimentary) nasal discharge (n = 11), salivation (n = 5), dysphagia (n = 4), aspiration pneumonia (n = 4), visible or palpable swelling of the cervical esophagus (n=3), fever (n = 3), regurgitation (n = 3), anorexia (n = 3), bruxism (n = 2) and lethargy (n = 2). In a few cases, the horses developed the habit of resting the head on a door post or another structure after having eaten (n = 3), probably in order to generate a straight and sloping position of the esophagus, facilitating passage of food.
Amongst Friesian horses presented to the Wolvega Equine Clinic case load and the Utrecht University Equine Clinic the prevalence of megaesophagus respectively 2.1% and 2.2%. In both clinic prevalence of esophageal problems in non-Friesian breeds was much lower (± 0.6%).

Discussion

Megaesophagus is quite rare in horses. In most cases it develops as a sequel to a previously occurring esophageal obstruction or laceration.[3,7,10] Friesian horses however, tend to show this disease with an increased incidence.[3]
It is worthy of note that there is a subset of horses that shows esophageal dysfunction, despite esophageal dilation not being observed at necropsy. It is possible that the esophageal dilation develops in a slowly progressive way over the course of several years since this subset includes only young horses (up to 1 year old) or foals. This is supported by the fact that histopathological abnormalities are also seen in this subgroup of young Friesians. When present, the esophageal dilation was almost always located in the thoracic cavity. However, as reported in a previous case,[10] there were two young Friesian horses with esophageal dilation along its full length. This suggests that the primary defect of this disease in Friesians affects the organ as a whole. Interestingly, concurrent gastric overload or gastric rupture, two other pathologies that seem to be encountered with increased frequency in Friesian horses, was diagnosed in any of these Friesian horses.

With respect to histopathology, an important difference between affected horses and control animals was the presence, in affected horses, of more abundant and morphologically changed collagen between the muscular layers of the esophagus. This may suggest a causative role for collagen in this disease and supports findings of a previous study that suggested a possible causative role for collagen, more specifically for collagen type IV.[10]

It is noteworthy that this aberrant collagen was not only present in horses with macroscopically visible dilation of the esophagus but also in horses only suffering from functional esophageal problems without any grossly visible esophageal dilation.

The increased collagen deposition was more pronounced in the non-dilated part of the esophagus of grossly affected horses. The histological appearance of collagen clumps may be affected by the stretching of the organ, explaining the apparently reduced amount of collagen in dilated regions. This supports the idea of an underlying disorder affecting the whole organ. During life the esophagus is repeatedly and extensively stretched and its functionality requires that it can resume its original shape. It is possible that in predisposed Friesian horses the aberrant collagen progressively loses its resilience and elasticity, leading to recurrent overstretch, finally culminating into overt functional esophageal failure and dilation. Esophageal dilation is most often encountered at the level of the thoracic cavity. This location might be a predilection site due to a combination of factors, including gravity, the absence of external pressure of adjacent organs and the presence of negative pressures during inspiration.

Horses with a grossly visible dilation showed a significant decrease in the number of neurons in chiefly the dilated part of the esophagus when compared with controls and also when compared with grossly non-affected Friesians. Primary neuronal degeneration causing megaesophagus has been observed earlier in mature Friesians.[9] In that study, aperistalsis in smooth muscle was the most remarkable finding, suggesting a loss of inhibitory neurogenic input resulting in secondary esophageal dysfunction. Neurogenic degeneration could be secondary to mechanical damage of the nervous system due to traction and repeated overstretch. However, a primary neuronal defect would lead to a diffusely dilated esophagus and this was rarely observed in the present study. In contrast, and in support of the idea that neuronal degeneration occurs secondary to repeated mechanical overstretch, no neuronal degeneration was seen in the esophagus of a three month old, clinically affected foal. The decrease in neurons in primarily the dilated portion supports the idea of secondary loss of neurons caused by extensive stretching of the esophageal wall. Furthermore, another retrospective study,[10] reports no indications of loss or inflammation of myenteric plexi.

The interstitial cells of Cajal (ICC) play a major role in generating and coordinating gastrointestinal motility patterns.[14] Loss of neurogenic input at the level of the ICC in the Friesian breed has been suggested as an underlying pathology in megaesophagus.[16] However, no differences in ICC expression were present between affected and control horses in our study. This makes a role for ICC dysfunction in this disease less likely, although a functional defect cannot be excluded.

No signs of a generalized neuronal or muscular problem were present in the affected horses, since histopathology of vagal nerve and brainstem and of semimembranosus muscles was normal in all cases. In humans, the neurodegenerative hypothesis for the pathogenesis of megaesophagus is based on the observation of loss of neurons within

the dorsal vagal motor nucleus and degenerative changes in the vagal nerve fibers.[4,8] This was not the case in the horses evaluated in this study. Additionally, defects in vagal innervation are likely to lead to additional clinical signs such as gastric emptying disorders, which were not observed in this study population.[5,6,11]

In a recent study describing 852 horses of different breeds,[10] only six horses were identified with megaesophagus, all of which were Friesian, lending support to the reported higher incidence of megaesophagus in this breed. In that study, five of the six cases (83%) had muscular hypertrophy of the distal part of the esophagus and a possible link with this change was proposed. This is not supported by the findings from the present study, as muscular hypertrophy was identified in only four out of 18 horses (22%) at necropsy. Idiopathic muscular hypertrophy in non-Friesian horses is usually seen in older animals.[1] This study shows that megaesophagus is also frequently encountered in much younger Friesians, of 2 years of age or less. It can be expected that the occurrence of distal muscular hypertrophy in a predisposed Friesian would worsen the clinical signs of megaesophagus. Moreover, the fact that esophageal dilation was not only present in the smooth muscle part of the esophagus but also affected the part of the esophagus with striated musculature, suggests that esophageal dilation in Friesians has no association with the distal muscular hypertrophy.

The results show that there were no gender differences with respect to prevalence of the disease, as reported earlier.[3] A predilection for the male gender was recently suggested; however, in that study only 6 Friesian horses were included. Furthermore, our study confirmed the predilection of Friesians for megaesophagus (prevalence 2.1 to 2.2%) compared to non-Friesians as was reported before by a growing number of studies.[2,3,10] This and the fact that histopathological abnormalities were also seen in Friesian foals, makes a genetic background for this disease very likely.

In conclusion, megaesophagus in Friesian horses most frequently occurs during the first five years of life. Most dilations are located at the level of the mid-to-caudal thoracic esophagus. Friesian horses can have severe functional problems without a grossly visible esophageal dilation. Histopathologically, there is an increased amount of aberrant collagen in chiefly the non-dilated parts of affected horses. This aberrant collagen was also identified in very young foals supporting the suggestion that this is a hereditary trait in Friesian horses. In only a minority of the Friesian horses muscular hypertrophy of the distal part of the esophagus is seen, making an association with megaesophagus not likely.

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Chapter 5

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

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In preparation

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Abstract
Aortic rupture is quite common in Friesian horses unlike in Warmblood horses and a hereditary trait is suspected. The aortic connective tissue shows histological changes such as medial necrosis and fibrosis with aberrant collagen morphology. However, ultrastructural examination of the mid-thoracic aorta was inconclusive when trying to further elucidate the pathogenesis of the disease. Therefore, several extracellular matrix components were assessed biochemically with the focus on collagen.
Affected Friesians (n = 18) were compared with unaffected Friesians (n = 12) and unaffected Friesians were compared with Warmblood horses (n = 30). 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 flexor tendon.
Total collagen content, posttranslational modifications of collagen formation including lysine hydroxylation, and hydroxylysylpyridinoline (HP), lysylpyridinoline (LP) and pyrrole cross-links were determined. Additionally, elastin cross-links, glycosaminoglycan content and matrix metalloproteinase (MMP) activity were assessed.
Significantly increased MMP activity and increased LP cross-linking, lysine hydroxylation and elastin cross-linking were found at the site of rupture in affected Friesians. Unaffected Friesians showed lower lysine hydroxylation and pyrrole cross-links of the tendons compared to Warmblood horses. In conclusion, increased elastin and LP cross-links and MMP activity at the rupture area of affected Friesians are probable due to processes involved in healing and aneurysm formation. Decreased lysine hydroxylation and pyrrole cross-links in tendons of healthy Friesians versus Warmbloods supports presence of differences in connective tissue metabolism between both breeds.

Introduction

Aortic rupture is known to be quite rare in Warmblood horses.[27] In most cases the horses present an aneurysm or tear of the aortic root.[20,30] Aortic rupture is more common in older stallions shortly after breeding and often clinically presents as sudden death.[26] In Friesian horses, however, aortic rupture is much more common and frequently presents clinically as a chronic disease characterized by pseudo-aneurysm formation.[23] Histopathological examination of the aortic tissues in Friesians suggests a possible connective tissue disorder.[24] Subsequent ultrastructural examination of the collagen fibers of the mid-thoracic aorta of affected Friesians compared to unaffected Friesians and Warmblood horses, however, showed no differences (Ploeg et al., unpublished data). In humans non-traumatic aortic rupture often occurs as a consequence of Ehlers-Danlos syndrome or Marfan's syndrome.[11,28] These syndromes are associated with connective tissue abnormalities and related with either the content or structural design of elastin and collagen.[33]

Collagen determines the tensile strength of the aortic wall and elastic fibers are necessary for stretching and recoil of arteries. [5] In human aortic abdominal aneurysms (AAA), elastin degradation has been implicated in the dilation and aberrant collagen structure that predispose to aneurysmal rupture.[9] Matrix metalloproteinases (MMP) have proteolytic activities and are able to degrade various extracellular matrix (ECM) proteins components.[15] In humans aberrant MMP activity has been found to be associated with increased risk for AAA[25] and aortic rupture.[36]

Collagen cross-linking is fundamental for tensile strength and also increases resistance of collagen fibers against proteolytic activity.[35] Enzymatic cross-linking of collagen through (hydroxy)lysine residues produces immature cross-links.[2] Subsequently, these mature into trivalent pyridinoline (HP and LP) and pyrrole cross-links.[14] Defects in cross-linking of collagen (or elastin) may lead to abnormal arterial structure and subsequent 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 aneurysms of patients with Marfan syndrome and AAA.[19] Additionally, patients with Marfan syndrome are also deficient in desmin (elastin) cross-linking and have strongly decreased elastin content.[1]

The purpose of this study was to investigate the biochemical composition of the aortic wall of affected Friesian horses compared to unaffected Friesians. Further, the possible existence of differences between unaffected Friesians and Warmblood horses was investigated as well, as a breed-related systemic ECM disorder as underlying cause cannot be excluded. For this same reason, samples from the deep digital flexor tendon (DDFT) from both breeds were included. The study focused at the assessment of collagen content, the post-translational modifications of collagen, collagen cross-

links, elastin cross-links, MMP activity and glycosaminoglycan (GAG) content at the rupture site and a usually non-affected site more distal in the thoracic aorta.

Materials & Methods

Animals

Eighteen affected Friesian horses (age: 3-10 years, mean age: 5.7 years) were included in this study. All affected Friesians were 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 of Utrecht University, the Netherlands (n = 9). Twelve unaffected Friesian horses (age: 0 - 10 years, mean age: 3.5 years) without a history of cardiovascular or orthopedic disease were used as control horses. Care was taken that there were at least three generations between the horses in this control population and any ancestors that were known to be affected. Additionally, 30 Warmblood horses (age: 1 - 9 years, mean: 6.2 years) without any history of connective tissue disease were used as a different control group.

Sampling procedure

In each horse the aorta was divided into five equal sections from the base of the heart (site AO 1/5) to the diaphragm (site AO 5/5). Tissue samples were taken at the level of aortic rupture (AO 1/5) and from two more caudal sections (AO 2/5 and AO 3/5). Additionally, a tissue sample of the DDFT at the level of the mid-metacarpus was taken. Samples were frozen and stored at -20°C until further analysis.

Sample digestion

Tendon and aorta samples were digested for approximately 20 hours by use of papain (Sigma Aldrich, St Louis, Missouri, USA) at 56°C in 200 µl of a 50 mM phosphate buffer (pH 6.5) containing 2 mM Na_2EDTA and 2 mM cysteine and elastase (Boehringer Mannhein, Alkmaar, NL) at 37°C in 200 µl of a 50 mM TRIS-HCl buffer (pH 8.5), respectively.

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

The samples for high performance liquid chromatography (HPLC) were prepared as described earlier.[31] Briefly, after lyophilizing for 24 hours, approximately 5 mg of aorta or tendon sample was hydrolysed in 600 µl 6 mol/l HCL at 110°C for 16 hours. After that, 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, hydroxylysine and the collagen crosslinks lysylpyridinoline (LP) and hydroxylysylpyridinoline (HP) were quantified by HPLC/MS analysis as described (Souza et al., 2010). This method was extended 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 hydroxyprolin (as a measure for total collagen) and mol hydroxylysine/mol collagen (lysyl hydroxylation as measure for the collagen glycosylation). The LP and HP collagen cross-links and desmosine and isodesmosine (elastin) cross-links were expressed as (iso)desmosin mol/g dry weight.

Pyrrole cross-links

The pyrrole cross-links were analyzed in the enzyme digests of the samples following the method of Thorpe et al.[32] Briefly, 20 µl of the digest was diluted with 180 µl H_2O, after which 40 µl of Ehrlich's reagent (500 mg 4-dimethyaminobenzalhyde in 4.4 ml 60% perchloric acid completed to 10 ml with H_2O) was added. The absorbance of the samples was measured at 558 nm and 650 nm (non-related wavelengths). 1-Methyl-Pyrrole was used to create a standard curve. Results were expressed as mol pyrrole per gram Lowry protein.

Metalloproteinase activity

General MMP activity was measured by means of a fluorimetric assay based on the cleavage of a fluorogenic peptide substrate FS-6 (Calbiochem, San Diego, CA, USA), as described earlier.[8]

Briefly, from each sample 0.1 g was added to 500 µl of cooled MMP buffer (0.1 M Tris, 0.1 M NaCl, 10 mM $CaCl_2$, 0.05% (w/v) Trition X-100, 0.1% (w/v) PEG6000, pH 7.5) and homogenized (MagNA Lyser homogenisator, Roche Diagnostics, Almere NL). Subsequently, 100 µl of this mixture was added to 100 µl of 10 µM FS-6 solution and the fluorescent signal was monitored for 30 minutes, using a Clariostar® fluorimeter (BMG-labtech (GMBH), Ortenberg, Germany). The slope of the resulting linear curve (relative fluorescence units/second, 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.[10]

Glycosaminoglycans

The GAG content in the digests of the samples was assessed spectrophotometrically with the modified 1,9-dimethylmethylene blue assay,[12] as used earlier for equine samples.[34] The amount of GAG was expressed as mg per g Lowry protein.

Statistical analysis

Statistical analysis was performed using a mixed model with the horse as random effect and location, type of horse and their interaction as categorical effects. 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 8 comparisons, to a comparisonwise significance level of 0.0063.

Results

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

Collagen content, measured by the amount of hydroxyprolin residues, showed no significant differences between the horse groups.

The degree of lysyl hydroxylation was significantly higher (Fig. 1A) in affected Friesians at location AO 1/5 when compared to unaffected Friesian horses
($P = 0.0026$).
Unaffected Friesians had a significantly lower degree of lysyl hydroxylation when compared to Warmblood horses (Fig. 1B) in samples from the DDFT
($P < 0.0001$).

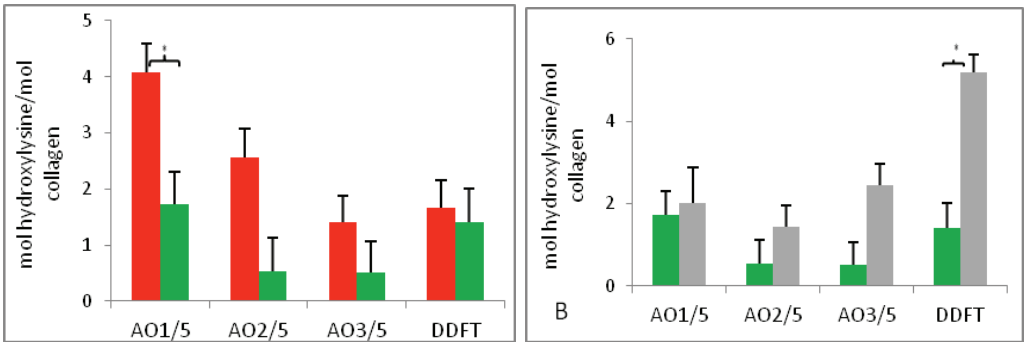

Figure 1. Lysyl hydroxylation within affected (red) versus unaffected Friesians (green) (1A) and in unaffected Friesians versus Warmblood horses (grey) (1B) for locations AO 1/5, AO 2/5 and AO 3/5 and deep digital flexor tendon (DDFT) samples. Mean and standard deviation; * denotes a significant difference.

The number of HP cross-links showed no significant differences between the horse groups.
The affected Friesians had a significantly higher number of LP cross-links (Fig. 2) when compared to unaffected Friesian horses for locations AO 1/5 ($P < 0.0001$) and AO 2/5
($P = 0.0047$).
There were no significant differences in number of LP cross-links between the Warmblood horses and unaffected Friesians in any of the samples.

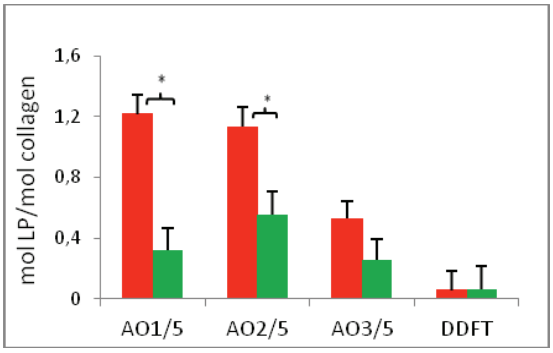

Figure 2. Number of LP cross-links within affected (red) and unaffected Friesians (green) for locations AO 1/5, AO 2/5 and AO 3/5 and deep digital flexor tendon (DDFT). Mean and standard deviation; * denotes a significant difference.

Affected Friesians had a significantly higher desmosin content when compared to unaffected Friesians (Fig. 3) for locations AO 1/5 ($P < 0.0001$) and AO 2/5 ($P = 0.0038$). Affected Friesians had a significantly higher isodesmosin content when compared to unaffected Friesians for locations AO 1/5 ($P < 0.0001$ isodesmosin) and AO 2/5 ($P=0.0053$) No significant differences in the elastin content were present between the unaffected Friesians and Warmblood horses.

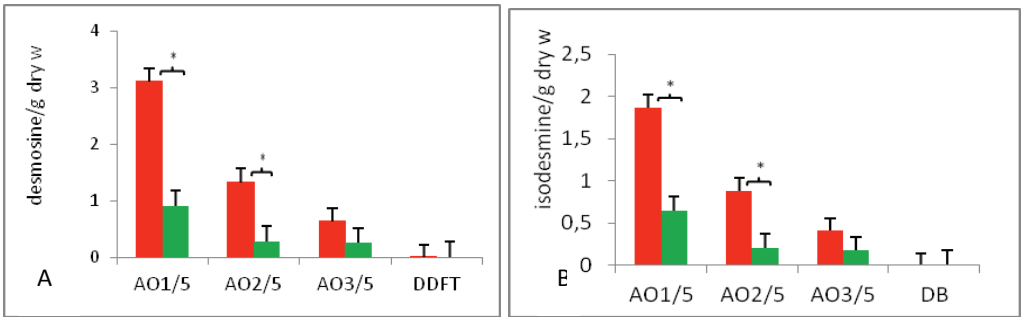

Figure 3. Desmosine and isodesmosine content, measured by the number of desmosine (3A) and isodesmosine (3B) cross-links per amount of tissue within affected (red) and unaffected Friesians (green) for locations AO 1/5, AO 2/5 and AO 3/5 and deep digital flexor tendon (DDFT). Mean and standard deviation; * denotes a significant difference.

Pyrrole cross-links

Pyrrole cross-links (Fig. 4) were significantly lower for the DDFT in unaffected Friesians when compared to affected Friesians (P = 0.0022) and Warmblood horses (P = 0.0007).

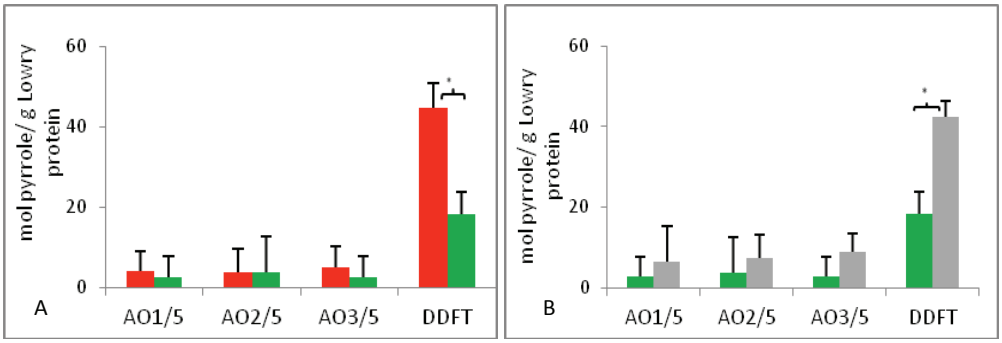

Figure 4. Pyrrole cross-links within affected (red) versus unaffected Friesians (green) (4A) and Warmblood horses (grey) versus unaffected Friesians (4B) for locations AO 1/5, AO 2/5 and AO 3/5 and deep digital flexor tendon (DDFT). Mean and standard deviation; * denotes a significant difference.

Metalloproteinase activity

MMP activity at AO 1/5 (rupture site) (Fig. 5A) was significantly higher in affected Friesians when compared to unaffected Friesians (P = 0.0033).

At location AO 2/5 Warmblood horses had a significantly higher MMP activity compared to unaffected Friesians (P < 0.0001) (Fig. 5B).

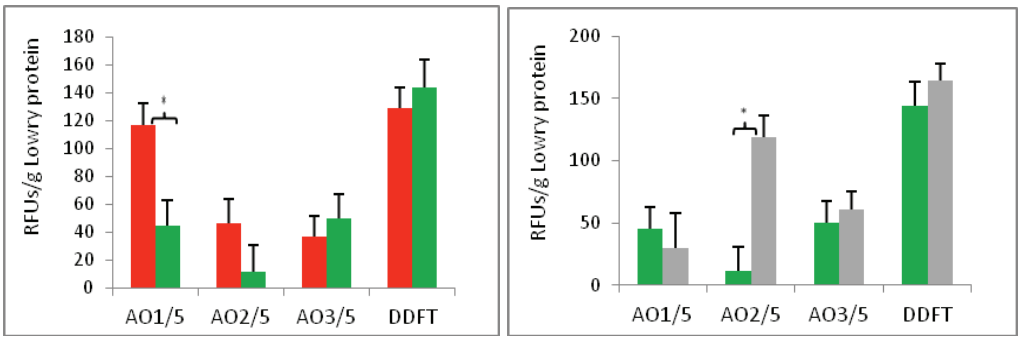

Figure 5. Metalloproteinase activity within affected (red) versus unaffected Friesians (green) (5A) and Warmblood horses (grey) versus unaffected Friesians (5B) for

locations AO 1/5, AO 2/5 and AO 3/5 and deep digital flexor tendon (DDFT). Mean and standard deviation; * denotes a significant difference.

Glycosaminoglycans

There was a general effect between the horse groups. The GAG contents was significantly lower in affected Friesians (P = 0.0085) and unaffected Friesians (P = 0.0096) when compared to Warmblood horses (Fig. 6).

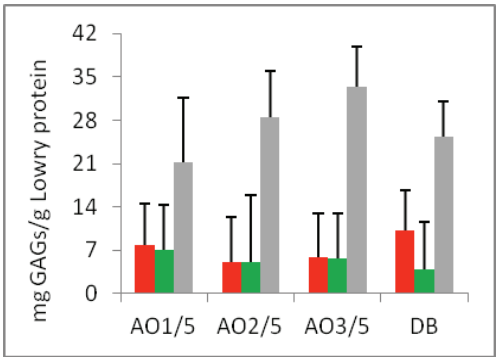

Figure 6. The GAGs content in unaffected Friesians (green), affected Friesians (red) and Warmblood horses (grey) and for locations AO 1/5, AO 2/5 and AO 3/5 and deep digital flexor tendon (DDFT). Mean and standard deviation; * denotes a significant difference.

Discussion

The ECM components of the aorta have an important role in maintaining the normal structure of the arterial wall.[3] Abnormalities of the ECM components, of their structure or their interaction can lead to an altered matrix turnover with consequences for aortic wall strength and subsequent vascular disease. The results of this study confirm that substantial changes of biochemical characteristics of the aortic wall are present, which are consistent with the occurrence of damage and subsequent remodeling at the site of rupture in affected Friesian horses. There are significant increases in LP (collagen) cross-linking, elastin cross-linking and MMP levels at the site of rupture in affected versus unaffected Friesians. Interestingly, there appeared to be also significant differences in flexor tendon ECM components between Friesian horses and Warmbloods, providing evidence for inherent differences between connective tissue ECM between both breeds. To our knowledge, this is the first study that used HPLC analysis for the study of equine aortic tissue with the aim to detect possible changes in horses suffering from aortic rupture, but also to detect possible breed-related systemic differences in connective tissue biology.

Recently, the presence of increased fibrosis, typically characterized by disorganization, fragmentation, or clumping of fibers, has been described at the site of aortic rupture in Friesian horses.[26] In the current study total collagen, assessed by quantifying hydroxyprolin and expressed as g collagen/dry weight showed no significant differences between unaffected and affected horses or horse breeds for aortic locations, nor (as expected) for flexor tendons. Whereas immunohistochemistry, as used in the earlier study, is more a method of visualization and a semi-quantitative measure at best, the HPLC technique used in this study is a fully quantitative method. It must be concluded, therefore, that the fibrosis detected by immunohistochemistry concerns more an aberrant and irregular configuration of the collagen fibrils than an absolute increase in collagen content. This conjecture is supported by the increased numbers of LP and elastin cross-links in affected tissues, which represent most likely a reactive response to the tissue damage. Further, the heterogeneity of the rupture site may have played a role. Immunohistochemistry suggested increased collagen deposition at the rupture site, but also areas of necrosis were visible. An increase in collagen at one site may therefore be masked by a decrease in an adjacent necrotic site in the same sample with collagen loss.

At the site of rupture, the affected Friesian horses showed a significantly higher MMP activity when compared to unaffected Friesians. This most likely reflects increased MMP activity within the context of increased repair and remodeling activity, as a sequel to the damage that has occurred. It is not known how many of the affected Friesians were chronic cases; however, previous research has shown that the chronic form of aortic rupture occurs quite often in Friesians and it is likely that many of the cases were chronic indeed. A considerable degree of remodeling of the aortic wall can be expected to be ongoing when a horse walks around with a rupture or even aorto-pulmonary fistulation for weeks to months. Under those conditions, a new equilibrium must have been formed that will be characterized by higher tissue turnover and hence increased MMP activity. This increased MMP activity seen at the site of the rupture is in concordance with what is seen in human aortic disease, where MMPs have a well-recognized role in the pathogenesis of degenerative abdominal and thoracic aortic aneurysm formation.[16,24] There were no breed differences between the unaffected Friesians and Warmblood horses, making aberrant MMP activity as a systemic Friesian disorder unlikely. The high MMP activity at location aorta 2/5 in the Warmblood horses was unexpected and cannot be readily explained. If it would be possible to repeat the observation, it might be considered to further investigate the phenomenon using qPCR and by measuring specific MMPs.

Other interesting findings at the site of rupture are the significantly increased elastin cross-links in affected Friesians versus unaffected Friesian horses. No differences could be found in the flexor tendon tissue. It is most likely that this increase of elastin

cross-links at the site of rupture in affected cases is the result of healing and remodeling, in an attempt to strengthen the rupture area as compensation for the loss of structural integrity of the aortic tissue and to deal with the whirling of blood within the pseudo-aneurysms. In humans with aneurysms, TGF-ß stimulation of chronically activated smooth muscle cells results in increased elastin formation and elastin cross-linking.[18] Likewise, a similar mechanism could explain the increased elastin cross-linking found at the site of rupture in affected Friesian horses. This "healing and remodeling" theory is supported by the increased MMP activity as discussed above.

Collagen hydroxylation (and possible subsequent glycosylation) are measures for collagen metabolism and remodeling. With respect to aortic tissue, collagen hydroxylation was shown to be significantly higher at the rupture site when compared to unaffected Friesians. There was no significant difference in collagen hydroxylation at the level of the flexor tendons of affected versus unaffected Friesian horses. However, very interestingly, there was a pronounced and significant lower degree of collagen hydroxylation in flexor tendons of unaffected Friesian horses when compared to Warmbloods. This means that there is an inherent and significant difference between both horse breeds with respect to the metabolism of collagen type I, which is the most abundant structural protein in the mammalian body. The exact role of collagen hydroxylation (and possible subsequent glycosylation) is still unknown.[4,21] It has been suggested that an increase of this post-translational modification is linked with a decrease in fibril diameter, [4,21] causing decreased tensile strength. It is possible that the increased hydroxylation in aortic tissue of Friesian horses affects the native structure and therefore the functionality of collagen. In humans aberrant glycosylation following hydroxylation is known to be implicated in connective tissue disorders such as cutis laxa and the progeroid type of Ehlers Danlos.[7,13] This observation merits further research. Whereas preliminary qualitative assessment of electron-microscopic data (Ploeg et al., unpublished data) did not show evident differences between affected Friesians, unaffected Friesians and Warmbloods, as mentioned before, no quantitative analysis of the ultrastructure of the collagen fibrils in terms of collagen fibrillar index or mass average diameter, as has been done earlier in the horse,[6] has been performed thus far.
Another ECM component that differed extremely and significantly in flexor tendon tissue between Friesian horses and Warmbloods was the pyrrole cross-linking, which again was significantly lower in Friesian horses when compared to Warmbloods. In aortic tissue, no difference at all, at any location could be detected across all groups. Pyrrole cross-linking is characterized by a connection between collagen fibrils thereby stiffening the tissue. So, The fact that both collagen hydroxylation and pyrrole cross-linking of the tendon were lower in unaffected Friesians compared to Warmbloods might reflect fundamental differences in collagen turnover between both breeds. The much lower collagen content of aortic wall tissue compared to tendon tissue may

explain why the breed differences are not detectable here. Interestingly, pyrrole cross-links in the tendons of Friesians affected by aortic disease increased to the normal level in Warmbloods. The reason for this is not clear, but may represent a generalized response to a local insult in the affected horses.
The final ECM component that showed a generally lower content in affected and unaffected Friesians compared to Warmblood horses for differences in both aortic tissue and flexor tendon tissue were the GAGs. GAGs have a strong water-binding capacity and a decreased GAGs content is expected to render the tissue, be it aortic wall or tendon, less resilient. Additionally, GAGs are a subset of the proteolyglan population, which molecules have a role in facilitating sliding movement between collagen fibrils.[30] This difference in GAG content between the ECMs of Friesians and Warmbloods is another hint at constituent differences in connective tissue properties between the two breeds.
There were no differences in GAG content between diseased and non-diseased Friesians. In the earlier study, histological examination of the aorta at the rupture site of affected Friesian horses showed accumulation of Alcian blue material mainly in the center of the aortic media.[26] This is again, as in the case of collagen, a difference between semi-quantitative and quantitative assessment methods and the same circumstance of inhomogeneous distribution of the material may apply. In human TAAD it has been suggested that GAG accumulations serve as initiation sites for dissection.[17] This seems to be less likely in Friesian horses with aortic rupture since only a few cases presented with a dissection of the aorta.[26]

In conclusion,
There is strong evidence of extensive remodeling at the site of rupture in affected Friesian horses. This is demonstrated by significant increases in LP cross-links, elastin cross-linking and MMP levels at the site of rupture compared to unaffected Friesians. None of these parameters significantly differed between unaffected Friesians and Warmbloods, supporting the hypothesis that their increased expression is secondary to the event of rupturing.
The important differences in collagen hydroxylation and pyrrole cross-links in flexor tendon tissue between Friesian horses and Warmbloods and the strong tendency towards differences in GAG content suggest the presence of constituent differences in connective tissue metabolism and homeostasis between both breeds with a possible hereditary background. These findings are potentially very interesting and warrant further research in this area.

Conflict of interest statement
None of the authors of the paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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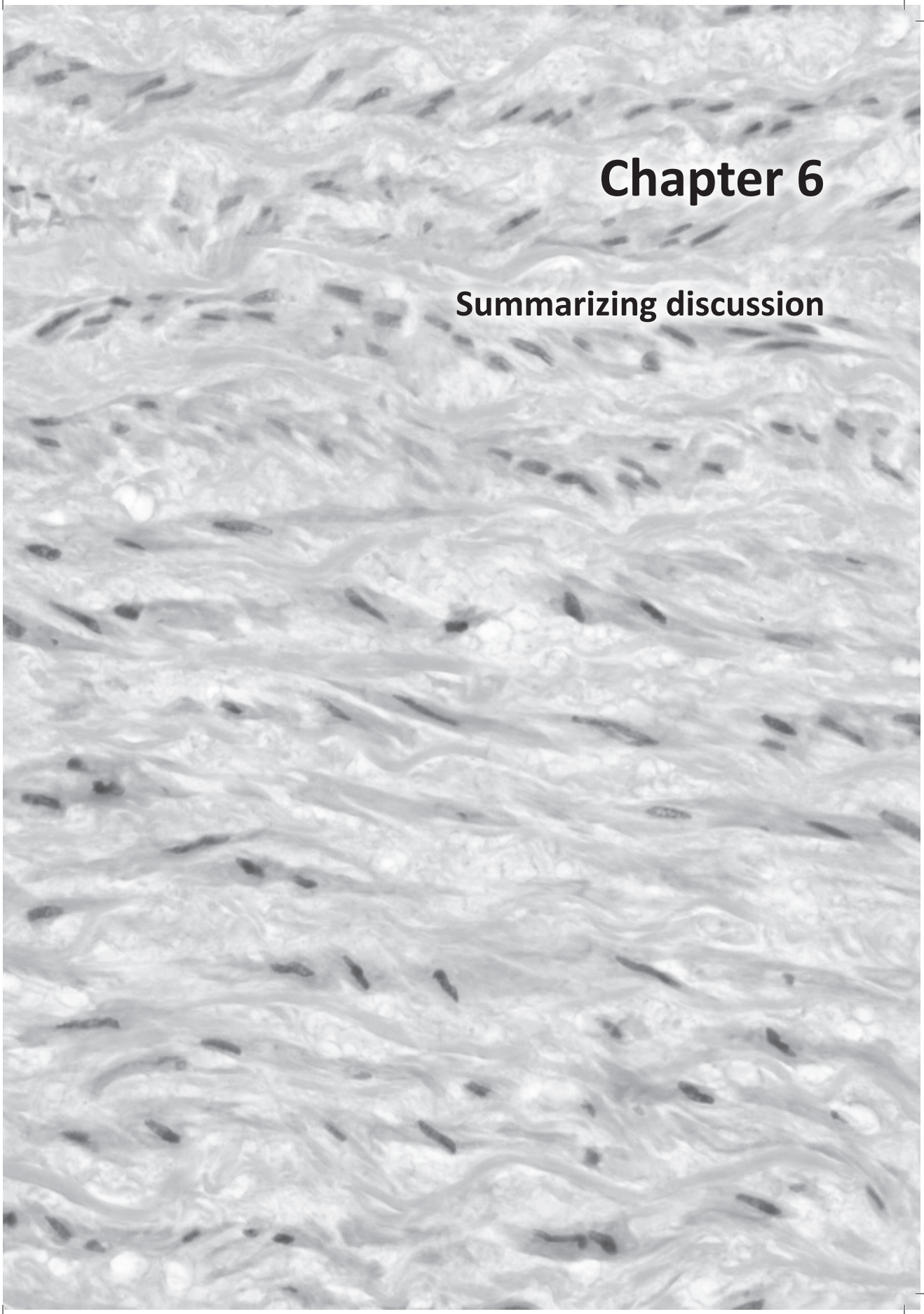

Chapter 6

Summarizing discussion

This summarizing discussion starts with an overview of thoracic aortic rupture in Warmblood horses with the emphasis on the clinical signs and morphological findings. Then similar topics regarding aortic rupture in Friesians are summarized and discussed with a focus on connective tissue-related disorders. Finally, the clinical signs and morphology of megaesophagus in Friesians will be discussed shortly with the hypothesis that both soft tissue disorders may have a common background, probably with a genetic basis.

THORACIC AORTIC RUPTURE IN HORSES

Clinical findings

Aortic rupture is quite rare in Warmblood horses[26] (prevalence ± 0.3%).[33] Due to its low incidence, only a limited number of studies and data are available in literature. Aortic rupture is best known as an acute and fatal rupture of the aortic root in older breeding stallions.[5,26] However, several cases of younger horses[10] have been described, which presented with additional gross findings comprising aneurysms and aortopulmonary shunting. This younger age group showed clinical signs such as acute discomfort, exercise intolerance, jugular pulse, tachycardia, sweating, depression and sudden collapse,[19,26] all compatible with heart failure, for several weeks prior to death.[10,38] During routine examination there is often a diastolic[26] or systolic[19]cardiac murmur. In most cases a loud continuous murmur with its maximal intensity at the level of the right fourth intercostal space is observed.[19] Diagnosis of aortic rupture in the not immediately fatal cases is based upon clinical signs, cardiac auscultation and cardiac ultrasound.[19]

Morphological findings

Based on gross lesions, cases of aortic rupture in warmblood horses are divided into a few categories.

Aortic ring rupture is characterized by a tear in the annulus fibrosis of the aortic valves. It has been described in 8 horses aged between 10 to 22 years.[26] The rupture reached directly into the right coronary sinus with subsequent dissection into either the right ventricle or the interventricular septum. Acute death probably occurs due to the accumulating blood in the dissection, causing damage of the atrioventricular bundle. [26] A possible explanation for the rupture location is the extra tension on the convex part of the aorta close to the aortic valves.[26]

At tissue level, aged Warmblood horses with aortic ring rupture showed necrosis of the medial layer in all cases.[26] The hemodynamic factors together with degenerative, inflammatory or sclerosing alterations in the aortic wall are probably the underlying pathogenetic factors that cause the aorta to rupture.[15,22,26,27] In one case histology suggested the lesion was chronic, based on the presence of fibrous tissue, calcification and bone.[26] Arterial calcification may be caused by increased levels of wall stress with subsequent increased risk of arterial wall failure, as is seen in racing horses.[36]

Aortic root disease refers to a tear or aneurysm of the aortic root.[33] Aortic root disease is reported in horses of varying age and sex and may be congenital or acquired.[33] In addition to the more common tear or aneurysm of the sinus of Valsalva, aorto-cardiac fistulas may develop as well and extend into the right atrium or ventricle. These aorto-cardiac fistulas seem to be more common in older stallions.[19] Furthermore, several cases with a dissecting aneurysm have been described. In these horse the dissection starts at the level of the aortic valve and continues up to the aortic arch, sometimes over a distance of 40 cm or more.[10,32]

Aortic rupture at the location of the aortic arch, near the ligamentum arteriosum in combination with a fistulation towards the pulmonary artery has been observed in only two young Warmblood horses (3-4 years).[10,38] Necropsy showed an aortic aneurysm surrounded by organized hematomas, either in combination with a rupture of the aorta and the pulmonary trunk or with presence of a dissecting aneurysm connecting with and rupturing into the left pulmonary artery.[10,38]
Chronic changes (granulation tissue and calcification) were also present in the aorta of Warmblood horses with an aorto-pulmonary fistulation[38] confirming that this type of aortic disease in Warmblood horses frequently has no acute course either.
Warmblood horses with an aortic dissection showed fibrosis around the rupture, degeneration and fragmentation of elastic fibers and increased amounts of mucopolysaccharides in the media.[7,10,32] These alterations, with the increase of mucoid material as key change, is defined as cystic medial necrosis (CMN)[31] and has in humans been shown to be related to aortic aneurysm, dissection and rupture.[41] CMN is a common pathological finding in several aortopathies and it has been proposed that CMN is not the cause but a consequence of a primary disorder such as fibrillin deficiency in Marfan's syndrome.[41]

AORTIC RUPTURE IN FRIESIAN HORSES

Clinical findings

Until the nineties, only 3 cases of aortic rupture in Friesian horses had been described in scientific literature. It has now become clear that this condition, which is diagnosed around an age of 4 years, is more frequent in the Friesian breed than in others (Chapter 2). The high prevalence in Friesians may be due to increased genetic susceptibility, as it is known that the breed has a narrow genetic base due to the fact that after World War II only a limited number of breeding stallions were left.[37] An underlying genetic defect may also explain the relatively young age of about 4 years at which aortic disease is commonly diagnosed in Friesians. This is comparable with the situation in human thoracic aortic aneurysms and dissection (TAAD). In young patients genetic diseases are the main cause of TAAD.[3]

Whereas Friesian horses with aortic rupture may develop acute forms, the majority of Friesians will display a subacute or chronic disease process. Friesians with a chronic form may show clinical signs, such as recidivating peripheral oedema, jugular pulsation or a strong bounding pulsation in the carotid artery, in the weeks to months prior to death (Chapter 2). These signs do not resemble the signs shown by Warmblood horses suffering from aortic rupture. This is important information for practicing equine veterinarians, who should be aware of this difference when dealing with Friesian horses.

Ante-mortem diagnosis of aortic rupture in Friesian horses is still quite challenging. In humans, the diagnosis of thoracic aortic disease is usually made by contrast-enhanced CT of the chest[34] or by transthoracic echocardiography.[28] Horses have a much bigger chest, precluding the use of these techniques. At this moment, ante-mortem diagnosis of aortic rupture in Friesian horses is based on recognizing the atypical clinical signs and transthoracic ultrasonography using non-classic cardiac windows.[39] However, a correct positioning of the probe that enables obtaining diagnostic views is challenging in heavily muscled Friesians. Recently, transesophageal ultrasound using a linear ultrasound probe has been introduced as a new diagnostic approach in standing Friesian horses.[4] This technique is a potentially interesting tool to monitor disease development in predisposed cases and may possibly allow for early preventive or therapeutic intervention.

Morphological findings

In Friesian horses the aortic rupture is encountered at the level of the aortic arch near the ligamentum arteriosum (Chapter 2), which is more caudally than seen in Warmblood horses (Fig. 1). Several cases showed presence of a longitudinal dissection of the aortic wall. A few Friesians with aortic rupture developed a circumferential cuff of blood around the aorta. All cases with chronic lesions in the aortic wall had an aorto-pulmonary fistulation in combination with a pseudoaneurysm. In human patients, aorto-pulmonary fistulation is rare[2] and associated with aortic dissection (with subsequent rupture), previous thoracic surgery or chronic aortic disease including aneurysm or giant cell arteritis.[24,37] In Friesians, however, only a few cases present with a dissection and with respect to etiological factors there are no indications for chronic infectious disease until now.

The reason why Friesians typically rupture at this specific site has not yet been elucidated. One possibility is an embryological defect developing during the fusion of the dorsal aorta with the developing heart base. Abnormalities in this development involving neural crest cells have in other species been shown to cause disorders of the aorta in conjunction with abnormalities of the aortic valves.[14] However, in the Friesian horses with aortic rupture, no such semilunar valve abnormalities have been observed

in any of the cases making this pathway as primary cause unlikely. Additional factors that may interfere with the location of rupture in Friesians are genetic disorders, connective tissue disorders affecting the vessel wall integrity, geometrical abnormalities including wall thickness, lumen diameter and abnormal blood pressure. Furthermore, the part of the aorta that ruptures in Friesians, is a relative mobile part compared to the more cranial aorta that is attached to the heart base and the more caudal aorta which is fixed to the thoracic wall. The aorta may have little support from the pulmonary artery during movement of the horse. However, repetitive tension from the pulmonary adventitious tissue on the aortic wall may cause tissue damage and can partly explain the fact that it is the concave side of the aorta in Friesians that is affected.

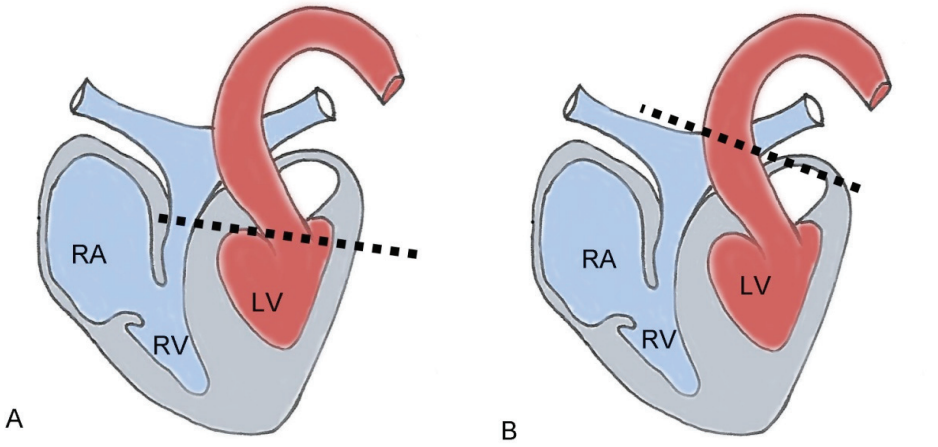

Figure 1: Aortic rupture (indicated by the dashed line) in Warmblood horses is typically located at the base of the heart in the area of the aortic valves (A). In Friesian horses the aortic rupture is located more caudally, near the ligamentum arteriosum (B). (RA, right atrium, RV, right ventricle)

Histological features at the site of aortic rupture in Friesian horses are medial necrosis, disorganization and fragmentation of elastic laminae, aortic medial smooth muscle hypertrophy and accumulation of mucoid material (Chapter 3).
One prominent finding is medial necrosis, which in humans is associated with several causes. Primary causes include ischemia[38], connective tissue disorders[31] and abnormal metalloproteinase (MMP) activity.[12] Furthermore, medial necrosis is known to be a secondary change to for example increased blood pressure,[31] inflammation[40] and toxins.[25]

The ruptured aortas of nearly all Friesians showed laminar medial necrosis (Chapter 3), defined by a laminar band with loss of nuclei,[31] which is in agreement with the descriptions by Van der Linde-Sipman and coworkers in 1985.[38] In that report thrombosis of the vasa vasorum of the aortic wall and secondary ischemia[38] was suggested as possible cause, but the results from the present study, which is based on a much higher number of cases, could not confirm this hypothesis.
Cystic medial necrosis, characterized by accumulation of ground substance,[41] can in humans also be caused by connective tissue abnormalities.[41] The histological features of CMN were present in aortas of all affected Friesians, which therefore is seemingly suggestive of a primary connective tissue disorder (Chapter 3). However, reality may be less simple than that. In humans, where aortic dilation associated with CMN is generally present in only the ascending aorta and seldom in the aortic ring or sinus of Valsalva,[41] CMN has been identified also in individuals without aortic disease in walls of the aorta as part of the ageing process.[31]
CMN may also be an adaptive response of elastic arteries to the high blood pressures in these vessels.[41] Not much is known about possibly high blood pressures at this specific location in Friesians. However, CMN seems to be a quite nonspecific finding as it is also seen in non-diseased horses.[25]

MMPs can have a primary role in medial necrosis of the aorta by increased expression as is seen in Marfan syndrome.[12] In humans with TAA overexpression of several MMPs together with a higher proteolytic state (ratio between MMPs and their inhibitors) was shown.[17] This imbalance causes pathological remodeling of the extracellular matrix (ECM) of the media and adventitia and subsequent aneurysm formation. [12]
Most MMPs are secreted freely into the ECM or anchored to the surface of cell membranes and are produced by many cell types including neutrophils, macrophages and fibroblasts. Tissue damage and subsequent inflammation cause activation of MMPs, thereby stimulating tissue remodeling and healing.[42] Within the Friesian population, all chronic cases and some acute cases showed infiltration of leukocytes in the aortic wall at the level of the rupture (Chapter 3). Therefore, it is probable that the inflammatory process activates MMPs with subsequent destruction and remodeling of the media.

Lastly, histology and ultrastructural examination of the mid-thoracic aortic media revealed smooth muscle hypertrophy in Friesian horses with aortopulmonary fistulation. This is suggestive of higher metabolic activity, which may represent a compensatory reaction to changed mechanical stresses.[29] However, elastin fragmentation is another trigger for smooth muscle cells to hypertrophy[18], which is seen in the media of all Friesian horses with aortic rupture as well.

Biochemical features

The most important structural components of the aortic wall and the morphological presentations of abnormalities have been mentioned above. These elements provide the structure of the artery and therefore have a key role in the biomechanical resistance.

Biochemical analysis of the aortic wall in Friesian horses revealed increased MMP activity only at the level of aortic rupture (Chapter 5). Furthermore, the elastin cross-links desmosine and isodesmosine were increased, probably due to the remodeling and healing process at the site of rupture. The total collagen content per dry weight, measured by the amount of hydroxyprolin residues showed no significant differences between the horse groups. Histopathology revealed increased deposition of collagen suggestive of fibrosis. For fibrosis, however, a decreased MMP activity was expected, which is not the case in Friesians, thereby supporting the role of MMPs in remodeling and a reactive collagen pattern.

Several biochemical differences between Friesians and Warmblood horses were demonstrated, including differences in the post-translational modifications of collagen in tendons and of GAG content in aorta and tendon tissue (Chapter 5). These findings suggest the presence of constituent differences in connective tissue metabolism and homeostasis between both breeds. Differences or changes in the ECM of the aorta of affected Friesians may have effects on the stiffness and strength of the aortic wall, and may also interfere with cell-matrix interactions and signaling cascades which are important for maintenance of the mechanical properties of the ECM.[11]

Although these differences do not seem to affect the overall biomechanical properties of the aorta between the breeds, the rupture site of the aorta in the affected Friesians had a lower load-bearing capacity compared to more distal parts, which difference was linked to the collagen content.[29] A local hereditary defect at the level of the ligamentum arteriosum has been suggested.[29]

Megaesophagus in Friesian horses

The aorta of Friesian horses showed several aberrant connective tissue elements, pointing towards a possibly hereditary breed-specific underlying cause. For this reason, a common background for soft tissue disorders including aortic rupture and megaesophagus was deemed not unlikely.

Indeed, clinically a high number of Friesians is suffering from megaesophagus (prevalence 2.2%), of which the majority is younger than 5 years (Chapter 4).

In the histological part of the study increased deposition of disorganized collagen and a decrease in elastin were found using semi-quantitative techniques. These changes were present in the dilated but also in the non-dilated parts of the esophagus and were similar to those found in the aortic wall of Friesians with aortic rupture and aortopulmonary fistulation. These findings provide indirect evidence for a possible underlying systemic connective tissue disorder. Another finding was the degeneration

of both smooth and striated muscle cells in the tunica muscularis of Friesians with megaesophagus. Involvement of both striated and smooth muscle in human esophageal disorders is rare and is associated with mixed connective tissue disease. This disorder is caused by autoantibodies directed to several receptors including receptors of fibrosis, thereby causing hypertrophy of collagen bundles.[1,23,30]

Connective tissue disorders in horses

Only a limited number of connective tissue disorders have been described in the horse. These include hereditary equine regional dermal asthenia (HERDA) or hyperelastosis cutis, which is histologically characterized by fragmentation and disorganization of collagen fibers.[8] The familial disease is caused by a mutation in the cyclophilin B gene, which is involved in the folding of collagen.[13]

Furthermore, one case was described with histological features similar to those in HERDA that additionally showed hemorrhage and inflammation within the skin. However, the symptoms in this case are probably caused by either inherited or spontaneous mutations in one of the collagen-producing genes and is known as Ehlers-Danlos syndrome.[35]

Another equine connective tissue disorder is degenerative suspensory ligament desmitis (DSLD), which primarily affects tendons and ligaments. This disease affects chiefly Peruvian Pasos and Peruvian Paso crosses and was shown to be a systemic disease.[20,21] DSLD is characterized by extreme accumulation of proteoglycans in organs with a high amount of connective tissues. Underlying findings are an abnormal form of decorin and changes in the processing of aggrecan.[9,16]

As shown in this work, Friesian horses have connective tissue abnormalities associated with aortic rupture and megaesophagus and a role for a hereditary genetic disease is suspected. In humans several genes have been identified in TAAD pathology, which are hence of potential interest for further research with respect to the pathology seen in Friesian horses.

All of these genes are involved in the metabolism of connective tissue and include genes involved in the TGF-β pathway, vascular smooth muscle and extracellular matrix components including fibrillin, collagen and glycoproteins.[6]

Conclusion

Aortic rupture is quite rare in Warmblood horses and tends to become manifest at older age. The rupture location is variable, but mainly involves the area located around or just caudal to the aortic valves. In Friesian horses, the rupture is usually diagnosed at a younger age and is located more caudally, near the ligamentum arteriosum. Morphological and biochemical assessment of the aortic wall showed abnormalities of several connective tissue elements. Similar morphological abnormalities were seen in the esophageal wall of young Friesians with megaesophagus. Together, these findings

are supportive of a systemic connective tissue disorder as underlying cause. Given the narrow genetic base of the Friesian breed and the significant differences found in extracellular matrix composition and metabolism between Friesians and warmbloods, a contribution of genetic factors to both Friesian disorders is likely and warrants detailed genetic research.

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Nederlandse samenvatting

Friese paard

Het Friese paard is vooral bekend om haar statige en sierlijke postuur. Onder de dierenartsen en vooral de paarden clinici staat dit ras helaas ook bekend om zijn vele afwijkingen en ziekten. Hieronder vallen de aortaruptuur en megaoesofagus, maar ook hydrocefalus (waterhoofd), dwerggroei en hyperflexibele pezen. In diverse paardenklinieken werden steeds vaker Friese paarden aangeboden met deze fataal aflopende ziekte (aortaruptuur), waarbij de diagnose door middel van sectie op het paard werd bevestigd. In de literatuur was slechts één artikel bekend met een beschrijving van een aortaruptuur in drie Friese paarden. Er werd een ras-specifieke ziekte van het hart en de bloedvaten verdacht. Door wetenschappelijk onderzoek kan er meer inzicht komen in deze ziekte en de oorzaak hiervan.

Aortaruptuur

Een van de afwijkingen die bij het Friese paard voorkomt is een scheur in de aorta, de belangrijkste lichaamsslagader, welke ontspringt vanuit de linker harthelft. Bij Warmbloed paarden is deze aandoening ook bekend, waarbij de oudere hengsten tijdens dekking een extreme hypertensie (hoge bloeddruk) ontwikkelen. Hierdoor scheurt deze grote slagader vlakbij de hartkleppen.
Andere afwijkingen die voorkomen in de wand van dit grote bloedvat zijn een dissectie, waarbij de wand zich splitst, een fistelvorming tussen de aorta en een ander bloedvat of het hart.

Aortaruptuur bij het Friese paard

In hoofdstuk 2 is onderzoek gedaan naar het herkennen van Friese paarden met deze ziekte. Daarbij zijn alle rapporten van het klinisch onderzoek en ook de verslagen van het postmortem onderzoek opnieuw bekeken en geëvalueerd. Hieruit is gebleken dat aortaruptuur veel vaker bij het Friese paard voorkomt dan bij andere paardenrassen en zich presenteert op relatief jonge leeftijd (ongeveer 4 jaar). Een mogelijke reden is dat er slechts een klein aantal Friese hengsten zijn en er door inteelt een grotere kans is op genetische defecten. De klinische symptomen die belangrijk zijn voor de dierenarts om deze ziekte in een zo vroeg mogelijk stadium te herkennen zijn beschreven en een therapie kan worden ingezet om het paard nog enkele maanden te stabiliseren.
Daarnaast is gebleken dat bij Friese paarden de aorta op een andere locatie scheurt, namelijk ter plaatse van het ligamentum arteriosum, daar waar de aorta in het veulen tijdens de dracht in verbinding staat met de longslagader. Andere bevindingen zijn de vorming van een circulair manchet van bloed rondom de aorta ter plaatse van de scheur en de vorming van een sterk verwijde aorta, welke frequent in verbinding staat met de longslagader via een opening in deze arterie (aorta-pulmonaire fistelvorming).

In hoofdstuk 3 is de wand van de aorta ter plaatse van de scheur microscopisch onderzocht bij 20 Friese paarden met een aortaruptuur. Hierbij zijn enkele karakteristieke afwijkingen gevonden zoals necrose (celdood) van het spierweefsel in de aortawand, afwijkende structuur van de eiwitten elastine en collageen en ophoping van mucoid materiaal. Deze afwijkingen verzwakken de aortawand en kunnen daardoor een scheur veroorzaken. De bevindingen in deze studie wijzen richting een onderliggend probleem in de steunweefsels, waarbij elastine en collageen een rol spelen. De aanwezige ontstekingsreactie is waarschijnlijk secundair aan de celdood in het middelste deel van de aorta. Daarnaast is het belangrijk dat er geen aanwijzingen zijn gevonden voor een infectieus agens als primaire oorzaak voor aortaruptuur bij Friese paarden.

In de literatuur wordt gesuggereerd dat er bij de Friese paarden mogelijk een systemisch weke delen probleem speelt. In hoofdstuk 4 wordt daarom een andere ziekte, namelijk megaoesofagus of verwijde slokdarm onderzocht op een manier zoals beschreven voor hoofdstuk 2. Het is vastgesteld dat dit verwijde slokdarm probleem ook vaker voorkomt bij Friese paarden dan bij warmbloed paarden.
Daarnaast zijn door microscopisch onderzoek van de wand van de slokdarm opnieuw afwijkingen gezien van het collageen, zoals ook beschreven bij de aorta's. Dit collageen is een van de belangrijkste componenten van steunweefsels in het lichaam. Deze bevinding ondersteunt het idee van een onderliggend systemische afwijking van de steunweefsels, wat de oorzaak zou kunnen zijn van meerdere ziekten die voorkomen bij het Friese paard. Het is bekend dat afwijkingen in de steunweefsels, waaronder het component collageen geassocieerd zijn met ziekten van de aorta in mensen.

De bovenstaande observaties hebben geleid tot de volgende onderzoeksstap, waarbij verschillende voornamelijk eiwitrijke onderdelen van de aorta, met name collageen, biochemisch zijn onderzocht. Hierbij is de matrix tussen de cellen in de aortawand op verschillende locaties (ter plaatse van de scheur en verderop) onderzocht en vergeleken met de aorta van warmbloed paarden. Daarnaast zijn de analyses ook toegepast op een stukje peesweefsel om te onderzoeken of er sprake is van een systemische afwijking in een van deze extracellulaire matrix componenten.

Specifieke eiwitten, matrix metalloproteinases, vertonen een toegenomen activiteit ter plaatse van de scheur in de aortawand. Op dezelfde locatie is een toename van het aantal verbindingen in het netwerk van het eiwit elastine gevonden. Beide bevindingen zijn waarschijnlijk onderdeel van het herstelproces secundair aan de scheur in de aorta.
Daarnaast is gebleken dat Friese paarden biochemisch verschillen van warmbloed paarden in de hoeveelheid glycosaminoglycanen in de aorta en peesweefsel en

verschillen in de vorming van collageen. Deze bevindingen suggereren verschil in het metabolisme van de steunweefsels tussen de Friese paarden en warmbloed paarden. aanvullend kunnen verschillen of afwijkingen in de extracellulaire matrix effect hebben op de stijfheid en sterkte van de aortawand.

In conclusie.
Aortarupturen bij Friese paarden presenteert zich op een andere locatie dan bij warmbloed paarden. Er zijn abnormaliteiten in de morfologie van de aorta van Friese paarden gevonden, waaronder afwijkend collageen. Dit afwijkende collageen is ook gevonden in de slokdarm van Friese paarden met een megaoesofagus wat suggestief is voor een systemisch probleem in de steunweefsels van Friese paarden. Biochemisch onderzoek van de aorta ondersteunt dit idee en mogelijk zijn de ziekten te verklaren door een genetische afwijking in een van de onderzochte componenten, bijvoorbeeld het collageen. Om dit te bevestigen is in de toekomst verder onderzoek nodig.

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Curriculum Vitae

Margreet Ploeg werd geboren op 23 maart 1979 te Ede. In 1996 behaalde zij het Havo diploma aan het Johannes Fontanus College te Barneveld, waarna zij in datzelfde jaar met de studie Hogere Laboratorium Opleiding te Utrecht begon. Margreet heeft deze studie afgerond met een jaar onderzoeksstage in het Children's Hospital Oakland Research Institute te Californië. Hierbij deed zij onderzoek naar "the role of protein kinase C in the mechanism of phosphatidylserine exposure in erythrocytes" bij patiënten met sikkelcel anemie . Zij heeft deze studie in 2000 afgerond met de afstudeerrichting Biochemie. Vervolgens heeft zij ruim een jaar ervaring opgedaan als onderzoeks- en diagnostisch analist in het Neurozintuigen laboratorium van het Amsterdam Medisch Centrum te Amsterdam. In september 2001 startte Margreet met de opleiding Diergeneeskunde aan de Universiteit te Utrecht. Als een van de eersten van het curriculum studeerde zij in november 2007 af met de differentiatie Gezelschapsdieren. Na als practicus te hebben gewerkt in Sterkliniek dierenartsen Hillegom en Dierenkliniek Sleeuwijk begon Margreet in Februari 2009 met de specialisatie Veterinaire Pathologie (faculteit Diergeneeskunde) te Utrecht. In 2011 startte zij tevens haar promotieonderzoek naar "Aortarupturen bij Friese paarden" onder begeleiding van met name dr. Catherine Delesalle en prof. dr. Andrea Gröne. In februari 2014 behaalde Margreet het Europese specialisten examen in de Veterinaire Pathologie, waarna zij haar werkzaamheden als patholoog kon voortzetten bij de afdeling Pathobiologie.

Scientific Publications

1. Disseminated toxoplasmosis in black-footed penguins (Spheniscus demersus). Ploeg M, Ultee T, Kik M. Avian Dis. 2011;55:701-703.
2. Osteochondral dysplasia of the coxofemoral joints in a Friesian foal: clinical findings and methods of diagnosis. Hermans H, Veraa S, Ploeg M, Boerma S, Hazewinkel H, Back W. Equine Vet Educ. 2014; DOI: 10.1111/eve.12210.
3. Aortic rupture and aorto-pulmonary fistulation in the Friesian horse: characterisation of the clinical and gross post mortem findings in 24 cases. Ploeg M, Saey V, de Bruijn CM, Gröne A, Chiers K, van Loon G, Ducatelle R, van Weeren PR, Back W, Delesalle C. Equine Vet J. 2013;45:101-6.
4. Axial osteitis of the proximal sesamoid bones and desmitis of the intersesamoidean ligament in the hindlimb of Friesian horses: review of 12 cases (2002-2012) and post-mortem analysis of the bone-ligament interface.
5. Brommer H, Voermans M, Veraa S, van den Belt AJ, van der Toorn A, Ploeg M, Gröne A, Back W. 2014;10:272.
6. Thoracic Aortic Rupture and Aortopulmonary Fistulation in the Friesian Horse: Histomorphologic Characterization. Ploeg M, Saey V, Delesalle C, Gröne A, Ducatelle R, de Bruijn M, Back W, van Weeren PR, van Loon G, Chiers K. Vet Pathol. 2014;52:152-159.
7. Esophageal Dysfunction in Friesian Horses: Morphological Features. Ploeg M, Gröne A, Saey V, de Bruijn CM, Back W, van Weeren PR, Scheideman W, Picavet T, Ducro BJ, Wijnberg I, Delesalle C**.** Vet Pathol. 2014; pii: 0300985814556780.

Contributions to Conferences

1. Hermans H, Belt AJM van den, Ploeg M, Boerma S, Hazewinkel HAW, Brommer H, Back W. Bilateral hip dysplasia in foals: another disease necessitating further genetic workup of the Friesian horse population? BEVA 2010.
2. Aorto-pulmonary fistulation in the Friesian horse: clinical characterization of 31 cases combined with histopathological features. Lifting a tip of the veil. Ploeg M, Gröne A, Saey V, Chiers K, van Loon G, de Bruijn MC, Ducatelle R, van Weeren PR, Back W, Delesalle CJG. Voorjaarsdagen 2011.
   Winner **BEVA award** for best performance in Equine clinical research session.
3. Aortic rupture and aortopulmonary fistulation: increased prevalence in Friesian horses and importance of early ante-mortem diagnosis. Van Loon G, De Clercq D, de Bruijn M, Decloedt A, Verheyen T, Saey V, Ducatelle R, Chiers K, Ploeg M, Back W. 39ièmes Journées annuelles de l'Association Vétérinaire Equine Française 2011.
4. Needle electromyography of the esophagus in Friesian horses with and without esophageal dysfunction. Van der Kolk, Ploeg M, Back W, Bruijn CM, Wijnberg ID. ACVIM 2011.
5. Use of transoesophageal ultrasound to visualize the aortopulmonary region in two normal Friesian horses and 3 Friesians with aortic rupture or aortopulmonary fistulation. De Bruijn CM, van Loon G, Ploeg M, Gröne A, De Clercq D, Decloedt A, van Weeren PR, Back W, Delesalle C. BEVA 2013.
6. Aortic media ultrastructure in a healthy Friesian horse and in a Friesian horse with aortopulmonary fistula. Saey V, D'Herde K**,** Ploeg M, Chiers K, Delesalle CJG, Gröne A, Back W, de Bruijn CM, van Loon G, Ducatelle R. ESVP/ECVP congress September 2013.
7. Morphological characterization of megaesophagus in Friesian horses. Ploeg M, Gröne A, Saey V, de Bruijn CM, Back W, van Weeren PR, Delesalle C. ECVP/ESTP congress 2014.
8. Differences in collagen type distribution in ruptured aortas of Friesian horses, vs aortas of healthy Friesian and Warmblood horses. Ploeg M, Gröne A, Duchateau L, Saey V, Chiers K, van Weeren PR, Back W, van Loon G, de Bruijn CM, Delesalle CJG. Voorjaarsdagen 2015.