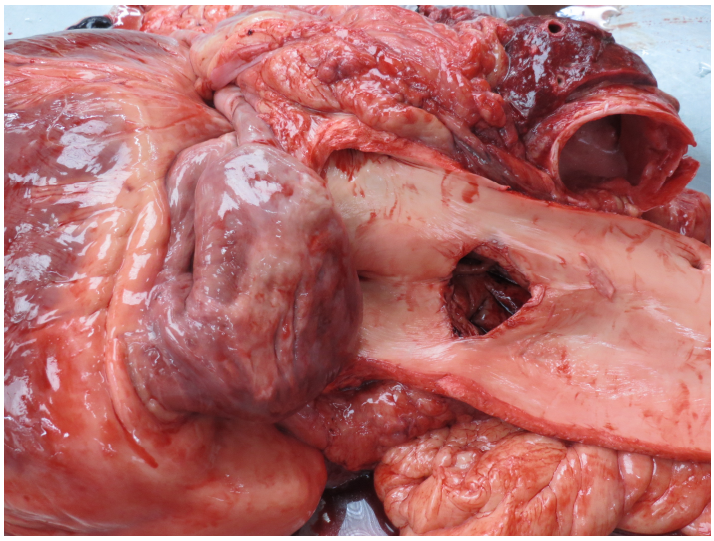

Aortic ruptures in the Friesian horse

The role of collagen type I and III

Student:	Sandra Havik
Student number:	0153044
Department:	Veterinary Pathology
Start research:	15-7-2013
Completion research:	04-10-2013
Supervisor:	Margreet Ploeg
Examinor:	Rachel Thomas

Index

Summary

This study explores the possibility that collagen type I and/or III plays a role in the development of aortic ruptures in the Friesian horse. A total of 70 horses were used, across three groups; Affected Friesians, Non-Affected Friesians and a control group of Warmbloods. Two sets of slides were stained with immunohistochemistry, one for collagen type I and the other for collagen type III, and examined for collagen amount and fragmentation. The results show a significant increase of the amount of collagen type I, but not type III, for Affected Friesians, which suggests that only collagen type I amount plays a role in the development of aortic ruptures. However due to differences in storage time of the slides, the results may not be entirely accurate. The results of fragmentation are surprising, as it shows no significant differences for collagen type I, but does show an increase of fragmentation in the control group of warmbloods. This may be attributed to the fact that the control group is, on average, older then the two Friesian groups.

Introduction

Recently aortic rupture in horses made headline news after the death of the stallion Hickstead, who was an Olympic champion in show jumping together with his rider Lamaze. Aortic ruptures at the level of the aortic root are a rare occurrence in the horse. However in Friesian horses, although still rare, ruptures are reported, and typically occur, in the more distal aorta, specifically at the level of the ligamentum arteriosum, where they are often accompanied by an aorto-pulmonary fistula. Previous studies have shown that the extra cellular matrix (ECM) component collagen may be involved in the development of these aortic ruptures. This study will look more closely at the different collagen types that compose the ECM and how they may contribute to aortic rupture in the Friesian horse.

Anatomy of the Aortic Wall

Figure 1 shows an overview of the three layers of the aortic wall.

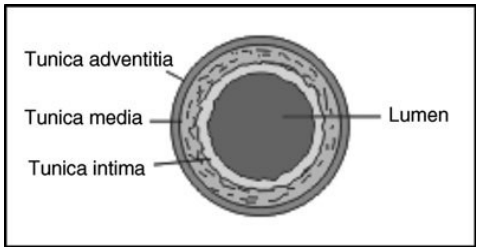

Figure 1: Layers of the Aortic wall; The *tunica intima* or *interna* (innermost layer), the *tunica media* (middle layer) and the *tunica adventitia* (outermost layer).[1]

The ***tunica intima*** consists of a layer of endothelial cells resting on a basement membrane supported by a subendothelial layer of connective tissue. It is directly in contact with the lumen of the aorta and its main function is to act as a barrier.

The ***tunica media*** is the thickest layer of the three and its main functions are structural support, vasoreactivity and elasticity. It is composed of laminar units (figure 2), which consists of two elastic lamellae encasing vascular smooth muscle cells and extracellular matrix (ECM) components. As well as elastin, the ECM between the elastic lamellae is mostly composed of collagen fibrils. The collagen fibrils form organized fibers, which give the aorta its tensile strength. The smooth muscle cells are supported by layers of collagenous microfibrils.[2]

There are several types of collagen fibers throughout the body. The function of the major types in the body can be found in table 1. In the aortic media the predominant types are I and III.[2, 5, 6]

The ***tunica adventitia*** has a supportive function. It consists of vascularized, loosely arranged collagenous connective tissue. The vascular component (small arteries and veins), also called the vasa vasorum, supplies blood to the aortic wall and also penetrates the outer half of the tunica media.[7]

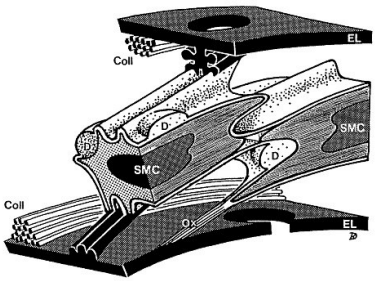

Figure 2: Scematic picture of a laminar unit in the *tunica media.*[2]

Aortic diseases

The three most common pathological diseases that involve the aortic wall and are associated with aortic rupture are presented below.

Aortic Rupture

The condition in which the aorta is torn or ruptured across the entire thickness is called an aortic rupture. The aorta is the main artery that brings blood from the heart to the rest of the body, and blood pressures within the aorta can be very high. As a result, if this large artery ruptures blood may be pumped out of the aorta at a high rate,[1] making aortic rupture a quickly fatal disease due to the extensive internal bleeding.

Aortic Aneurysm

An aortic aneurysm is the widening of the aorta, affecting all three wall layers. Currently the term aneurysm is used when the diameter is 50-150% greater then is to be expected based on age, gender and species.[1, 8, 9]

Type	Description	Function	Location
I	Most abundant collagen, forms thick wavy fibers.	Tensile strength	Bone, dermis, tendon, ligaments, cornea
II	Loosely woven network of collagen fibers	Resistance to intermittent pressure	Cartilage
III	Reticular fibers, made first in granulation tissue before type I	Maintaining structure in loosely shaped organs	Skin, vessel wall, reticular fibres of most tissues (lungs, liver, spleen, etc.)
IV	Doesn't form fibers but thin amorphous membranes	Support, binding site, filtration	Basement membrane
V	Closely associated with and forms fibers with type I	Associated with Type I collagen	Lung, cornea, bone, foetal membranes
VI	Forms microfibrillar fibers	Possible anchoring point for other networks and cells	Widespread: Dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc

Table 1: Major types of collagen in the body.[3, 4]

Human medicine classifies an aortic aneurysm based on the location in the body. The Abdominal Aortic Aneurysm (AAA) is the most common form in humans, accounting for about 75% of the cases whilst the Thoracic Aortic Aneurysm (TAA) accounts for about 25% of the cases.[1]

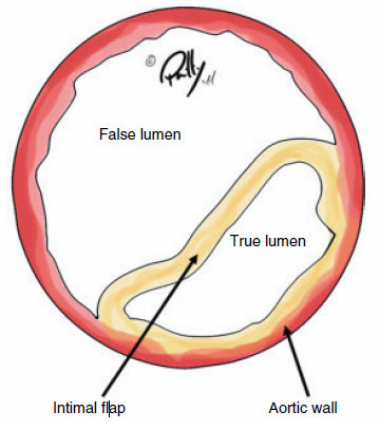

Figure 3: The two lumens of an aortic dissection.[14]

In equine medicine an aortic aneurysm is rare and if it occurs it is usually located in the aortic root or ascending thoracic aorta.[10-13] There are no known cases of abdominal aneurysms in horses.

Aortic Dissection

Dissection of the aortic wall occurs when blood is allowed to flow in between the layers of the wall. Typically the blood flows through a defect in the inner lining of the aorta. Damage of the aortic wall is caused by the high blood pressure and this typically occurs at the proximal or distal aortic arch in humans.[14, 15] Due to the high blood pressures that exist in the aorta, the blood forces the layers apart creating a second, false lumen of unpredictable length.[14-16] The true lumen is still surrounded by the inner wall, but is often compressed by the false lumen (see figure 3). Blood flow in the false lumen is almost always ante grade, but can also be retrograde.[14, 15]

The most commonly used classification of this lesion is the Stanford system that distinguishes the dissections based on the location of the false lumen, and whether it involves the ascending aorta (type A) or not (type B).[14, 15]

Equine Aortic rupture

As mentioned previously, in horses aortic rupture typically occurs just distal to the aortic valve, in a location that is known as the aortic root. Aortic root disease is described as an aneurysm of the sinus of Valsalva or a tear in the aortic root.[13, 17, 18] It has been reported to occur in breeding stallions during, or shortly after, coitus[18] and is an uncommon finding in race horses.[19, 20] When the aortic root ruptures, it typically does so into the right ventricle, or less frequently into the right atrium, forming aorto-cardiac fistulas, leading to congestive heart failure.[10, 13]

When the rupture does not involve the heart it may cause an acute haemorrhage into the pericardial sac, causing cardiac tamponade.[17]

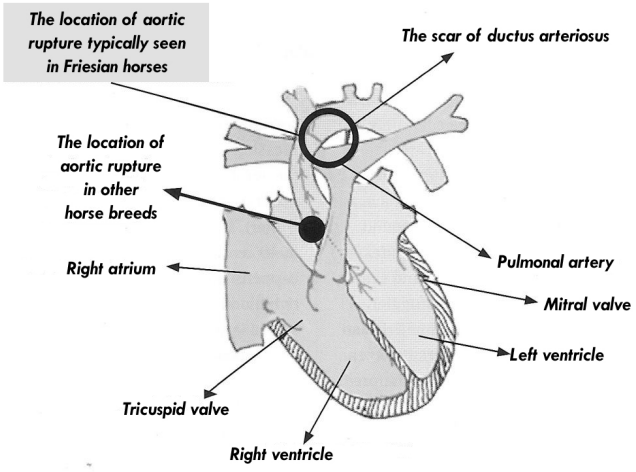

Figure 4: Diagram of aortic rupture in horses.[21]

Horses can survive the acute stage of rupture if the site of the rupture does not lead to exsanguination or a fatal arrhythmia.[10, 18]

A more distal location (see figure 4) has been reported by van der Linde-Sipman *et.al.*[11] in 3 Friesian horses. This location is in close proximity to the ligamentum arteriosum at the level of the aortic arch. Other researchers have since then reported similar findings, and a recent study done by Ploeg *et.al.*[17] characterized the phenotypical appearance of this aortic rupture.
On post mortem examination a circumferential cuff of blood was seen around the aorta in one third of the cases. These periaortic haemorrhages were initially regarded as dissecting aneurysms, but were later shown to be formed by leakage of blood out of the rupture site into the connective tissue surrounding the aorta. This feature was also reported by van der Linde-Sipman *et.al.*[11]
More than 50% of the horses also had aorto-pulmonary fistulas, where the route of fistulation was described as a connection between the aorta and pulmonary artery with multiple pockets. Other notable findings were pleural effusion, with or without haemothorax, pulmonary oedema, and peripheral oedema. Congestion of the liver was also reported in over a third of the cases, demonstrating this disease to be a chronic process. Where some Friesian horses developed an acute tear with haemothorax and died within minutes, others formed a tear and/or aorto-pulmonary fistulation with a cuff of haemorrhage around the aorta and/or pulmonary artery stabilizing the condition for several days to weeks. A further group of horses formed a stable aorto-pulmonary fistula that may have lead to right-sided heart failure after weeks to months. Preliminary histological findings in these Friesian horses demonstrate an abnormal collagen deposition in about half of the chronically affected group; specifically, an increase in collagen content. (Data not yet published)

Comparing to Human Medicine

In contrast to horses, in humans a non-traumatic aortic rupture is usually secondary to either an aneurysm or a dissection.[22] The spontaneous aortic rupture without prior formation of aneurysm or dissection, as seen in horses, is rare[23] and atherosclerotic plaques are typically found at the site of rupture, implicating them in the pathogenesis. Atherosclerotic disease is not recognized in horses, however research in racehorses has demonstrated the presence of arterial calcifications in the pulmonary artery[24] as well as in the aorta[25] to some degree. Such lesions were not reported in the Friesian horses examined in the previous study.[17]
Although there is rarely evidence of aneurysm or dissection at the site of equine aortic ruptures, study of the mechanisms that are behind the rupture of a dissection or aneurysm in humans may give some insight into the pathology of the aortic wall in horses.

Risk factors

In humans the exact mechanisms behind aortic dissection and aneurysm forming are still unknown; however several risk factors have been found to play a role. They can be divided into categories: patient signalment, social history, medical history, status of the cardiovascular system and heritable disorders.

Patient signalment:

Age: Generally the condition is not seen in people younger than 50.[8, 8, 9, 26] In horses it is unknown if age plays a role.

Gender: Aortic aneurysms and dissections seem to be more common in human males.[1, 8, 9, 27] Some studies suggest that aortic ruptures are more common in stallions,[10, 18] but others describe a higher prevalence in mares.[11, 17]

Social history:

Smoking: Even though several studies have shown that smoking increases the risk of developing an aneurysm[8, 28] in humans, this is unlikely to be a predisposing factor in the equine disease.

Medical history:

Previous aortic pathology: Patients with thoracic aneurysms, repaired or not, have a higher risk of aortic dissection[15]. Only a few cases of ruptured dissecting aneurysms have been reported in horses[12].

Status of the cardiovascular system:

Hypertension: While maybe not a direct cause of an aneurysm or dissection, hypertension could increase the risk of rupture amongst patients with pre-existing aneurysm.[9, 14, 29] In horses, blood pressure is not frequently measured, but increased heart rate and peripheral oedema have been seen in horses with aortic ruptures.[10, 13, 17, 18]

Atherosclerosis: The destruction of the aortic wall caused by atherosclerosis can cause weakening of the wall possibly resulting in an aneurysm.[8, 9, 28] Atherosclerotic intimal destruction may also cause dissection.[14] Atherosclerosis is not recognized in horses and does not seem to be linked to aortic ruptures.[17]

Heritable disorders:

Family members: While the genetic mechanisms involved in the process are unknown, studies have shown that first degree family members of a person with a diagnosed aneurysm or dissection have a higher life-time risk of developing an aneurysm or dissection themselves.[8, 27, 30] Three of the Friesian horses reported in a previous study[11] had the same sire, so heritability can not be excluded, however, to date, no published studies have investigated this aspect of the disease.

Predisposing heritable disorders: These include, but may not be limited to, Marfan Syndrome,[31-33] Vascular Ehler-Danlos syndrome (type 4),[33, 34] and Bicuspid Aortic Valve.[33] The first two are connective tissue disorders which are discussed later and the latter is a congenital defect which has not yet been reported in horses.

Current Theories

The focus of this study is the role of collagen in the pathogenesis of aortic ruptures in the Friesian horse. The aortic wall consists of several layers and currently studies show that the *tunica media* plays a major, if not the main, role in the stability of the aorta.[2, 35, 36] In the *tunica media*, elastin is most commonly responsible for the elastic properties of the aorta and collagen fibers generally ensure that the aorta is not overstretched.[5, 37]

Histological examination of non-aneurysmal ruptured aortas in the Friesian horses[11] showed clear signs of necrosis and fibrosis of the *tunica media* and, in all but one case, signs of inflammation. This was not only found near the site of the rupture, but across the entire

thoracic aorta. Destruction of the elastin and collagen fibers within the aortic wall could thus lead to instability and subsequent rupture.
However, in human medicine several studies have been done on the histological profile of both ruptured aneurysms and intact aneurysms. In most cases it was found that the overall collagen protein content was higher than in the control group, while the overall elastin protein content was lowered compared to the control, as expected.[38-40] Other studies show the same decrease in elastin proteins, but did not find a change in collagen fibers.[41, 42] More detailed studies found that while the relative protein content of collagen type I and III was less then in the control group, the content of collagen type V and XI was increased. Overall content of collagen is not reported.[6] It is uncertain as to whether altered collagen content in diseased compared to non-diseased aortas precedes or follows disease. For example, increased collagen synthesis may be secondary to changes in the haemodynamics or due to inflammatory processes.[43]

Despite these possibilities, the role of collagen in the development of an aortic rupture cannot be excluded. The previously mentioned connective tissue disorders have long been associated with an increased risk of rupture in the vascular system. Marfan syndrome has been related to elastin malformation, but also collagen defects,[37] where Ehler-Danlos syndrome type 4 involves a mutation that leads to abnormal collagen type III synthesis. A study of patients with both Marfan syndrome and AAA showed that aneurysms may be associated with defects of the microarchitecture of collagen fibers instead of only abnormalities in the amount of collagen.[37] Similarly, it is has been shown that collagen type III is necessary for proper fribrillogenesis of collagen type I,[44] which explains why Ehler-Danlos syndrome is associated with the risk of vascular rupture. None of the above described syndromes are recognized in the Friesian horse, but a currently unrecognized heritable disorder can not be excluded based on the results of previous studies.

This study

In this study we will explore the possibility of a collagen imbalance in the Friesian horse, which may lead to aortic rupture near the ligamentum arteriosum. In this study, aortas of affected Friesian horses are compared to aortas of non-affected Friesians and non-Friesians by means of immunohistological staining. Both fragmentation and amount of collagen will be scored in a semi-quantitative way.
The first hypothesis is that there will be a difference in amount of collagen type I and III in affected Friesian horses compared to non-affected Friesian horses and non-Friesian horses. This is based on the assumption that collagen plays a major role in the stability of the aorta, and changes in the concentration or amount of collagen would lead to either fragility (decrease)[6] or stiffness (increase)[38-40]. Both changes could lead to increased risk of ruptures in the aorta. The second hypothesis is that there will be more fragmentation of collagen types I and III in affected Friesian horses compared to non-affected Friesian horses and non-Friesian horses. This is based on the findings in humans[37], which show that the microarchitecture of collagen is dramatically altered in AAA and Marfan syndrome patients and based on the effects that the necrosis and inflammation seen in previous Friesian horses[11] may have on the collagen fibers.

Material and Methods

Animals

All animals were presented for post-mortem examination to the pathology department of either the University of Utrecht in the Netherlands or the University of Gent in Belgium. Only horses that died less than 24 hours before examination were used in the study. A total of 70 horses were categorized into one of three study groups: Friesian horses with an aortic rupture confirmed or examined during the post-mortem examination (Affected Friesians, or AF), Friesian horses without an aortic rupture (Non-Affected Friesians, or NAF), and a control group of warmbloods without aortic rupture (WB). An overview can be seen in appendix A.

Immunohistochemistry

During the post-mortem examination the aorta from the heart base to the diaphragm was sectioned transversely into five equal parts and samples were taken from of each part and subsequently formalin fixed. Paraffin embedded sections of the third part were cut into 3µm slices and fixed on a silan-coated microscope slide and dried over night at 55 ºC and stored at room temperature until stained. The slides were grouped in six batches for each collagen type, for a total of 12 batches. Each batch contained 10-13 testing slides plus a positive and negative control slide of the tissue on which the protocol was optimized. The positive control was included to show that the staining succeeded for that batch. The negative control was used to show any background staining during that batch. All staining was performed by one individual over the course of three weeks.

Staining protocol: Slides were first deparaffinized in xylene (2 x 5 minutes) and rehydrated in graded alcohol 100%, 96%, 70% and aquadest, each 2 x 3 minutes. For antigen retrieval 800ml of 10 mM citrate buffer pH 6.0 was preheated in an 1100 Watt microwave at 100% for 10 minutes, after which the slides were treated in the citrate for 15 minutes at 70% in the microwave. The slides were set to cool at room temperature for 30 minutes.

Slides were subsequently rinsed in phosphate buffered saline (PBS) for 3 x 5 minutes. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 30 minutes, followed by another rinse in PBS/tween for 3 x 5 minutes.

Non-specific binding was blocked by treatment with Normal Horse Serum at 1:10 solution with PBS for 20 minutes. Primary antigen (SIGMA C2456-2ml collagen type I or ABCAM 6310 collagen type III) was then applied at 1:200 in PBS and incubated overnight at 4 ºC. The negative control received PBS instead of the primary antigen.

After rinsing with PBS/tween for 3 x 5 minutes the slides were incubated with the second antibody, biotinylated horse anti-mouse (VECTOR LABS BA-2000) for 30 minutes. The ABC/PO complex solution elite (VECTOR LABS, PK-6100) was prepared at least 30 minutes in advanced, and after rinsing the slides in PBS/tween for 3 x 5 minutes they were incubated for 30 minutes. The slides were rinsed again in PBS followed by visualization with freshly made 3,3'-Diaminobenzidine (DAB) solution in 0,05 mM Tris/HCl (pH 7.8) for 30 minutes. Slides were rinsed for 5 minutes in running water and then counterstained in

haematoxylin for 30 seconds. After rinsing in running water for 10 minutes the slides were dehydrated in graded alcohol (70%, 96%, 100%, each 2x3 minutes) and xylene (2 x 5 minutes). Slides were then sealed with Eukitt and covered.

Method of Scoring

All scoring of the longitudinal section of the aorta was done by a single pathologist over two sessions, one for collagen type I and one for collagen type III, to prevent inter- and intra-observer variance. All scoring was carried out with a light microscope to visualize the tunica media at a magnification of 200x. Fragmentation was scored by using a scale of 0 for no fragmentation to 3 for severe fragmentation. Amount of collagen was scored by determining the % of area covered in a single view. This grading system uses a scale from 0 for 0-20%, 1 for 20-45%, 2 for 45-75% and 3 for 75-100%. Photos were made of representatives for the categories, except for grade 3 of amount of collagen as there was no representative. (figure 5)

Statistics

SPSS was used for statistical analysis. To compare both fragmentation and amount between the three different groups, six independent t-tests were performed. For both types of collagen (type I and III) the fragmentation and amount is compared in the first two tests between WB and NAF; the second two tests NAF with AF; and the last 2 tests WB with AF. Significance was assumed for p less than 0.05. As our scoring take the shape of a likert-scale, these tests may indicate intergroup differences, but does not quantify these differences.

Furthermore SPSS was used to perform descriptive analyses on the three groups for both age and gender and to create crosstabs to show the staining results for each group as a percentage within the group.

Results

Descriptive analysis

Mean age and frequencies of genders across the groups are shown in table 2. The mean ages of the different groups differ significantly. ($p = 0.05$ for WB vs NAF, $p < 0.01$ for WB vs AF and $p = 0.019$ for NAF vs AF) The distribution of gender did not differ across the groups. ($\chi^2(6, N = 70) = 5.65$, $p = 0.463$)

Collagen type I

An overview of the results for fragmentation and amount of collagen type I can be seen in table 3 and table 4, respectively. Two slides in the AF group did not stain properly and it was not possible to score them. These were not included in the total.

No significant difference in fragmentation of collagen type I between the groups was determined. (WB vs NAF, $p = 0.543$; WB vs AF, $p = 0.932$; NAF vs AF, $p = 0.482$)

However, the amount of collagen type I within the AF group is significantly more in comparison to the amount within the other two groups. (WB vs NAF, $p = 0.95$; WB vs AF, $p = 0.016$; NAF vs AF, $p = 0.005$)

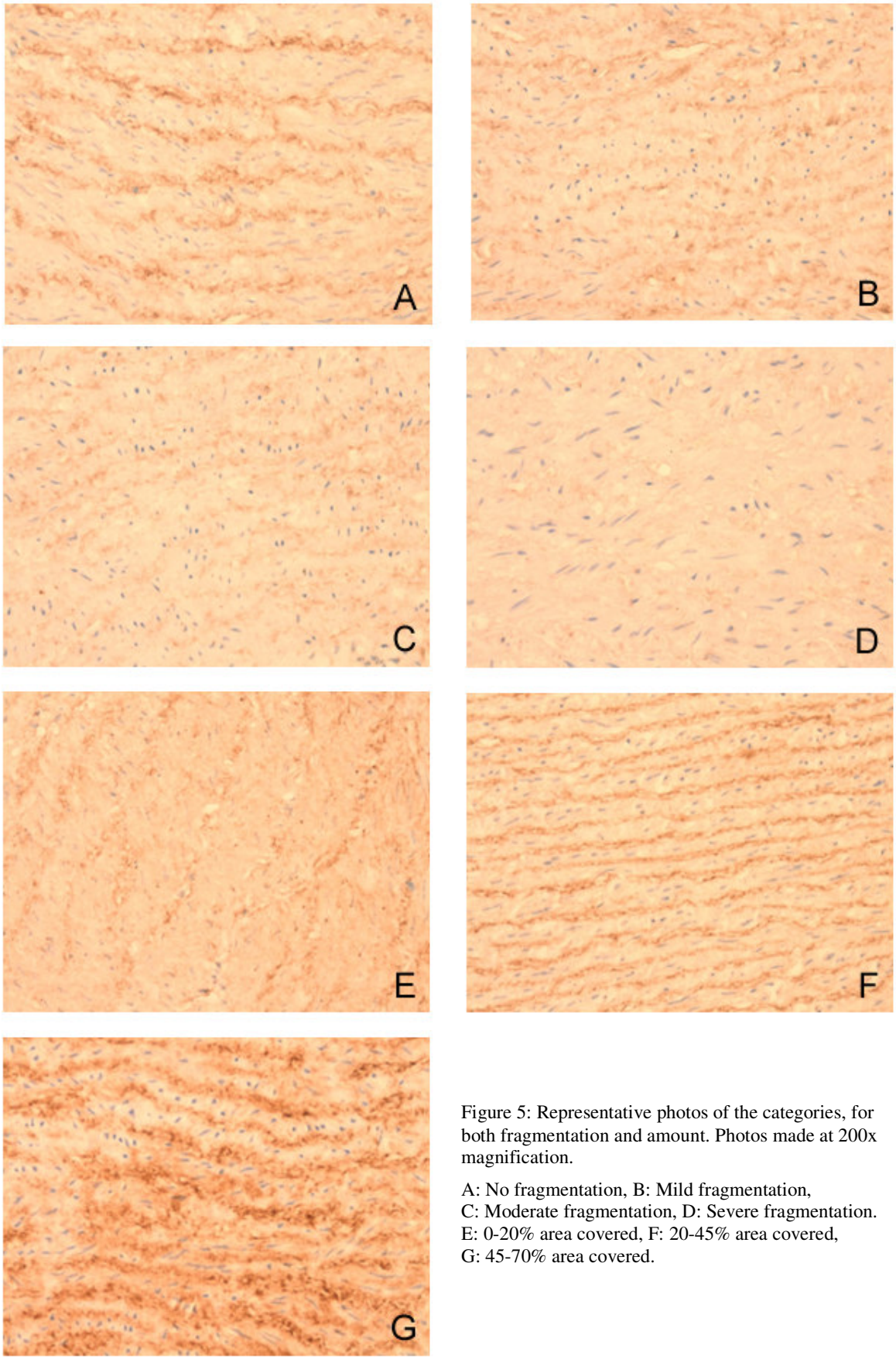

Figure 5: Representative photos of the categories, for both fragmentation and amount. Photos made at 200x magnification.

A: No fragmentation, B: Mild fragmentation,
C: Moderate fragmentation, D: Severe fragmentation.
E: 0-20% area covered, F: 20-45% area covered,
G: 45-70% area covered.

Collagen type III

An overview of the results for fragmentation and amount of collagen type III can be seen in table 5 and table 6 respectively. Six slides did not stain properly and it was not possible to score them. Three were part of the WB group, two were in the NAF group, and one was in the AF group. These were not included in the group totals.

		Mean Age	Gender				Total
			Stallion	Gelding	Mare	Unknown	
Group	Warmblood	12,3	2	3	15	0	20
	Non-Affected Friesian	8,4	6	5	17	2	30
	Affected Friesian	5,4	1	6	12	1	20
Total		8,7	9	14	44	3	70

Table 2: Frequencies of gender across the different groups and average age.

Collagen type I		Fragmentation				Total
		None	Mild	Moderate	Severe	
Group	Warmblood	43,8%	26,2%	23,8%	6,2%	100%
	Non-Affected Friesian	45,0%	21,7%	20,0%	13,3%	100%
	Affected Friesian	44,4%	29,2%	22,2%	4,2%	100%
Total		44,5%	25,0%	21,7%	8,8%	100%

Table 3: Fragmentation of collagen type I scores as a percentage within each group.

Collagen type I		Amount			Total
		0-20%	20-45%	45-75%	
Group	Warmblood	18,8%	76,2%	5,0%	100%
	Non-Affected Friesian	17,5%	78,3%	4,2%	100%
	Affected Friesian*	2,8%	93,1%	4,2%	100%
Total		14,0%	81,6%	4,4%	100%

Table 4: Amount of collagen type I scores as a percentage within the each group.
* Significantly different in comparison to the other groups ($p < 0.05$)

Collagen type III		Fragmentation				Total
		None	Mild	Moderate	Severe	
Group	Warmblood*	16,2%	39,7%	38,2%	5,9%	100,0%
	Non-Affected Friesian	29,5%	50,9%	19,6%	0,0%	100,0%
	Affected Friesian	32,9%	35,5%	28,9%	2,6%	100,0%
Total		27,0%	43,4%	27,3%	2,3%	100,0%

Table 5: Fragmentation of collagen type III scores as a percentage within the each group
* Significantly different in comparison to the other groups ($p < 0.05$)

Collagen type III		Amount			Total
		0-20%	20-45%	45-75%	
Group	Warmblood	7,4%	83,8%	8,8%	100,0%
	Non-Affected Friesian	8,9%	81,2%	9,8%	100,0%
	Affected Friesian	14,5%	75,0%	10,5%	100,0%
Total		10,2%	80,1%	9,8%	100,0%

Table 6: Amount of collagen type III scores as a percentage within the each group.

The results show significantly more fragmentation of collagen type III in the WB group when compared to the other groups. (WB vs NAF, $p < 0.01$; WB vs AF, $p = 0.022$; NA vs AF, $p = 0.348$)
There was no significant difference in amount of collagen type III between the groups. (WB vs NAF, $p = 0.929$; WB vs AF, $p = 0.480$; NAF vs AF, $p = 0.483$)

Discussion

This study aimed to investigate the role of collagen type I and III in the development of aortic rupture in the Friesian horse. These are the main collagen types inside the aorto and play a crucial role in the stability and flexibility of the aortic wall.

Amount results

The first hypothesis that was made during the setup of this study stated that the AF Friesians would have a different amount of collagen compared to the other two groups. This was based on the assumption that collagen plays a major role in the stability of the aorta, and that changes in the concentration or amount of collagen would lead to either fragility (decrease)[6] or stiffness (increase)[38-40]. Both changes could lead to increased risk of ruptures in the aorta.
The results of this study do not seem to support this hypothesis completely as no significant difference was found in the amount of collagen type III between the groups (table 6). This significance was found in collagen type I as the amount of collagen in the AF group was greater when compared to the other two groups (table 4). This suggests that collagen type I may indeed play a role in the development of aortic ruptures in the Friesian horse.

Fragmentation results

At the start of this study it was hypothesized that the AF group would have more fragmentation of both collagen type I and III in comparison to the other groups. This was based on: 1) previous studies done in humans[37], where it was found that the microarchitecture of collagen was dramatically altered; and, 2) the possible effects on the collagen fibers due to the necrosis and inflammation seen previously in Friesian horses with aortic rupture.[11] The results of this study do not support this theory. In fact, our results actually show that there was more fragmentation of collagen type III in the WB control group than in both Friesian groups (table 5). It is possible that this unexpected result may reflect the study design's limitations.

Limitations

Slide age

Due to the collaboration with the University of Gent, some of the slides were cut several weeks before the start of this study. This means they were stored up to 12 weeks before staining. According to several studies[45-51] storage time of unstained paraffin slides may have an impact on the reactivity of the antigens to the staining protocol. Not all antibodies are as sensitive as others, and furthermore, there seems to be a correlation with storage temperature: decrease of reactivity when slides are stored at warmer temperatures,[48-51] and the light

intensity during storage: decreased reactivity when slides are stored in high light intensity.[51] It has also been suggested that not all antigen/antibody combinations are affected.[47, 51]
According to some studies this decrease in immunoreactivity can be countered by a different antigen retrieval protocol[48] or increased antibody concentration[47], but can not be countered in another study[45]. The mechanism for this loss of antigen-reactivity is not yet known, though there are several hypotheses. Firstly, that over time there is more masking of the antigens, due to atmospheric influences. The exposure of the slides to air could lead to oxidation of antigens or epitopes, but methods to protect the slides from exposure does not seem to influence reactivity.[48, 49] Secondly, it has been hypothesized that the change in reactivity is not due to antigen loss but to antigen alteration. This may be supported by studies[45, 51] showing an increase in reactivity for some antigen/antibody combinations after a long storage time, instead of a decrease. A recent study[50] on the effects of endogenous and exogenous water influence showed that improper fixation of tissue before embedding in paraffin or improper paraffin imbedding could result in endogenous water inside the tissue, which increases the rate at which the antigens degrade. Aside from that even properly fixed and embedded sections show an increased rate of decay if the slides are stored in higher humidity.[50]

These phenomena might explain the fact that some of the slides used in this study did not stain correctly, as all of the slides that could not be scored came from the University of Gent. Furthermore, all the slides that came from the University of Utrecht were no older than four weeks, and as a result may not have been affected by the loss of reactivity. Scoring slides of different ages without knowing this may have influenced the results.

Age difference of the subjects
According to the literature[52-56] the amount and concentration of collagen changes with age. However, the precise nature of these age-related changes is not clear. Some studies report an increase of collagen[52, 53], while others report no change[57] or a decrease[52, 53, 56]. These changes may be due to the means of measuring the collagen. There is a difference between the total collagen content in the aorta and the concentration of the collagen if the aortas are of different thickness. According to this study[52] the dry weight of the human aorta decreases with age, which suggest that the reported decrease of total collagen content corresponds to an increase of collagen concentration. It is speculated that even though the amount of collagen decreases with, some other aortic components decrease at a greater rate.
There also seems to be a change in collagen composition with increasing age. Two studies[53, 58] found that the ratio between collagen type I and III changes with advancing age in rats; both studies found that with increased age the type III: type I ratio increases.
In addition to these age-related changes in concentration or amount of collagen, it has also been shown that the structure of the aorta looses stability as the body ages. This study[59] found that in the human thoracic media the collagen fibers are more irregularly arranged at older ages.
Furthermore these changes do not seem to occur in a linear fashion with increasing age. Most studies[52, 53, 56] found that the changes only became severe when a certain age was reached. This age is, of course, species-dependent. Whilst to our knowledge there are no such studies

in horses, this phenomenon may have influenced our results as the control group (WB) was, on average, older then both Friesian groups. (see table 2)

Effects on results

The WB group is on average older then the other two groups, which as mentioned earlier may affect the fragmentation of collagen in the aorta. To make comparison of these groups more reliable, results should be corrected for age.

Furthermore, due to the fact that all the slides in the WB group and some in both the NAF and AF groups came from the University of Gent, which were stored much longer then the slides that came from the University of Utrecht, it is possible that the staining was not uniform across all slides. A decrease in reactivity due to slide aging may have been mistaken for a decrease in amount or increase of fragmentation. This might mean some of the slides from Gent were wrongly scored. The uncertainty surrounding the extent to which aging affects the fragmentation of collagen in horses, and the nature of the effects of storage time and conditions on the immunoreactivity of the slides limits interpretation of our findings.

Conclusions

In conclusion, the found increase of amount of collagen type I in affected Friesian horses supports the hypothesis that the amount of collagen is different in Friesian horses with an aortic rupture when compared to non-affected Friesians or non-Friesians. This suggests that collagen type I, but not type III, plays a role in the development of aortic ruptures.

Our hypothesis that the fragmentation of collagen type I and III is higher in affected Friesians when compared to non-affected Friesians and non-Friesians has not been supported by our results. In fact, the opposite was seen, as the results show an increase in fragmentation of collagen type III in the non-Friesians. This may be explained by the fact that the non-Friesians is on average older, which can increase the fragmentation of collagen. Why the same has not been found in collagen type I is not known.

However, due to differences in storage time of the slides, these results may or may not be entirely accurate. Further testing may need to be done to asses the influences slide age has on our protocol.

References

1. White. 2012. *Acute aortic emergencies-part 1: Aortic aneurysms.* Advanced emergency nursing journal. 34: 216-29
2. Dingemans KP, Teeling , Peter, Lagendijk ,Jaap H., Becker AE. 2000. *Extracellular matrix of the human aortic media: An ultrastructural histochemical and immunohistochemical study of the adult aortic media.* Anat. Rec. 258: 1-14
3. Kierszenbaum Kierszenbaum AL. 2007. *Histology and cell biology : An introduction to pathology,* Philadelphia, PA: Mosby Elsevier
4. Carneiro. 2006. *Functionele histologie,* Maarssen: Elsevier gezondheidszorg
5. Kong CH, Lin XY, Woo CC, Wong HC, Lee CN, Richards AM, Sorokin VA. 2013. *Characteristics of aortic wall extracellular matrix in patients with acute myocardial infarction: Tissue microarray detection of collagen I, collagen III and elastin levels.* Interactive CardioVascular and Thoracic Surgery. 16: 11-5

6. Toumpoulis IK, Oxford JT, Cowan DB, Anagnostopoulos CE, Rokkas CK, Chamogeorgakis TP, Angouras DC, Shemin RJ, Navab M, Ericsson M, Federman M, Levitsky S, McCully JD. 2009. *Differential expression of collagen type V and XI α-1 in human ascending thoracic aortic aneurysms.* Ann. Thorac. Surg. 88: 506-13
7. Woodruff CE. 1926. *Studies on the vasa vasorum.* Am. J. Pathol. 2: 567,570.5
8. Robinson D, Mees B, Verhagen H, Chuen J. 2013. *Aortic aneurysms screening, surveillance and referral.* Aust. Fam. Physician. 42: 364-9
9. Lavall D, Schafers HJ, Bohm M, Laufs U. 2012. *Aneurysms of the ascending aorta.* Dtsch. Arztebl Int. 109: 227-33
10. Marr CM. 1998. *Aorto‐cardiac fistulas in 7 horses.* Veterinary radiology ultrasound. 39: 22-31
11. van der Linde‐Sipman JS. 1985. *Necrosis and rupture of the aorta and pulmonary trunk in four horses.* Vet. Pathol. 22: 51-3
12. Shirai W, Momotani E, Sato T, Kashima T, Saito T, Itoi Y. 1999. *Dissecting aortic aneurysm in a horse.* J. Comp. Pathol. 120: 307-11
13. Sleeper MM, Durando MM, Miller M, Habecker PL, Reef VB. 2001. *Aortic root disease in four horses.* J. Am. Vet. Med. Assoc. 219: 491,6, 459
14. Krüger T, Conzelmann LO, Bonser RS, Borger MA, Czerny M, Wildhirt S, Carrel T, Mohr FW, Schlensak C, Weigang E. 2012. *Acute aortic dissection type A.* Br. J. Surg. 99: 1331-44
15. White A, Broder J, Mando-Vandrick J, Wendell J, Crowe J. 2013. *Acute aortic emergencies--part 2: Aortic dissections.* Adv. Emerg. Nurs. J. 35: 28-52
16. Weidenhagen , Weidenhagen R, Bombien , Bombien R, Meimarakis , Meimarakis G, Geisler , Geisler G, Koeppel A. 2012. *Management of thoracic aortic lesions - the future is endovascular.* Vasa. 41: 163-76
17. Ploeg M, Saey V, de Bruijn CM, Grone A, Chiers K, van Loon G, Ducatelle R, van Weeren PR, Back W, Delesalle C. 2013. *Aortic rupture and aorto-pulmonary fistulation in the friesian horse: Characterisation of the clinical and gross post mortem findings in 24 cases.* Equine Vet. J. 45: 101-6
18. Rooney JR, Prickett ME, Crowe MW. 1967. *Aortic ring rupture in stallions.* Pathologia Veterinaria Online. 4: 268-74
19. Baker JR, Ellis CE. 1981. *A survey of post mortem findings in 480 horses 1958 to 1980: (2) disease processes not directly related to the cause of death.* Equine Vet. J. 13: 47-50
20. Johnson BJ, Stover SM, Daft BM, Kinde H, Read DH, Barr BC, Anderson M, Moore J, Woods L, Stoltz J. 1994. *Causes of death in racehorses over a 2 year period.* Equine Vet. J. 26: 327-30
21. Ploeg M, Saey V, de Bruijn CM, et al. 2012. Aortic rupture in friesian horses: 3 scenarios revealed. *Phryso,*
22. Hirai S. 2006. *Spontaneous rupture of the ascending thoracic aorta resulting in a mimicking pseudoaneurysm.* Annals of Thoracic and Cardiovascular Surgery. 12: 223-7
23. Kopadis G, Maltezos C, Tzortzis E, Marakis J, Dayantas J. 2003. *Spontaneous rupture of a non-aneurysmal non-inflammatory atherosclerotic aorta.* EJVES Extra. 5: 106-8
24. Teeter MG, Arroyo LG, Bakker JD, Hayes MA, Viel L, Runciman RJ. 2010. *Finite element analysis of wall stress in the equine pulmonary artery.* Equine Vet. J. 42: 68-72
25. Arroyo, L G Hayes, M A Delay, J Rao, C Duncan,B Viel, L. 2008. *Arterial calcification in race horses.* Vet. Pathol. 45: 617-25
26. Estes JE,Jr. 1950. *Abdominal aortic aneurysm; a study of one hundred and two cases.* Circulation. 2: 258-64
27. Grootenboer N, Bosch JL, Hendriks JM, van Sambeek MRHM. 2009. *Epidemiology, aetiology, risk of rupture and treatment of abdominal aortic aneurysms: Does sex matter?* European Journal of Vascular and Endovascular Surgery. 38: 278-84
28. Lee AJ, Fowkes FGR, Carson MN, Leng GC, Allan PL. 1997. *Smoking, atherosclerosis and risk of abdominal aortic aneurysm.* European Heart Journal. 18: 671-6
29. Eagleton MJ. 2012. *Inflammation in abdominal aortic aneurysms: Cellular infiltrate and cytokine profiles.* Vascular. 20: 278-83

30. Nicod P, Bloor C, Godfrey M, Hollister D, Pyeritz RE, Dittrich H, Polikar R, Peterson KL. 1989. *Familial aortic dissecting aneurysm.* J. Am. Coll. Cardiol. 13: 811-9
31. Ferguson MJ, Clemente AR. 1959. *Rupture and dissection of aorta in marfan's syndrome.* Am. J. Cardiol. 4: 543-6
32. Hirst Jr. AE, Gore I. 1973. *Marfan's syndrome: A review.* Prog. Cardiovasc. Dis. 16: 187-98
33. Gleason TG. 2005. *Heritable disorders predisposing to aortic dissection.* Semin. Thorac. Cardiovasc. Surg. 17: 274-81
34. Moon J, Lee S, Kang TS. 2012. *The vascular aneurysms of Ehlers–Danlos syndrome type IV.* European Heart Journal. 33: 415-
35. Abdul-Hussien H, Soekhoe RGV, Weber E, von der Thüsen JH, Kleemann R, Mulder A, van Bockel JH, Hanemaaijer R, Lindeman JHN. 2007. *Collagen degradation in the abdominal aneurysm: A conspiracy of matrix metalloproteinase and cysteine collagenases.* The American Journal of Pathology. 170: 809-17
36. Wills A, Thompson MM, Crowther M, Sayers RD, Bell PRF. 1996. *Pathogenesis of abdominal aortic aneurysms — cellular and biochemical mechanisms.* European Journal of Vascular and Endovascular Surgery. 12: 391-400
37. Lindeman JH, Ashcroft BA, Beenakker JW, van Es M, Koekkoek NB, Prins FA, Tielemans JF, Abdul-Hussien H, Bank RA, Oosterkamp TH. 2010. *Distinct defects in collagen microarchitecture underlie vessel-wall failure in advanced abdominal aneurysms and aneurysms in marfan syndrome.* Proc. Natl. Acad. Sci. U. S. A. 107: 862-5
38. Eberlová L, Tonar Z, Witter K, Křížková V, Nedorost L, Korabečná M, Tolinger P, Kočová J, Boudová L, Tř□eška V, Houdek K, Moláč□ek J, Vrzalová J, Pešta M, Topolčan O, Valenta J. 2013. *Asymptomatic abdominal aortic aneurysms show histological signs of progression: A quantitative histochemical analysis.* Pathobiology. 80: 11-23
39. Kadoglou NPE, Papadakis I, Moulakakis KG, Ikonomidis I, Alepaki M, Moustardas P, Lampropoulos S, Karakitsos P, Lekakis J, Liapis CD. 2012. *Arterial stiffness and novel biomarkers in patients with abdominal aortic aneurysms.* Regul. Pept. 179: 50-4
40. Meng, Yh Tian, C Liu, L Wang,L Chang, Q. 2013. *Elevated expression of connective tissue growth factor, osteopontin and increased collagen content in human ascending thoracic aortic aneurysms.* Vascular.
41. Koole D, Zandvoort HJA, Schoneveld A, Vink A, Vos JA, van den Hoogen LL, de Vries JPM, Pasterkamp G, Moll FL, van Herwaarden JA. 2013. *Intraluminal abdominal aortic aneurysm thrombus is associated with disruption of wall integrity.* Journal of Vascular Surgery. 57: 77-83
42. Sokolis DP, Kritharis EP, Giagini AT, Lampropoulos KM, Papadodima SA, Iliopoulos DC. 2012. *Biomechanical response of ascending thoracic aortic aneurysms: Association with structural remodelling.* Comput. Methods Biomech. Biomed. Engin. 15: 231-48
43. Lemarié CA, Tharaux P, Lehoux S. 2010. *Extracellular matrix alterations in hypertensive vascular remodeling.* J. Mol. Cell. Cardiol. 48: 433-9
44. Liu X, Wu H, Byrne M, Krane S, Jaenisch R. 1997. Type III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular□development. *Proceedings of the National Academy of Sciences.* 94: 1852-6
45. Bertheau P, Cazals-Hatem D, Meignin V, de Roquancourt A, Verola O, Lesourd A, Sene C, Brocheriou C, Janin A. 1998. *Variability of immunohistochemical reactivity on stored paraffin slides.* J. Clin. Pathol. 51: 370-4
46. Mirlacher M, Kasper M, Storz M, Knecht Y, Durmuller U, Simon R, Mihatsch MJ, Sauter G. 2004. *Influence of slide aging on results of translational research studies using immunohistochemistry.* Mod. Pathol. 17: 1414-20
47. Olapade-Olaopa E, Ogunbiyi JO, MacKay EH, Muronda CA, Alonge TO, Danso AP, Moscatello DK, Sandhu DPS, Shittu OB, Terry TR, Wong AJ, Habib FK. 2001. *Further characterization of storage-related alterations in immunoreactivity of archival tissue sections and its implications for collaborative multicenter immunohistochemical studies.* Applied Immunohistochemistry & Molecular Morphology. 9: 261-6
48. Wester K, Wahlund E, SundstrÃ¶m C, Ranefall P, Bengtsson E, Russell PJ, Ow KT, MalmstrÃ¶m P, Busch C. 2000. *Paraffin section storage and immunohistochemistry: Effects of time,*

temperature, fixation, and retrieval protocol with emphasis on p53 protein and MIB1 antigen. Applied Immunohistochemistry & Molecular Morphology. 8: 61-70
49. Jacobs TW, Prioleau JE, Stillman IE, Schnitt SJ. 1996. *Loss of tumor marker-immunostaining intensity on stored paraffin slides of breast cancer.* Journal of the National Cancer Institute. 88: 1054-9
50. Xie R, Chung J, Ylaya K, Williams RL, Guerrero N, Nakatsuka N, Badie C, Hewitt SM. 2011. *Factors influencing the degradation of archival formalin-fixed paraffin-embedded tissue sections.* Journal of Histochemistry & Cytochemistry. 59: 356-65
51. Ramos-Vara JA, Webster JD, DuSold D, Miller MA. 2013. *Immunohistochemical evaluation of the effects of paraffin section storage on biomarker stability.* Veterinary Pathology Online.
52. Cattell MA, Anderson JC, Hasleton PS. 1996. *Age-related changes in amounts and concentrations of collagen and elastin in normotensive human thoracic aorta.* Clinica Chimica Acta. 245: 73-84
53. Mays PK, Bishop JE, Laurent GJ. 1988. *Age-related changes in the proportion of types I and III collagen.* Mech. Ageing Dev. 45: 203-12
54. Tsamis A, Krawiec JT, Vorp DA. 2013. *Elastin and collagen fibre microstructure of the human aorta in ageing and disease: A review.* Journal of The Royal Society Interface. 10:
55. Andreotti L, Bussotti A, Cammelli D, di Giovine F, Sampognaro S, Sterrantino G, Varcasia G, Arcangeli P. 1985. *Aortic connective tissue in ageing--a biochemical study.* Angiology. 36: 872-9
56. Vogel HG. 1991. *Species differences of elastic and collagenous tissue — influence of maturation and age.* Mech. Ageing Dev. 57: 15-24
57. Smith EB. 1965. *The influence of age and atherosclerosis on the chemistry of aortic intima: Part 2. collagen and mucopolysaccharides.* J. Atheroscler. Res. 5: 241-8
58. Brüel A, Oxlund H. 1996. *Changes in biomechanical properties, composition of collagen and elastin, and advanced glycation endproducts of the rat aorta in relation to age.* Atherosclerosis. 127: 155-65
59. Toda T, Tsuda N, Nishimori I, Leszczynski DE, Kummerow FA. 1980. *Morphometrical analysis of the aging process in human arteries and aorta.* Acta Anat. (Basel). 106: 35-44

Appendix A

Group 1: Affected Friesian horses

Nr	Animal	Localization	Gender	Age	Slide Nr
1	A/3	Thorac1	Mare	2	A17046-7
2	A/4	Thorac1	Mare	1	A19315
3	A/6	Thorac1	Gelding	5	B7205
4	A/7	Aorta 3/5	Mare	6	B8139
5	A/8	Aorta 3/5	Gelding	7	B10603
6	A/14	Aorta 3/5	Stallion	10	C6047
7	A/15	Aorta 3/5	Gelding	4	C11193
8	A/20	Aorta 3/5	Gelding	10	D5075
9	A/Abe	Aorta 3/5	Gelding	4	D7901
10	A/Mona	Aorta 3/5	Mare	4	E388
11	A/	Aorta 3/5	Mare	4	3111108001
12	A/	Aorta 3/5	Mare	?	3111116008
13	A/	Aorta 3/5	Mare	7	3111129031
14	A/	Aorta 3/5	Mare	4	3120510031
15	A/	Aorta 3/5	Mare	7	3130201024
16	A/	Aorta 3/4	?	?	3130201022
17	A/	Aorta 3/4	?	?	100102
18	A/Tomas	Aorta 3/5	Gelding	7	E7627
19	A/Ditte	Aorta 3/5	Mare	4jr	E9102
20	A/	Aorta 3/5	Mare	9	3130906028

Groep 2: Non-Affected Friesian horses

Nr	Animal	Localization	Gender	Age	Slide Nr
1	NA/2	Thorac1	Mare	17	B1938
2	NA/4	Aorta 3/5	Mare	5	C4183
3	NA/5	Aorta 3/5	Stallion	0	C4207
4	NA/7	Aorta 3/5	Mare	5	C4212
5	NA/11	Aorta 3/5	Mare	14	C7848
6	NA/14	Aorta 3/5	Mare	14	C11164
7	NA/15	Aorta 3/5	Mare	4	C13417
8	NA/16	Aorta 3/5	Mare	20	C13460
9	NA/17	Aorta 3/5	Mare	3	C14340
10	NA/18	Aorta 3/5	Stallion	3	C15154
11	NA/19	Aorta 3/5	Mare	0	C15190
12	NA/22	Aorta 3/5	Mare	8	D927
13	NA/24	Aorta 3/5	Stallion	6	D999
14	NA/25	Aorta 3/5	Mare	9	D1035
15	NA/26	Aorta 3/5	Stallion	1	D5436
16	NA/27	Aorta 3/5	Gelding	3	D5119
17	NA/28	Aorta 3/5	Mare	12	D6429
18	NA/41	Aorta 3/5	Gelding	14	D7524

19	NA/44	Aorta 3/5	Mare	15	D10987
20	NA/45	Aorta 3/5	Mare	21	D11414
21	NA/48	Aorta 3/5	Stallion	11	D13579
22	NA/49	Aorta 3/5	Mare	9	D13609
23	NA/	Aorta 3/5	Gelding	9	E1016
24	NA/	Aorta 3/5	Gelding	11	3111214034
25	NA/	Aorta 3/5	Gelding	8	3111220019
26	NA/	Aorta 3/5	?	?	3120104036
27	NA/	Aorta 3/5	Mare	6 mo	3120111056
28	NA/	Aorta 3/5	?	3	3111216038
29	NA/	Aorta 3/5	Stallion	10	3121102001
30	NA/62	Aorta 3/5	Mare	16	E6240

Group 3: Warmbloods

Nr	Animal	Localization	Gender	Age	Slide Nr
1	C/8	Aorta 3/5	Stallion	0	C12778
2	C/9	Aorta 3/5	Stallion	1	C127781
3	C/11	Aorta 3/5	Gelding	10	C12845
4	C/3	Aorta 3/5	Mare	5	C6820
5	WB1 5/7	Aorta 3/5	Mare	15	D9754
6	WB2 5/7	Aorta 3/5	Mare	19	D9758
7	WB3 5/7	Aorta 3/5	Mare	9	D9762
8	WB4 5/7	Aorta 3/5	Mare	26	D9766
9	WB5 5/7	Aorta 3/5	Mare	18	D9770
10	WB6 5/7	Aorta 3/5	Mare	18	D9774 bis
11	WB1 23/8	Aorta 3/5	Mare	7	D11200
12	WB2 23/8	Aorta 3/5	Mare	14	D11202
13	WB3 23/8	Aorta 3/5	Mare	26	D11204
14	WB4 23/8	Aorta 3/5	Mare	4	D11206
15	WB6 23/8	Aorta 3/5	Mare	12	D11210
16	WB7 23/8	Aorta 3/5	Mare	9	D11212
17	WB8 23/8	Aorta 3/5	Mare	14	D11214
18	WB9 23/8	Aorta 3/5	Mare	4	D11216
19	WB110301	Aorta 3/5	Gelding	18	D13308
20	WB120305	Aorta 3/5	Gelding	17	D13337