PYRROLE CROSS-LINKS AND GLYCOSAMINOGLYCANS IN COLLAGEN AND THEIR ROLE IN AORTIC RUPTURE IN THE FRIESIAN HORSE

Amarins Sijsling
Student number: 3593649

Supervisor: Drs. Ing. Margreet Ploeg
Utrecht University, Pathology Department Faculty of Veterinary Medicine

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ABSTRACT

Aortic rupture in horses is a rare condition, although relatively common in the Friesian breed. This study searches for abnormalities in pyrrole cross-link and glycosaminoglycan concentration at the site of the aortic rupture. Three locations of the aorta were used, including the site of rupture. These were compared with each other and with the tendons of the horses, for systemic conditions.
The goal of this study is to ascertain if there are specific differences in the composition of the aortic wall in Friesian horses with an aortic rupture, Friesian horses without an aortic rupture and non-Friesian horses. A significant difference in concentration in one or more of the measured groups, comparing affected Friesian horses with other horses, is expected. When there is a systemic problem in the Friesian breed, a significant difference is expected when comparing the affected and non-affected Friesian horses with the Warmblood horses. This study found that the tendons of the Warmbloods contained a significant higher concentration of pyrrole cross-links compared to the non-affected Friesians, and the non-affected Friesians contained a lower significant concentration than the affected Friesian horses. In this research a trend was found towards a difference of the GAG concentration when comparing the aortas 2/3, 3/5 and tendons from the Warmbloods with the affected and non-affected Friesian horses. This could mean that there is a systemic problem in the Friesian breed concerning the GAG concentrations of the collagen The differences found in the concentration of GAGs in the aortic walls of Friesian horses compared to Warmbloods, could be used as a new insight in the reason behind aortic rupture. Also other systemic diseases in the Friesian horse could be related to the GAGs, which makes them an important topic for further research.

Keywords: Aortic rupture, Friesian horse, Extracellular matrix, Collagen, Pyrrole cross-link, Glycosaminoglycans

INTRODUCTION

STRUCTURE OF THE AORTA

The aorta is classified as an elastic artery (McGavin, M.D., Zachary, J.F. 2007). The elasticity of the aorta aids in the propulsion of the bloodstream to the systemic vasculature (Tsamis, Krawiec et al. 2013). The aortic wall consists of three layers or tunics: the tunica intima (interna), the tunica media and the tunica adventitia (externa) (Eurell, J., Frappier, B.L. 2006).

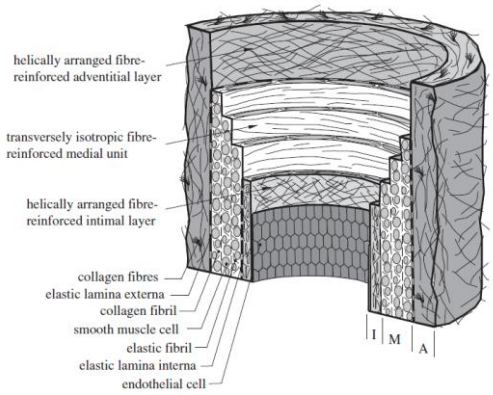

Fig. 1 The three layers of the aorta. *Tunica intima (I), tunica media (M) and tunica adventitia (A)* (Tsamis, Krawiec et al. 2013).

The lumen is surrounded by the tunica intima, which consists of single layer of endothelial cells, a thin basal membrane and a subendothelial layer of connective tissue (Tsamis, Krawiec et al. 2013). The subendothelial layer consists predominantly of collagen fibrils. The outer layer of the tunica interna is the internal elastic membrane. This membrane permits diffusion of nutrients into the tunica media (McGavin, M.D., Zachary, J.F. 2007, Eurell, J., Frappier, B.L. 2006).

The thick middle layer is the tunica media. The basic element of the tunica media is the "lamellar unit" (Fig. 2), consisting of fenestrated elastic laminae with smooth muscle cells, a network of elastic and collagen fibrils, and "ground substance" (McGavin, M.D., Zachary, J.F. 2007, Tsamis, Krawiec et al. 2013, Eurell, J., Frappier, B.L. 2006).

The outer layer, the tunica adventitia, consists predominantly of thick bundles of collagen fibrils arranged in helical structures (Tsamis, Krawiec et al. 2013) and elastic fibers with penetrating blood vessels, termed the vasa vasorum. The vasa vasorum is supplying nutrients and extends into the outer layers of the tunica media (McGavin, M.D., Zachary, J.F. 2007, Eurell, J., Frappier, B.L. 2006).

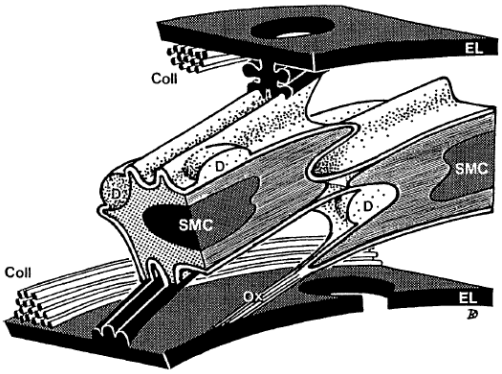

Fig. 2 Lamellar Unit. *The basic element of the tunica media. Schematic representation of two smooth muscle cells (SMC), two elastic lamellae (EL) and their interconnections. Thick collagen fibers (Coll) are closely associated with the EL* (Dingemans, Teeling et al. 2000).

EXTRACELLULAR MATRIX

The extracellular matrix (ECM) is primarily composed of two main classes of macromolecules: fibrous proteins (collagen and elastin for example) and glycoproteins (Mouw, Ou et al. 2014). This study mainly focuses on type pyrrole cross-links in collagen and the proteoglycans in the ECM.

COLLAGEN

Collagen proteins are dominantly present in the ECM (Mouw, Ou et al. 2014).The type of collagen in the aortic wall consists predominantly off type I and type III (Barnes 1985, Bode 2000), which are fibril-forming collagens (Bode 2000). All types of collagen consist of a triple helix of three α-polypeptide chains, containing repetitions of proline rich tripeptide Gly-X-Y. The triple helix can be formed by three identical chains (type III) or two or more different chains (type I). The α- chains assemble around a central axis in a way that all glycine (Gly) residues are positioned in the center (Gelse, Pöschl et al. 2003). The X and Y positions are proline and lysine residues (Bode 2000), and depending on the type of collagen, the specific proline and lysine residues are modified by enzymatic hydroxylation (Gelse, Pöschl et al. 2003).

Collagen is produced by fibroblasts. These cells secrete a precursor form of collagen, called procollagen.
This procollagen contains additional peptides at each end that obstruct assembly into collagen fibrils (Alberts, Bray et al. 2010). The procollagen is imported into the rough endoplasmic reticulum, which ensures that the α-chains form triple helical procollagen (Mouw, Ou et al. 2014).
The next step is the modification of procollagen in the Golgi apparatus and its packaging into secretory vesicles (Mouw, Ou et al. 2014). In the extracellular space, the procollagen is cleaved by proteinases, to form the collagen molecule and allowing the fibril forming of collagen (Alberts, Bray et al. 2010). The fibril forming collagens are further stabilized by the formation of covalent cross-links (Gelse, Pöschl et al. 2003).
The type of cross-link formed depends on the degree of lysine hydroxylation in the helical part of the molecule and non-helicaltelopeptide region (Thorpe 2010).

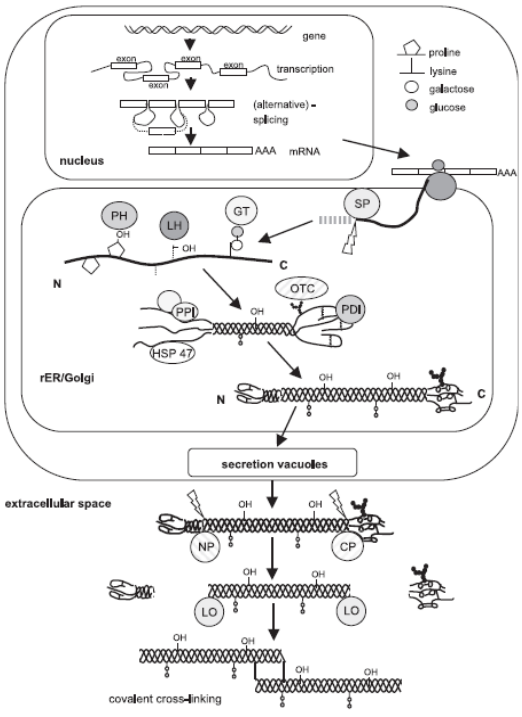

Fig. 3 Collagen synthesis. *Schematic representation of collagen synthesis starting from the nuclear transcription of collagen genes to the final steps of fibril formation.* (Gelse, Pöschl et al. 2003).

Extracellular lysyl oxidase catalyzes the cross-linking between collagen molecules to stabilize the supramolecular collagen structure. (Mouw, Ou et al. 2014).The hydroxylation of the proline and lysine residues is catalyzed by prolyl 3-hydroxylase, 4-hydroxylase and hydroxylase. The hydroxylysine residues are able to form stable intermolecular cross-links of collagen molecules in fibrils (Gelse, Pöschl et al. 2003). The hydroxylation state of the telopeptide lysine residues is crucial in defining collagen cross-links (Gelse, Pöschl et al. 2003).

The lysine and hydroxylysine residue of the aminoterminal telopeptide can be oxidized into aldehyde by lysyl oxidase (Bode 2000). Lysine aldehydes and hydroxylysine aldehydes undergo a spontaneous reaction with lysine or hydroxylysine residues in the helical region of neighbouring molecules to form the immature bivalent cross-links. These immature cross-links undergo further spontaneous reactions to form mature trivalent pyridinoline and pyrrole cross-links.
Extensive hydroxylation of lysine residues results in the formation of hydroxylysyl-pyridinoline (HL-Pyrrole). In tissue where less hydroxylation of the helical or telopeptide lysine residues occurs, the mature cross-links lysyl-pyridinoline (L-Pyrrole) and pyrrole form respectively (Thorpe 2010). The L-Pyridinoline and pyrroles are preferentially located at the N-terminus of the collagen molecule (Bode 2000).

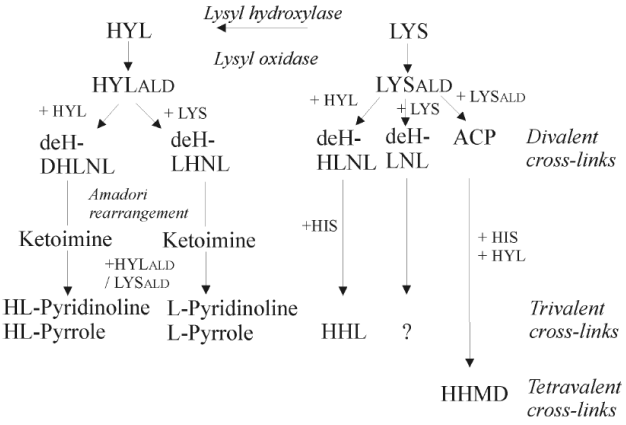

Fig. 4 Forming Pyrrole cross-links. *Schematic representation of enzymatic formation and maturation of HL-Pyrrole and L-Pyrrole cross-links* (Bode 2000).

GLYCOSAMINOGLYCAN

Proteoglycans form the basis of higher order ECM structures around cells (Mouw, Ou et al. 2014). While collagen provides tensile strength to resist stretching, the proteoglycans in the ECM provide the resisting of compression and they serve as space-fillers. The proteoglycans are extracellular proteins linked to a special class of complex negatively charged polysaccharides, the glycosaminoglycans (GAGs). They consist of a protein core with one or more GAG sidechains. Because the GAGs are negatively charged, they attract a cloud of cations that are osmotic active, causing large amounts of water to be sucked in to the matrix. This creates a swelling pressure that is balanced by tension in the collagen fibers interwoven with the proteoglycans. When the amount of collagen and GAG in the ECM is large, such a matrix is tough, resilient and resistant to compression (Alberts, Bray et al. 2010).

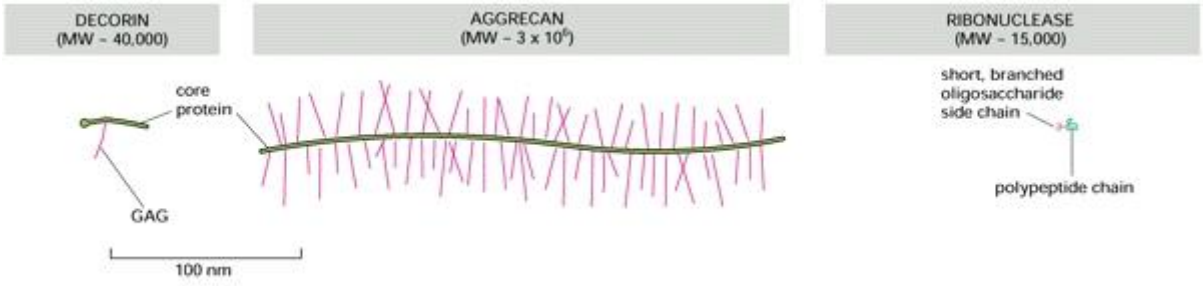

Fig. 5 Example of a large and small proteoglycan found in the extracellular matrix. *The protein core of the proteoglycan with the glycosaminoglycan (GAG) sidechains* (Alberts, Bray et al. 1994) .

AORTIC RUPTURE

RECENT RESEARCH

Aortic rupture in humans mostly occurs in the abdominal part of the aorta. The thoracic rupture, as observed in horses, is predominantly linked to hereditary diseases and very uncommon. Alterations in elastin (Marfan syndrome), firbillin (Ehlers-Danlos syndrome type IV) and proteoglycans (biglycan gene deficiency), in the connective tissue, have been determined in the preexistence of thoracic aortic rupture. The functional connection between the ascending aorta and pulmonary artery described in the Friesian horse, is seen rarely in cases of human erosion and/or rupture of a chronic process of pseudoaneurysms of the aorta (Ploeg, Saey et al. 2014).

The Friesian horse population appears to be the only animal species in which aorto-pulmonary fistulation is diagnosed. In recent research of the thoracic aortic rupture and aorto-pulmonary fistulation in the Friesian horse, an indication for abnormalities in the extracellular matrix was found, referring to aberrant collagen and elastin present in the aortic wall. In order to understand the pathogenesis of this condition, biochemical examination of the extracellular matrix in the aorta of affected Friesians should be performed (Ploeg, Saey et al. 2014).

In a study of Thorpe (2010), they tried to find a reason for the wide variation in mechanical strength of the superficial digital flexor tendon between individual horses. The high mechanical strength of tendons relies on the correct orientation of collagen molecules within fibrils, as well as cross-linking, it gives mechanical strength to the collagen and guarantees firmness. In this study, a positive correlation was found between mechanical strength of the superficial flexor tendon in horses and pyrrole cross-links of the tendon (Thorpe 2010).

In a study of the biochemical differences of the tendons between Friesians and Warmbloods, the hypothesis was tested that the different concentrations of pyrrole and pyridinoline cross-links between these horses, could be a cause for the differences in elasticity. The result indicated that the higher content of pyrrole cross-links in Friesians tendons could be the cause of the biochemically differences in elasticity (Hazeleger, Back et al. 2013). This could be an indication for a systemic problem in the Friesian breed.

This study searches for abnormalities in pyrrole cross-link and glycosaminoglycan concentration at the site of the aortic rupture, to find a specific reason for this condition of aortic rupture in the Friesian horse. The goal of this study is to ascertain if there are specific differences in the composition of the aortic wall in Friesian horses with an aortic rupture, Friesian horses without an aortic rupture and non-Friesian horses. The differences between these groups could explain why the specific aortic rupture exists in the Friesian horse.

Initially, the main goal was to measure the pyrrole cross-links, but due to the importance of the glycosaminoglycans in the ECM, they were also included in this study.
A significant difference in concentration in one or more of the measured groups, comparing affected Friesian horses with other horses, is expected. Especially when comparing the affected Friesian horses with the Warmblood horses. When there is a systemic problem in the Friesian breed, a significant difference is expected when comparing the affected and non-affected Friesian horses with the Warmblood horses.

MATERIAL AND METHODS

COLLECTION MATERIAL

SAMPLE COLLECTION

Samples of three groups of horses were used in this study, described below as follows;

-Group 1: Affected Friesian horses with acute, mildly or chronic signs of aortic rupture (Aff)
-Group 2: Non affected Friesian horses without aortic rupture (NA)
-Group 3: Non-Friesian horses, Warmblood, without aortic rupture (WB)

Only horses with a maximum of 10 years of age are used to make the groups more comparable.

Seventeen affected Friesian horses 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 and the Faculty of Veterinary Medicine of Utrecht University, the Netherlands. Thirteen nonaffected Friesian horses without a history of cardiovascular or orthopedic disease were used as control horses. Additionally, nineteen Warmblood horses from the same faculties or slaughterhouses were used as control horses. The age and mean of the different horses is presented in table 1.

Group	**Amount of horses**	**Age in years**	**Mean**
Aff	17	3 -10	5.7
NA	13	0-10	3.5
WB	19	1-9	6.1

Table 1 Overview of the different test groups and ages

Samples were taken from the tendons and aortas of horses which fit in group 1, 2 and 3, as described above. The aorta was measured (in length) starting from the cardiac base up to the diaphragm and was subsequently equally divided into five parts. The 1/5 location is the location of the aortic rupture, which is compared to the 2/5 and 3/5 aorta location in this study. The tendon samples were collected from the deep digital flexor tendon (D.B.). A systemic problem within the Friesian breed, as suggested above, could be demonstrated when comparing the tendons and aortas of the Friesian horses with the Warmblood horses. After collection, the samples were frozen at -20°C solid to preserve them and kept at the pathology department of the Utrecht Faculty of Veterinary Medicine.

For this study, 0.1 grams sample weight of each sample was used to do the measurements described below. Samples were freeze dried prior to measurements, as to calculate the correct concentrations in the samples. After that, nitrogen freezing was used to ensure a state of hardening at which the samples could be crushed by hammering.

Due to crushing, the surface area at which the enzymes could work was increased ensuring better digestion. Samples were then transferred to cups including a buffer (described below) and placed in a MagNaLyser (Roche Diagnostics, Almere, NL) instrument for homogenization of the sample. In order to optimize the measurements, it was important that samples maintained cooled. In order to achieve that, the samples were cooled 2 minutes in between the MagNaLyser runs. This process was repeated four times. Before the measurements could be performed, all samples had to be digested using enzymes. Because the buffer has already been added to the sample, only the active enzyme had to be added for digestion. To accomplish the best result, different test runs have been carried out and eventually developed the method described below.

SAMPLE DIGESTION

Elastase digestion

For digestion of the aorta samples the enzyme elastase (Boehringer Mannhein, Alkmaar, NL) was used. The sample already contained a 1000 µl 1,0 M TRIS·HCl buffer of pH 8,5 to which 50 µl elastase with buffer was added (10 ml buffer with 10 mg elastase). The samples were incubated overnight in a shaking water bath, at a temperature of 37 °C. After overnight incubation, the samples were not yet fully digested. Therefore, 20 µl trypsine (Sigma Aldrich, St Louis, Missouri, USA) was added.

Papain digestion

For the digestion of the tendon samples the enzyme papain (Sigma Aldrich, St Louis, Missouri, USA) was used. The sample already contained a 1000 µl papain buffer solution (90 ml H_2O and 10 ml papain buffer: 0,5 M $NaH_2PO_4 \cdot H_2O$, 20 mM $Na_2EDTA \cdot 2H_2O$, 5M NaOH with pH 6,5), to which 50 µl papain enzyme was added (3.35 ml buffer solution: 3,015 ml buffer with 10x Cysteine·HCl and 335 µl papain). The samples were incubated overnight in a shaking water bath, at a temperature of 56 °C.

ASSAYS

The assays were performed by preparing the samples in 96-well microplate. By measuring the optical density of the samples at the selected wavelength, the concentrations can be calculated. The pyrrole cross-links, GAGs and protein assays performed using a VersaMex microplate spectrophometer (Molecular Devices Corporation, California, USA). The DNA assay was performed using a CLARIOstar microplate spectrophometer (BMG Labtech, Ortenberg, Germany).

Pyrrole cross-link assay

After digestion, the samples were centrifuged at 3000 RPM for 15 minutes. The clear supernatant containing the digested samples was used for further analysis. A 1:1 dilution was made with MiliQ H_2O, thus 100 µl sample with 100 µl H_2O. A reference line was made with 1-methyl-Pyrrole (100 µl 50 mM1-methyl-Pyrrole with 250 ml MiliQ H_2O). The diluted samples and the reference line were mixed with 40 µl of Erlich's Reagent (500 mg 4-dimethyaminobenzaldehyde in 4,4 ml 60% perchloric acid, made up to 10 ml with deionised H_2O). The samples were left for 10 minutes at room temperature before measuring the absorbance at 558 nm and 650 nm (non-related wavelength). 1-methyl-Pyrrole Standard Dilution was used to make a standard curve. Results were expressed as micrograms pyrrole per milliliter.

Glycosaminoglycan assay

The concentration of GAGs was measured by a method described by Farndale et al (Farndale, Buttle et al. 1986). This method measures the sulphated glycosaminoglycan content. 20 µl of diluted sample is mixed with 10 µl of 3% bovine serum albumin. Immediately following, 250 µl dimethylmethylene blue reagent is added. The well is mixed and left at room temperature for 20 minutes. The plate is measured with an absorbency wavelength of 525 nm and a reference wavelength of 595 nm. Chondroitin Sulphate was used to make a standard curve. Results were expressed as micrograms GAG per milliliter.

DNA assay

The DNA concentration was determined by a fluorescent dye described by Kim et al (Kim, Sah et al. 1988). A diluted sample concentration of 20 µl was used to determine the amount of DNA. Calf thymus DNA was used to make a standard curve. After adding 200 µl dye solution, the DNA concentration was measured immediately at excitation of 352 nm and emission of 455 nm. Results were expressed as microgram DNA per microliter.

Protein assay

The protein concentration of the samples was measured to compare the concentration of the assays on an equal basis. The concentration DNA, GAG and pyrrole assays were equalized by the protein concentrations of each sample.

The protein concentrations of the samples was determined by a method described by Lowry et al (Lowdry, Rosebrough et al. 1951). 20 µl of diluted sample was mixed with a 150 µl 1:100 buffer solution (B: 100 ml 4% $CuSO_4$ and A: 1 L 1% SDS, 2% Na_2CO_3, 0,4% NaOH and 0,16% K/Na-tartate). The solutions is mixed and left at room temperature for 30 minutes. Then 80 µl of diluted Folin-reagens is added (1:7, Folin-reagens with solution A). After mixing, the solution was left for 20 minutes at room temperature before measuring at 640 nm. 0,5 µg bovine serum albumin/µl in 0,1 M NaOH was used to make a standard curve. Results were expressed as micrograms protein per milliliter.

STATISTIC ANALYSIS

The results were analyzed statistically by Luc Duchateau at the University of Gent. There were twelve comparisons made between three different horse groups and four reperate locations. Therefore, a P-value of 0.05/12 = 0.0042 was considered to be significant.

The statistic analysis was based on the mixed model with horse as a random effect and location and type of horse and their interaction as categorical fixed effects.
At first it is determined if an interaction between a study group and a location of lesion is present. When this is the case, the results are further examined if and what the significant differences mean.

The analysis is performed for the difference between horse groups on different variables. These variables are the result of the concentrations found when measuring the concentrations of pyrrole cross-links, GAGs and DNA and were corrected by measuring the protein concentration per sample.

RESULTS

STATISTIC RESULTS

Table 2 shows the mean and standard error of the concentrations found at the different locations and groups. Figures 5, 6 and 7 show the concentrations of pyrrole, GAG and DNA. The pyrrole and GAG concentrations were corrected as described above.

Mean and Standard Error

		WB		**NA**		**Aff**	
		Mean	*StdErr*	Mean	*StdErr*	Mean	*StdErr*
Pyrrole (10^{-4} µg/µl)	**AO1/5**	6,59	*8,81*	2,71	*5,1*	4,24	*4,9*
	AO2/5	7,23	*5,88*	3,76	*8,81*	3,85	*5,88*
	AO3/5	8,9	*4,58*	2,7	*5,1*	4,97	*5,32*
	D.B.	42,47	*4,05*	18,33	*5,58*	44,58	*6,24*
GAG (10^{-4} µg/µl)	**AO1/5**	21,27	*10,41*	7,08	*7,31*	7,85	*6,74*
	AO2/5	28,38	*7,54*	5,14	*10,72*	5,09	*7,34*
	AO3/5	33,47	*6,42*	5,72	*7,31*	5,78	*7,12*
	D.B.	25,41	*5,64*	3,88	*7,73*	10,26	*6,39*
DNA (µg/µl)	**AO1/5**	0,18	*0,1*	0,46	*0,06*	0,23	*0,06*
	AO2/5	0,66	*0,06*	0,41	*0,1*	0,28	*0,07*
	AO3/5	0,36	*0,05*	0,63	*0,06*	0,26	*0,06*
	D.B.	0,12	*0,05*	0,15	*0,07*	0,12	*0,05*

Table 2 Mean and Standard Error (StdErr) of the concentrations found. *With the different aorta locations (1/5, 2/5 and 3/5) and tendons (D.B.). The different groups are shown: Warmbloods (WB), non affected Friesians (NA) and affected Friesians (Aff).*

Pyrrole cross-links

Within the pyrrole cross-link concentrations, there was a significant effect seen when comparing the D.B.'s of the different groups:

- WB is increased vs. NA,
  P = 0.0007
- NA is decreased vs. Aff,
  P = 0.0022

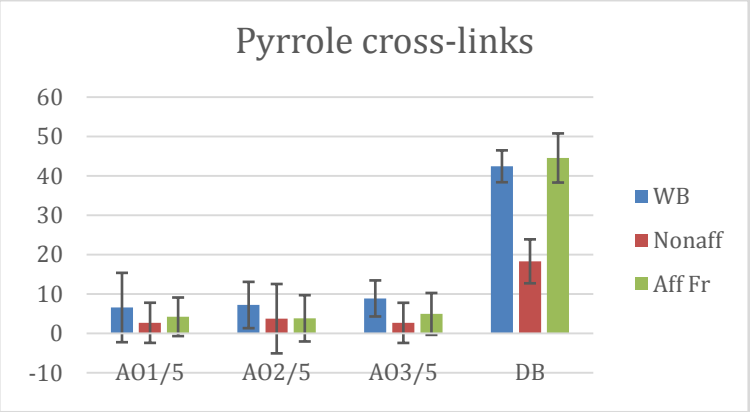

Fig. 6 Pyrrole concentration found in the different groups. Pyrrole/protein x 10^-4 ug/ul

Glycosaminoglycans

Within the GAGs concentrations, an almost significant effect was seen comparing all groups, except AO 1/5:

- AO 2/5: WB is increased vs. Aff,
  P =0.0290
- AO 3/5: WB is increased vs. NA,
  P = 0.0056
- AO 3/5: WB is increased vs. Aff,
  P = 0.0048
- DB: WB is increased vs. NA,
  P = 0.0271

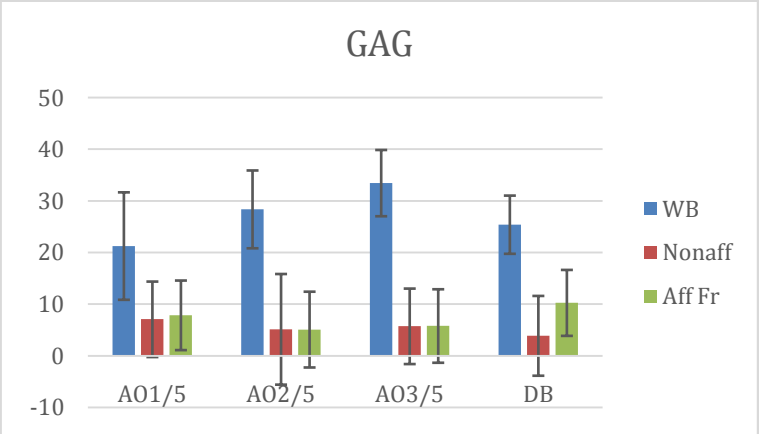

Fig. 7 GAG concentration found in the different groups. GAG/protein x 10^-4 ug/ul

DNA

Within the DNA concentrations, a significant effect was seen in the different groups of aortas:

- AO 1/5
  - WB is decreased vs. NA, P = 0.0218
  - NA is increased vs. Aff, P = 0.0071
- AO 2/5
  - WB is increased vs. NA, P= 0.0387
  - WB is increased vs. Aff, P < 0.0001
- AO 3/5
  - WB is decreased vs. NA, P= 0.0015
  - NA is increased vs. Aff, P < 0.0001

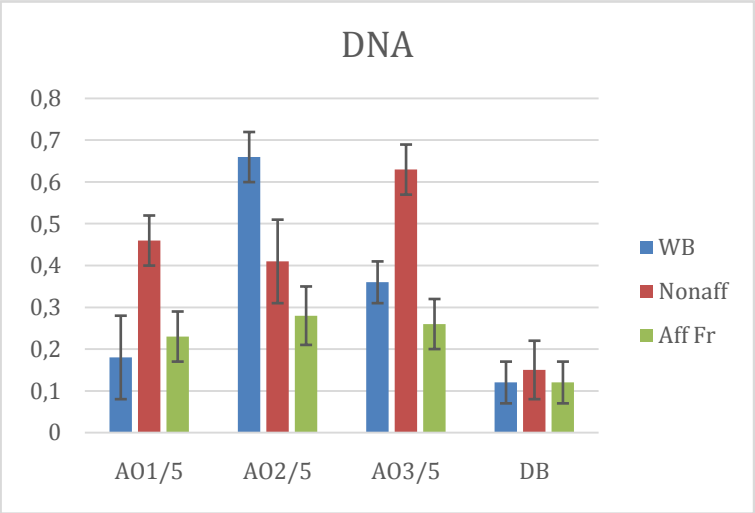

Fig. 8 DNA concentration found in the different groups.

Within groups

Within the group of the Warmbloods, there is a significant effect for DNA when comparing different locations of the aortas. This way, there were six more comparisons (three aorta locations and three groups), making the significance level 0.05/(12+6) = 0.0028.

- AO 1/5 is increased vs. AO 2/5, P = 0.0001
- AO 2/5 is increased vs. AO 3/5, P = 0.0003

Effect of Age

In the above-mentioned statistic results, only the horses with a maximum age of 10 years were used. When comparing the results of the different groups of age, only the pyrrole cross-link within the Warmbloods showed a significant effect:

- WB ≤ 10 vs. >10 years old, mean (StdErr)
  - AO 1/5 -1.99 (0.82), slope negative with P = 0.0178
  - AO 2/5 -1.56 (0.75), slope negative with P = 0.0417
  - AO 3/5 -1.91 (0.70), slope negative with P = 0.0091
  - D.B. 2.51 (0.69), slope positive with P = 0.0007

DISCUSSION

This study is designed to further investigate the differences in the existence of the aortic rupture of the Warmblood and the Friesian horses. There are several theories in the pathogenesis of the aortic rupture and the most important are discussed below. This information is also important to demonstrate the difference between the Friesians and the Warmboods, and finally to find the specific cause of aortic rupture in the Friesian breed.

The aortic rupture of a non-Friesian horse, which often results in sudden death, is rare and predominantly described as a condition in breeding stallions. Sudden death occurs mainly during coitus or shortly thereafter (Sleeper 2001). In these horses the aorta ruptures at the base: the sinus of Valsalva (Ploeg, Saey et al. 2013). An explanation for this location, is the fact that the aortic wall is thinner in this area than anywere else (Linde-Sipman 1985). At the end of the systole (closure of the aortic valve), the recoil of the column of blood tends to push the heart down, while the aorta is firmly held in place (by its large branches and attachments to the ventral surfaces of the thoracic vertebrae). This process is putting tension on the convex part of the aorta, close to the aortic valve. Excitement, with its elevation of heart rate and blood pressure, increases this process and can lead to rupture, with (exceptionally) or without pre-existing necrosis of the tunica media (Rooney 1967).

Typically, when the aortic root or an aneurysm of the aortic sinus ruptures, it does so into the right ventricle. Less frequently, the rupture occurs into the right atrium or tricuspid valve (Sleeper 2001). The rupture can result in acute hemorrhage into the pericardial sac, leading to signs of acute cardiac failure (Ploeg, Saey et al. 2013). The acute distress is frequently mistaken for pain of abdominal origin (Marr 1998).

Linde-Sipman (1985) describes the rupture of the aortic arch, combined with a rupture in the pulmonary trunk. This results in a left to right shunt between the aorta and pulmonary trunk. Three of those four patients were Friesian horses (Linde-Sipman 1985). In contrary to the aortic rupture in warm blood horses, in Friesian horses the rupture takes place at the site of the ligamentum arteriosum, which can result in an aorto-pulmonary fistulation (Ploeg, Saey et al. 2013). In addition, there may also be hemorrhage, mainly found around the aorta. These are circumferential cuffs of perivascular hemorrhage, and is probably formed by leakage of blood out of the ruptured site into the connective tissue surrounding the arteries. Some affected Friesian horses develop an acute tear resulting in a hemothorax and subsequent death within minutes, whereas others form an aortic tear with an aorto-pulmonary fistulation or with a cuff of perivascular hemorrhage. This process is allowing stabilization for several weeks in some cases. There can also form a quite stable aorta-pulmonary fistulation which leads to right-sided heart failure, after weeks to months (Ploeg, Saey et al. 2013). In contrast to the aortic rupture in Warmblood horses, no sex predilection is found in the Friesian horses (Ploeg, Saey et al. 2014). Hepatic fibrosis found in Friesian horses, which was considered to be a sign of chronic heart failure, indicates that aortic rupture and aorto-pulmonary fistulation can manifest not only as an acute condition but also as less acute (subacute and chronic). In these non-acute cases, complaints such as recurrent colic, epistaxis, coughing and intermittend manifestation of peripheral oedema in the weeks prior to the final stage of cardiac failure were reported (Ploeg, Saey et al. 2013).

Linde-Simpan (1985), suggested alterations in the vasa vasorum as a possible cause for aortic medial necrosis and the scar of the former ductus arteriosus was considered to be predisposed to rupture and fistulation. All three affected Friesian horses from that study, showed medial wall necrosis. This necrosis was suggested to weaken the aortic wall and in this way causing the affected vessels to dissect and rupture spontaneously (Linde-Sipman 1985). This process is also been described as a predisposing factor in the aortic rupture of non-Friesian horses, however, chronic histological lesions such as fibrosis of the adventitia and periadventitial tissue with infiltration of hemosiderin-laden macrophages, have only been described in Friesian horses (Ploeg, Saey et al. 2014).

The partial or total obliteration of the vasa vasorum could cause hypoxia or anoxia in the vessel walls, which could lead to local comprised circulation necrosis, and finally rupture of the aortic wall (Linde-Sipman 1985). The study of Ploeg, Saey et al. (2014), showed that only 5 of the 20 horses had mild to moderate intimal thickening of the vasa vasorum, without complete occlusion. Medial fibrosis in 2 to 5 vessels (of 20) of the vasa vasorum was infrequently present in subacute and chronic cases.

Because the big difference in both studies, ischemic damage as underlying cause is unlikely. Ploeg, Saey et al (2014), suggested that the alterations observed in the vasa vasorum are a secondary phenomenon related to blood flow changes, such as severe circulatory disturbance, caused by ruptured aortas or pseudoaneurysms. Another cause of medial necrosis are abnormalties in the connective tissue, where this study focuses on (Ploeg, Saey et al. 2014).

In the study of Hazeleger, Back et al (2013), they found a significant higher concentration of pyrrole cross-links in the tendons of the Friesian horse, compared to the Warmbloods and Thoroughbreds. In the GAG concentrations, there was no significant difference when comparing the Friesians with the Warmbloods and Thoroughbreds. They used 12 horses from each breed. In the contrary, this study found that the tendons of the Warmbloods contained a significant higher concentration of pyrrole cross-links compared to the non-affected Friesians, and the non-affected Friesians contained a lower significant concentration than the affected Friesian horses. This study is more likely to demonstrate a systemic problem by comparing two different kind of tissues, the aortas and tendons.

This research also found an almost significance difference of the GAG concentration when comparing the aortas 2/3, 3/5 and tendons from the Warmbloods with the affected and non-affected Friesian horses. Due to the small amount of samples, there might be not enough statistical power to demonstrate a significant effect. This results could be an indication for a systemic problem in the Friesian breed, assuming that there will be a significant difference when using a bigger sample size. This results might be similar to the lower GAG concentration of the collagen as seen in the Ehlers-Danlos syndrome (EDS) in humans. EDS is caused by genetic defects that are characterized by joint and skin laxity, and tissue fragility. A subtype of this condition is caused by the abnormal collagen biosynthesis and recently abnormal GAG synthesis and incorrect post-translation modification of GAG in proteoglycans (PGs) was identified. The hydrodynamic function of the GAGs enable tissues to absorb large pressures changes, providing tissue elasticity. Due to the loss in function of the GAGs in EDS, cardiovascular conditions as rupturation of subcutaneous arteries of veins and (congenital) heart defects can occur (Miyake, Kosho et al. 2014). The increased levels of GAG of the Warmbloods compared to the Friesian horses (affected and non affected) could be a reason for the tissue fragility and an indication for a systemic problem in the Friesian horse.

Because all the horses older than 10 years were left out of the statistic analysis, the study groups became smaller than originally intended. This affected the values of the mean and standard error of the study groups. The non-significant difference seen in the measurements of GAGs in aorta group 1/5 could be due to this fact. Therefore, the result is unreliable and a significant difference in GAGs may still be present.

When comparing the pyrrole cross-links of the different parts of the aortic wall, as well as comparing these between different groups, no significant differences in concentration were found. But the number of horses used for the samples of this study was small, namely 49 samples. Thus, to say for certain that pyrrole cross-links do not play a role in aortic rupture in the Friesian horse, the number of samples need to be extended. This way significant differences can be ruled out for certain.

The differences found in the concentration of GAGs in the aortic walls of Friesian horses compared to Warmbloods, could be used as a new insight in the reason behind aortic rupture. Also other systemic diseases in the Friesian horse could be related to the GAGs, which makes them an important topic for further research.

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