THE ROLE OF MMP'S IN AORTIC RUPTURE IN THE FRIESIAN HORSE

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Index

Abstract

Aortic ruptures are seen quite frequently in the Friesian horse compared with Warmblood horses. Because of the increased prevalence and the characteristic findings in pathologic examination it is suggestive that aberrant collagen is the underlying cause for rupturing the aorta. In human, mice and rats similar aorta pathologies were found and they were proven to be connected with an increased matrix metalloproteinase (MMP) level. Because MMP's have demolishing influence on collagen it is thought that increased levels could lead to weakening of the aortic wall with a bigger risk of rupture as result. To prove this theory samples from different parts of the Friesian and Warmblood aortas were taken and the levels of MMP's were determined with the aid of fluorescence. Finallythe results of this experiment were compared with each other. As a control group also deep flexor tendons of the distal limbs were included in this research. Only in the 1/5 part of the aorta the MMP-levels were increased in Friesian horses, apparently this is not enough evidence for MMP's being the cause of aortic rupture. In the other samples the levels of MMP were higher in Warmblood horses as they were in Friesians, which might be suggestive that Friesians have a lower MMP-level in general which could lead to less renewal of tissue.

Introduction

Aortic rupture in the Friesian horse

Aortic ruptures are seen quite frequently in Friesian horses compared with horses of other breeds.[31,33]
In most of the cases of non-Friesian horses the rupture is located close to the heart at the aortic root.[31,35]
To date more than 40 Friesian horses were diagnosed with an aortic rupture and most of them had a rupture near the ligamentum arteriosum. Preceded symptoms were diverse and there was not an age- or sex predilection.[31]
Friesians with an aortic rupture show a great range of clinical signs such as anorexia, depression, peripheral oedema, tachycardia, recurrent colic and so on. It is also possible that no clinical signs were noticed before heart failure and that the rupture leads to sudden death.In the research of Ploeg et al. in 2013 16 out of the 24 Friesian horses used underwent echocardiography to find out if there were visible pathologies in the heart or the vessels as well. Only a few had a dilated pulmonary artery, a dilated right heart, a pleural or pericardial effusion and/or valvular insufficiencies.[30]
When they looked at the post-mortem findings more similar findings were found. All cases had an aortic rupture that was located very close to the ligamentum arteriosum. The blood that leaked out of the rupture formed a cuff of blood in 8 cases. 13 out of the 24 Friesian horses did have an aorto-pulmonary fistulation as well and findings like pleural effusion, pulmonary oedema and liver congestion were present in many cases.[30]
Because of the similar findings at post mortem investigation it is suggested that the pathogenesis of aorta rupture is unique for the Friesian breed. Van der Linde-Sipman recognized earlier that the location of rupture is situated proximal to the scar of the former outlet of the ductus arteriosus. They believed that tension of the ligamentum arteriosum, what is a vestige of the ductus arteriosus, could affect damaging of the aorta or pulmonary wall. This because the scar tissue should be predisposed to prolapse or rupture.[31,41]

Building of the aortic wall and histologic pathologies found in ruptured aortas.

The aorta consists of three layers which are called the intima, media and adventia. The intima is covered on the inside with endothelium and supported by the basal lamina. After that, a subendothelial layer follows, this layer is called the lamina elastic interna and has an high amount of elastin. This layer also contains collagen fibers, fibrocytes and smooth muscle cells. In the aortas of large domestic animals this layer in relatively thick. The most outside part of the tunica intima is called the internal elastic membrane. This part consists of elastin prevailingly and has some gaps which makes diffusion of nutrients into the media possible. The tunica intima is a nonvascular layer so all the nourishment is native from the circulating blood and transendothelial transport. The media is the thickest layer of all and consists of a broad layer of elastic membranes and fibers, collagen fibers and several layers of smooth muscle, because of this the wall is quite stiff and strong. The most inner part of the tunica media is fed by nutrients from the intima, the outer part is fed by little vessels that nourish the aortic wall, these are called vasa vasorum. The tunica adventia consists of elastic and collagen fibers prevailingly. This layer also consist the vaso vasorum that takes care of the nourishment of the adventia and the superficial part of the media.[16,29]

In the literature ruptured aortas from Friesian horses were scored for necrosis of the media, accumulation of mucoid material, orientation of smooth muscle, fragmentation of elastin, fibrosis and/or mineralization of the aorta, presence of fibrin and inflammation in the media

and/or adventia. Also the amount of vasa vasora in the media and adventia were examined. This is based on the protocol determined by Schlatmann et al.[31,34]

All the cases showed media necrosis, mucoid accumulation, elastin fragmentation, fibrosis and inflammation. None of the investigated aortas showed a difference in muscle orientation, but muscle hypertrophy was present in all of them.[31]
The necrosis might be a result of hypoxia because of alterations in de vasa vasorum, at the end only 5 out of 20 horses showed alterations at the vasa vasorum and all of them were coming from the subacute or chronic group. Because of this it is quite unlikely that changes in de vasa vasorum has an direct effect on rupturing of the aorta.[31,41]
The mucoid material accumulation present in all investigated horses could be evidence for a primary connective tissue disorder that may be the trigger for a rupture. This accumulation makes that the original structure of the aortic wall is interrupted and because of this the wall becomes extended and less strong. In human mucoid accumulation is already linked with denegerative changes in the aortic wall.[45]
This accumulation is also seen is all cases of aortic rupture in this research, so that is why there is a premonition that underlying connective tissue disorders might have an effect on the prevalence of aortic rupture.[31]
Finally, necrosis can also be caused by mediators native from the media. In humans it is already proved that matrix metalloproteinase (MMP) release could cause damage of the medial part of the aorta.[18,31]
There is some morphologic evidence that the problem might be in the destruction of collagen in affected Friesians as well. In a lot of subacute or chroniccases of aortic rupture disorganization and fragmentation of collagen fibers is noticed. (Figure 1)
This might be connected with each other because of the assaulting activities of MMP's on the collagen network. (Table 2)[32]
Because of the cleaning activities of MMP's in injured tissue the presence of them is always together with an inflammatory reaction which leads to the infiltration of immune cells and possibly to fibrosis. This proteolytic activities could explain the disorganization and fragmentation of collagen and elastin as well.[11,31]

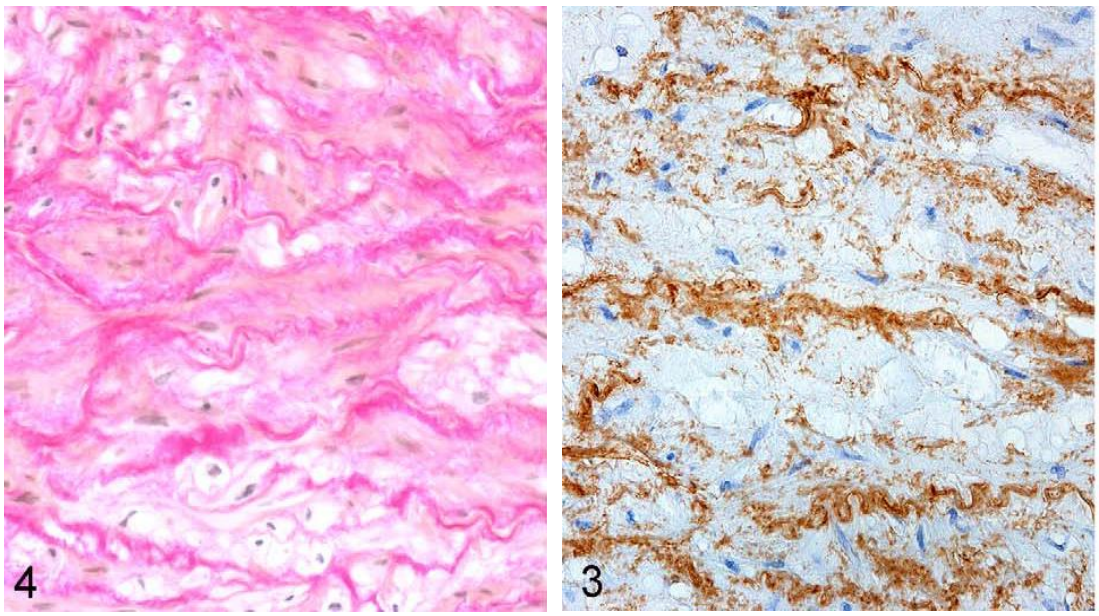

Figure 1 - Histological provement for collagen disorganization (left) and elastin fragmentation (right)in subacute and chronic cases of aortic rupture.[31]

Matrix metalloproteinases (MMP's) and their function

MMP's are enzymes with various proteolytic activities, one of them is to break down the collagen structure in multiple tissues in the body. The family of MMP's has 25 adherents and 24 of them are established in mammals. All the members of the MMP family have a signal sequence protein that regulates the release of the MMP's. Some MMP's will be released in the extracellular space while others do not lose contact with the cell surface.[32]
Different types of MMP's could be involved with the arise of an aortic rupture in diverse ways. The table below shows the most important MMP's and their role in the pathogenesis of Abdominal Aortic Aneurysm (AAA) in humans.

Matrix metalloproteinase	Cells of origin	Role in AAA
MMP-1	Fibroblasts	Degrades collagen (I,II,III,VII,VIII,X) and is associated with increased risk for AAA rupture.[32]
MMP-2	Vascular smooth muscle cells, fibroblasts	Elastin degradation. This dominant gelatinase is necessary in the early stages of AAA formation.[21]
MMP-3	Macrophages	It promotes angiogenesis by activating vascular endothelial growth factor A. It also degrades collagen (II,III,IV,IX,X,XI).[32]
MMP-8	Neurophils	Degrades collagen (I,II,III,V,VII,VIII) and has been associated with AAA rupture, but gene deletion of MMP-8 did not have any overall effect on aneurysm formation.[32,44]
MMP-9	Macrophages, neurtophils	Degrades elastine and collagen (IV,V,VII,X,XIV). It is the most important gelatinase in later stages of AAA formation. High levels are associated with increased aneurysmal diameter and a higher risk for AAA rupture.[32,44]
MMP-12	Macrophages	Elastolytic activity. It is not detected in normal aortas.[7]
MMP-13	Vascular smooth muscle cells	Degrades collagen (I,II,III,IV) and is associated with aortic rupture.[32]
MMP-14	Macrophages and vascular smooth muscle cells	Activates pro-MMP-2 and is involved in macrophage migration.[32]

Table 1 - The most important MMP's and their role in AAA in human.[32,36]

The expression of MMP's is regulated in several ways, one of them is by gene expression. MMP's are not expressed all the time, they become induced when injured tissue starts to remodel and repair. MMP's are also regulated by zymogen activation. They become released as pro-peptins and need to be cleaved, modified or activated by allosteric influence before they could act on the tissue. Not only the release but also the activity of the MMP's is regulated quite precise. When the actions of the MMP's are too much or too strong, proteolytic enzymes could cleave the active MMP's again and make them unactivated.[32]
The inhibition of MMP's is also enabled by different tissue factors, it is proven in rats that an overexpression of these tissue factors could prevent AAA. So when these factors are not or less present in the aortic tissue it might be a reason for too much catalytic activity that could lead to aortic damage and rupture.[1,8,32]
This hypothesis is also confirmed in human aortic disease by the research of Zhang et al. in 2014, they found high MMP-1 and MMP-9 levels in tissue samples from people with chronic Thoracic aortic dissection (TAD). Zhang et al. showed that the levels of tissue inhibitors of MMP's (TIMP's) were relatively decreased too.[46]
Also in horses the disbalance between MMP's and TIMP's could be an important factor in different diseases, for example Arosalo et al. showed that kidney damage and colic might be results of too much MMP activity.[2]

Because MMP's attack the extracellular matrix they could affect many different types of tissue, diseases as laminitis, osteoarthritis, poor healing skin wounds and degenerative central nervous system diseases are proven to be related with a derailment of the MMP maintenance.[6]

Measuring MMP activity by fluorescence-quenched peptide substrate.

Fluorescence is a frequently used method for the provement of the presence of MMP's in different biological samples. In a lot of assays the use of FS-1 MMP substrate (sequence Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2) is applicable. But, FS-1 is not that suitable for showing collagenases in a sample because they are not that specific. That is why we have chosen to use another substrate which has more substrate properties and improved specificity. This FS-6 substrate has another Lys in his sequence: Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2. By adding the extra Lys in the sequence of FS-1, the new formed FS-6 is more efficient in the hydrolytic step in the reaction between substrate and enzyme. Beside of that FS-6 is more soluble in water which decreases the impact of unfamiliar nonphysiologic effects that might be caused by organic solvents.[25]
This method is based on a short sequenced peptide that could be broken by an enzyme. The enzyme break down the bond between a fluorescenced amino-acid and his quencher by hydrolysis. When this bond is broken the splitted sequence becomes fluorescent.[17,24]
In this case the Mca-group ((7-methoxycoumarin-4-yl)acetyl) is fluorescent and the Dpa-group (N"-Dnp-t_-2.3-diaminopropionic acid) is the quencher. When the enzymes which are in this case the MMP's, were admitted they break the Gly-Leu bond and as a result of that the inhibitor of fluorescence disappears.[17]

The improvement in fluorescence could be measured with the help of a luminescence photometer.
The Photometer uses a light source that could excitate electrons from molecules which are part of the protein. When this excitated molecules returns in their ground state light is released, this light is also known as fluorescence. Logically for detecting fluorescence a substance that could do that is necessary, that is why the FS-6 substrate is used in this case. The light coming from electrons returning in ground state is measured while the excitation light is filtered, this ensures a usable value for fluorescence. The wavelength of the light bundle which takes care that the electrons become excitated is called the excitation wavelength, the amount of light that is released during falling back in ground state is the emission wavelength.[14,19]
Following the results of the research of Neumann et al. the maximal excitation and emission for FS-6 are 325 nm and 400 nm respectively.[25]

Determining the protein-fraction by the Lowry assay.

The Lowry method for protein fraction measuring is a quite specific assay. These method is based on spectrophotometric grounds and contains of two reactions. In the first reaction copper native from the used copper-sulfate dilution binds with the protein under influence of a alkaline environment. After the first reaction the mixture turns blue. Chromogenic groups in the sequence of the protein like tyrosine, tryptophan, cysteine and histindine ensures that the mixture become even more characteristic blue in the second reaction. In the second reaction Folin reagens, which is a phosphomolybdic-tungstic mixed acid, were added and this takes care that the aromatic amino acids in particulary, become oxidated. The blue color is the effect of the loss of 1 or more oxygen atoms from tungstate or molybdate. This process is facilitated by an copper-catalysing mechanism, the copper enables the electron transfer from the aromatic amino acids in the protein to the chromogen which is in this case the Folin reagens.

Because you need electrons for reduction of the Folin reagent and for losing of his oxygen atoms, this part of the reaction makes that the protein mixture becomes more sensitive for getting the blue color.[22,23,27]

When all unknown protein mixtures are placed in the ELISA wells plate they were putted in a ELISA micro plate reader. This machine measures the protein fractions on the basis of the colorations happened in the antecedent reactions. The quantification of the protein is measured by absorbance. A light bundle with an altered wavelength shines on each well of the 96-wells plate while a light absorbance meter on the other side registers the light that is not absorbed by the protein mixture. The linked computer convert this apperceptions in numeric values.[26,37]

Hypothesis:

Because of the morphologic evidence that aberrant collagen is the base of aortic rupture and because MMP's are proven collagen affectors, it is expected that they are playing a role in the pathogenisis of aortic rupture.
Beside of that, the presence of MMP's in mammals, the high levels of MMP's found in human, mice and rat with AAA or TAD and the correlation of MMP's with different equine diseases are good arguments for the hypothesis that MMP's are involved in aortic rupture in the Friesian horse as well.

Material and Methods:

Samples were obtained from horses that were send to the pathology department in Utrecht or Gent after death. Samples were taken from 20 affected Friesian horses (AF), 12 non-affected but predisposed Friesians (NA/P), 10 non-affected and non-predisposed Friesians (NA/NP) and from 28 Warmblood horses (WB). Each horse included in this research delivered a sample from the 1/5, 2/5 and 3/5 part of the aorta (AO1/5, AO2/5 and AO3/5) and a sample of the deep flexor tendon of the distal limb (DFT). The deep flexor tendon samples of the Warmblood horses were also submitted by 21 other horses coming from the slaughterhouse. All samples were numbered and weighed. From each sample about 0.1 gram is necessary for the further measurement and soon after weighing the samples were frozen again.

The samples were prepared for measuring by homogenization of the tissue, to make the tissue fine enough the frozen samples were packed in aluminum foil and putted in liquid nitrogen. After taking the samples out of the nitrogen they were smashed with a hammer, this method is advised by Dr. F. van Asten.[39] All little parts were collected with a pincet and putted in a green MagNA Lyser tube together with 500 µl of cooled buffer which is made following this recipe: 6,05 gram Tris-HCl, 2,92 gram NaCl, 0,735 gram $CaCl_2$ H_2O, 1,25 ml 20% Triton and 0,5 gram PEG-6000 by pH = 7,5.[25,40]

This tubes were placed in the MagNA Lyser homogenisator (Roche Diagnostics, Almere NL), 4 runs of 15 seconds with 2 minutes of cooling in between is enough for total homogenization. After homogenization the samples were pipetted in eppendorf cups and stored at -20°C.

For measuring the MMP-activity the fluorogenic FS-6 substrate (M-2350, Bachem AG, Budendorf CH) is used, this substrate is high sensitive to MMP-1,-2, -8, -9, -13 and -14. Stock solutions were made in water and become durable by a few hours of freeze drying and after that maintaining at -20°C. To make it useable 20µl 10mM substrate were dissolved in 2 ml of the buffer used before.

When the samples were thawed, 100 µl of it is putted in an eppendorf cup together with 100 µl substrate and 2 µl 100 mM PMSF in iso-propanol (stored at -20°C). This mixture is divided in 2 parts of 101 µl, the first part is added with 100 µl 10 mM EDTA pH 7,4 immediately. The one with EDTA before incubating is the Blanco and the other one is the Sample. Both cups were incubated at 37°C for 30 minutes and after that EDTA is added at the Sample cups as well. Because of the great number of samples in this research, the samples were frozen again after incubating and before measuring.

Measuring is done with the aid of a PerkinElmer LS50B luminescence photometer and the FL Winlab software (Perkin Elmer, Waltham USA). The $excitation_{max}$ is trimmed at 325 nm while the $emission_{max}$ is trimmed by 400 nm. The slit width is altered at 8 mm and 8mm. Both the Blanco and the Sample cups were measured and the difference in fluorescence were determined. ($F_{delta} = F_{sample} - F_{blanco}$)

This F_{delta} value were corrected for the protein concentration of the sample. ($F_{corrected} = F_{delta}$ / protein fraction) This fraction is measured with the help of the Lowry protein fraction determination. During the determination the protocol used by Flores (1978) is followed. In order to that 1 L of solution A consists of 10 g 1% SDS, 20 g 2% Na_2CO_3, 4 g 0,4% NaOH and 1,6 g 0,16% K/Na-tartrate. 100 ml of solution B is made of 4 g 4% $CuSO_4$.When all amenities are complete 100µl of solution A is added to 20 µl of the sample in a 96-wells plate. For every protein mixture two wells are used. Solution B have to be mixed with solution A in a 1:50 dilution before it is useful in this determination, after that 100 µl of this mixture is also added. After about 10 minutes 20 µl of Folin reagens in a 1:1 dilution with water could be added too. This composition has to incubate at room temperature for about 45 minutes. For valid results a Bovine Serum Albumine (BSA) standard ranging is made for 1000, 800, 600, 400, 250, 100, 50 and 0 µg.[10,40]

The protein concentrations were measured with the aid of a VersaMax ELISA microplate reader. The linked computer that is necessary for converting the apperceptions in numeric values used the SoftMax Pro 5 computer program. (Molecular Devices, Silicon Valley USA) The quantification of the protein is measured by 750 nm.

All the obtained values were collected and the statistical analysis between all groups is done by a Kruskal Wallis analysis of variance of ranks. For more specific results a comparison following Dunn's method is done between the separate groups. During the statistical analysis a confidence interval of 95% is used.

Results

The values obtained from the experiments we did gave quite surprising outcomes after the statistical analysis. For all exact values used for the analysis see the attachment at the end of this paper.

AO1/5	AF	NA/P	NA/NP	WB
Mean	114,039	45,582	36,113	27,683
Median	80,125	41,594	33,823	7,286

AO2/5	AF	NA/P	NA/NP	WB
Mean	38,482	21,032	30,357	91,134
Median	41,231	16,325	21,368	94,846

AO3/5	AF	NA/P	NA/NP	WB
Mean	37,874	54,270	28,301	52,573
Median	14,011	38,190	19,903	33,897

DFT	AF	NA/P	NA/NP	WB
Mean	125,200	172,642	104,109	162,338
Median	111,382	157,135	73,677	144,958

Table 2- Means and Medians of the used values

The first group of samples analyzed were the AO1/5, AF samples. This group has an mean of 114,039 with a dispersion from 10,808 till 395,139. The associated median is 80,125. The NA/P samples had a mean of 45,582 with a range of 8,163 till 106,244. The median of this group is 41,594. The range of the NA/NP samples was 4,729 till 62,983 with a mean of 36,113 and a median of 33,823 while the WB group had a mean of 27,683 and a median of 7,286 by a dispersion of 4,836 till 99,356. As expected the group of samples native from the first part of the aorta in the affected horses (AF) was significant for the group of NA/NP samples (P = 0,040), for the group of NA/NP samples (P = 0,014) and for the group of WB samples as well (P =0,006)

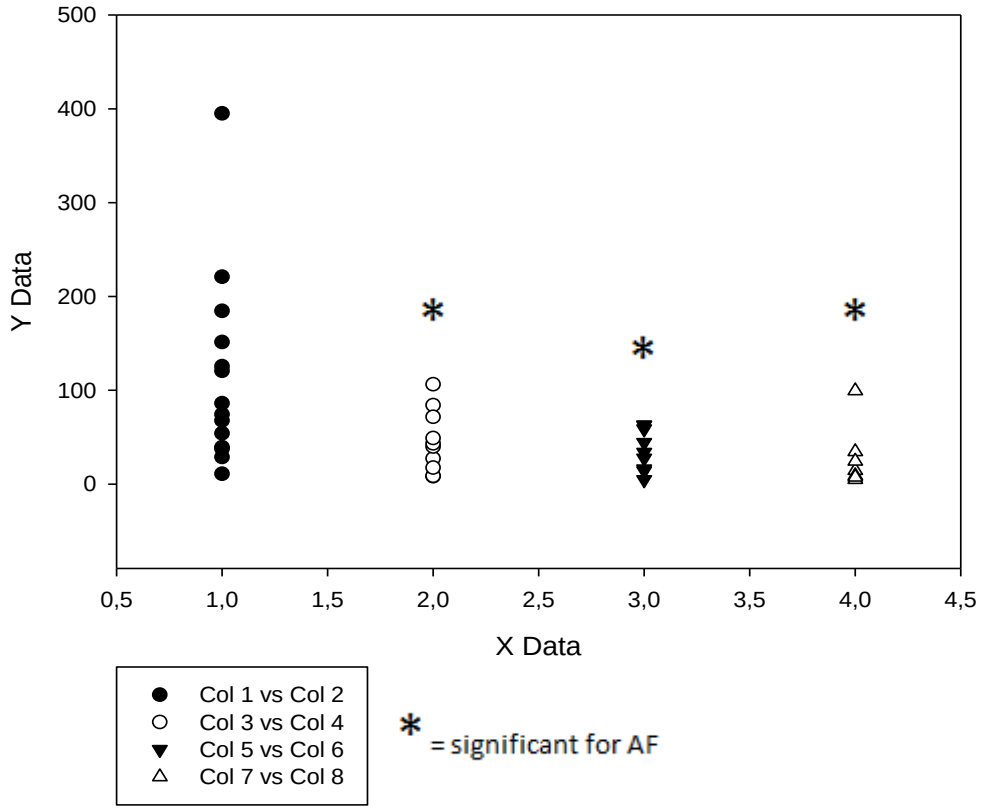

Figure 2 - Scatterplot of AO 1/5 samples

The analysis of the samples of the 2/5 and 3/5 part of the aorta showed a different but analogical pattern. In the 2/5 group AF was not significant for NA/P and NA/NP (P = 0,079 and P = 0,550) while the WB samples were significant for AF (P = 0,001). Also in the 3/5 group AF was not significant for NA/P and NA/NP (P = 0,082 and P = 0,955) but the WB sample were significant for AF (P = 0,029). These values are based on the range of 8,486 till 86,250 for AO2/5, AF which has a mean of 38,482 and a median of 41,231. The AO2/5, NA/P samples had an range of 2,137 till 67,626 with a mean of 21,032 and a median of 16,325. For the NA/NP samples of this group the mean is 30,357, the median is 21,368 by a dispersion of 3,750 till 87,502. The values of the AO2/5, WB samples were spread from 18,061 till 199,918 and have a mean of 91,134 and a median of 94,846. For the AO3/5, AF samples the dispersion was from 1,350 till 258,226 and the mean and median were 37,874 and 14,011 respectively. For the NA/P samples of this group the mean was 54,270 while the median was 38,190 by a range of 9,531 till 206,596. The values for the mean and median of the NA/NP samples were 28,301 and 19,903 by a range of 2,800 till 64,895. The last group, the AO3/5, WB samples were spread from 0,869 till 263,872 with a mean of 52,573 and a median of 33,897.

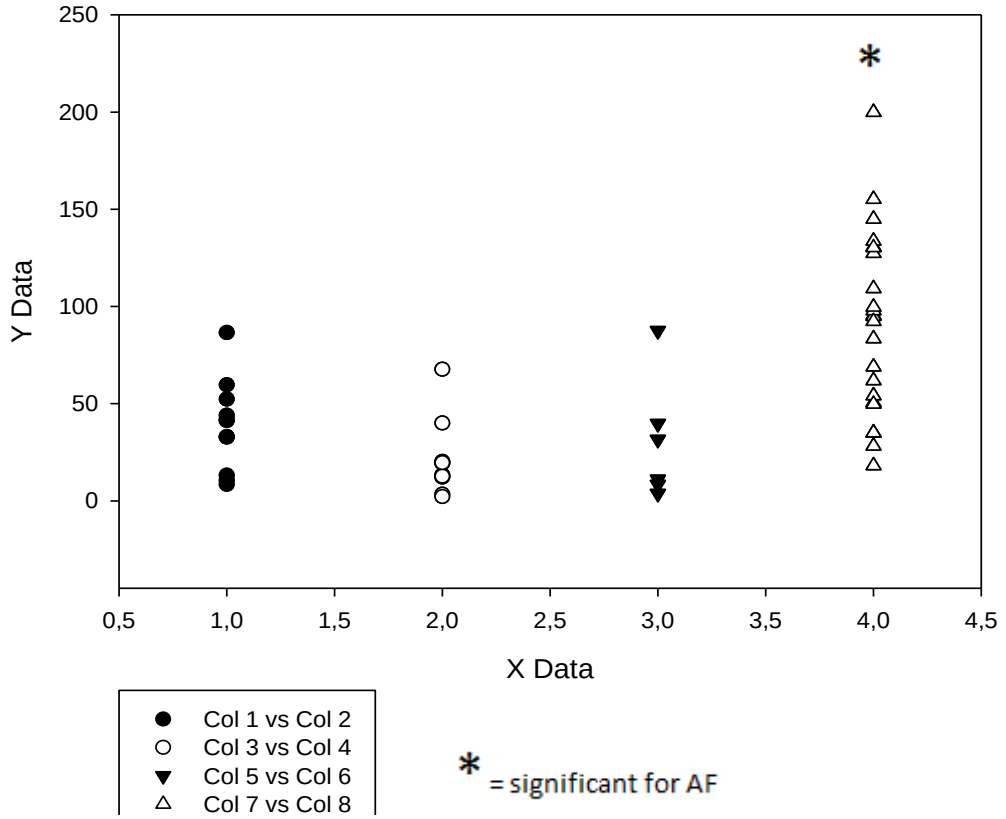

Figure 3 - Scatterplot of AO 2/5 samples

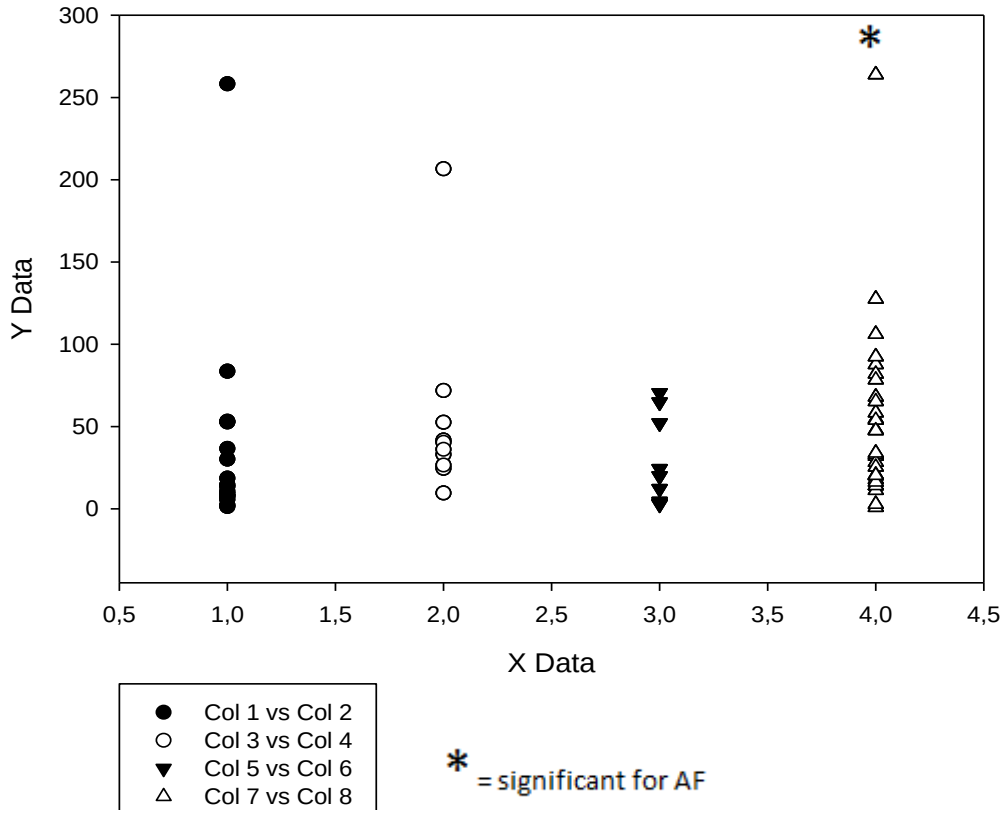

Figure 4 - Scatterplot of AO 3/5 samples

Finally the deep flexor tendon samples were analyzed and there seems to be a significant difference between AF and NA/P (P = 0,013) and between AF and WB (P = 0,006). AF and NA/NP were not significant for each other (P = 0,439). This analysis is done by the aid of the following values: DFT, AF had a mean of 125,200 and a median of 111,382 while the results were spread from 70,491 till 233,183. The NA/P values were spread from 112,085 till 250,377 with a mean of 172,642 and a median of 157,135. For the NA/NP values the dispersion was 58,157 till 152,970 while the values for the mean and median were 104,109 and 73,677. Finally the WB samples were spread from 101,660 till 324,215 and had a mean of 162,338 and a median of 144,958.

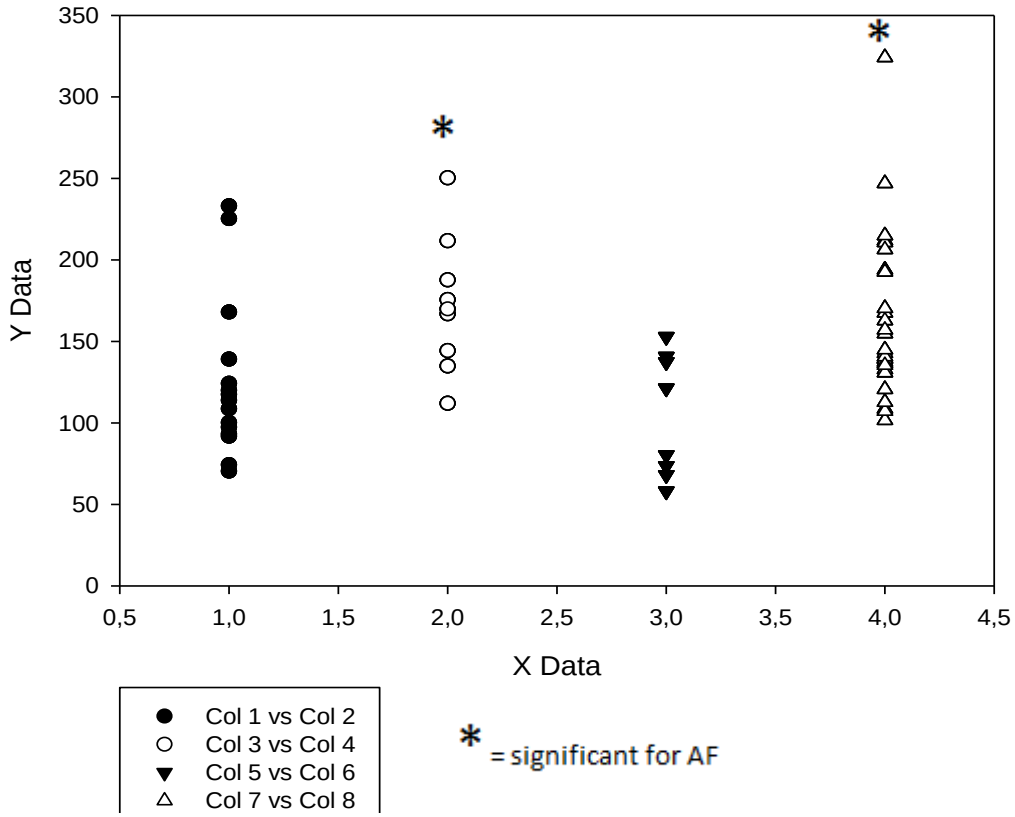

Figure 5 - Scatterplot of DFT samples

Conclusion

As a result of this analysis we could conclude that MMP's did not have that influence in aortic rupture in the Friesian horse as they have in human aortic aneurysm and rupture. There are some significant results but they were reversed from what is expected based on the research done in human, mice and rats. Beforehand it was expected that the amount of MMP's would be increased in horses that were died because of aortic rupture. However, the results of this research showed that it is more likely that the levels of MMP's are decreased in Friesian horses in general compared with Warmblood horses.

Discussion

The results are quite satisfying but even all the experiments are done with great care there is always a possibility for little mistakes caused by human failure or just coincidence. A big point of irritation during the first phase of the research were the missing monsters, because the obtaining from samples was done both in Gent and in Utrecht as well, a lot of samples were lost during transportation or just lost by an unknown cause. An good example of that are the DFT samples from the Warmblood horses obtained in a Belgian slaughter house, this whole set of samples is lost and could not be involved in the research. The problem of not enough control samples is solved by making a other set of DFT samples native from slaughter horses. Also not all samples delivered were suited to be part of this research, some samples or even the whole horse were decomposed too much to could purvey usable values.

During preparing and categorization the samples were defrosted and frozen again several times, a reason for that is the spreading of the samples between the different cooperative universities but also between the different departments involved in this research. Following the research of Becktel and Jiang freezing and thawing has an effect on the stability of protein mixtures and on the activity of the proteins as well. Both the stability and the activity decreases when a sample become frozen and increases when it becomes thawed again. But, following research the activity increases back to about just 90% dependent of the protein concentration. So when samples with different protein concentrations are frozen and thawed several times like we did, it is imaginable that this could have an effect on the reliability of our results.[4,15]

Because of the missing samples some groups of horses became even smaller than they already were. Unfortunately, the groups of affected, not affected but predisposed and not affected plus non predisposed horses were quite small. A reason for that might be the fact that not all 'sudden death Friesians' were send for section or that they were send too late and that they could not be included in the research anymore. For that reason it is decided to not make a difference in age, which would mean that some groups included just one horse. Beside of that, the research of Ploeg et al. showed that an age predisposition is quite unlikely in the prevalence of aortic rupture in the Friesian horse.[31]

Also during running the protocol a few unforeseen things happened; for example, there was no standard and normally used protocol for homogenization of aortic tissue. Therefore it is possible that not the most optimum protocol has been used. Even after freezing in liquid nitrogen, smashing with a hammer and run 4 rounds of 15 seconds in the MagNA Lyser instrument not all tissue became pulp. In some samples a few little lumps remained and because they could not be pippeted, this lumps of tissue were not included in the measurement. Running more rounds or longer rounds in the MagNA Lyser is tried but the samples became too hot, which might have an negative effect on the further determinations.[4] Because of the remaining lumps the increasing fluorescence could not be corrected for the weight of the used samples, in order to that a Lowry protein quantification is done which ensures that the amount of protein in the sample could be determined quite precise. Correcting the increasing fluorescence for the protein fraction would give more reliable results than when they were corrected for the weight.

During the preparing of the FS-6 substrate used for enabling the measurement of fluorescence a little mistake is made. For making the stock solutions of 10 mM the substrate has to be solved in 800 µl water, because of human failure the substrate were solved in 600 µl water. Because of this the concentration of the substrate is higher than the concentration used in the guiding protocol.[25]
Unless the protocol could not be achieved as prescribed we decided to use this 'wrong' solution because FS-6 was not in stock anymore and the higher concentration would not envenom our determinations. The FS-6 substrate become fluorescent after cleavage by MMP's so an abundance of substrate would not make any difference.[17,40]

As mentioned before a Lowry protein quantification is done for getting more reliable results, nevertheless this essay has his own disadvantages. At first some components used in the buffer could interfere with the results for the protein quantification. Triton X-100 and Tris are an example of that. These components could disturb the measurement but a defined concentration is necessary too.[26,27]
The same applies for EDTA which is used to stop the reaction between FS-6 substrate and the MMP's in the sample. EDTA could interfere with copper that is originally necessary for the coloring reactions.[27,43]
These problems are solved by adding sodium dodecylsulfate (SDS) to the used solutions. SDS prevents the precipitation of the different components after reacting with each other and it ensures the solution of proteolipid substances. Even though it is proved that SDS has positive effects on the assay and does not interfere, an error in measuring could never be excluded. This could cause distorting proportions that might be a reason for deviating results.[10,28]
Lowry himself reported that the used solutions need to be prepared fresh every day. We did not do that in our research so it might be possible that our results are not fully reliable.[22]
In spite of that, we did add NaOH, which is proven to make the solutions more stable.[12,27]

Also in this part of the research we deviated from the guiding protocol of Flores.[10]
Flores et al. determined their protein fractions at 640 nm while in our research we determined the fraction by 750 nm, this in order to the advice from Dr. C.H.A van de Lest and different articles.[23,27,40]
The blue color that has to be measured has a maximal wavelength of 745-750 nm and a minimal of 405 nm, according to that measuring by 750 nm is acceptable but the results would not be the same as in Flores' research.[27]

It has to be said that it is possible that this research is not complete. The used FS-6 substrate has a high specify for MMP-1, -8, -13 and -14 and in a lower amount also for MMP-2 and -9.[25]
If we use the results of Rizas et al. as our guideline it is also possible that MMP-3 which breaks down collagen and MMP-12 which degrades elastin are involved in aortic rupture.[32]
Because of the properties of the FS-6 substrate those MMP's are not or less included in our results which might imply that these results are incomplete. To be sure another substrate with a more high specificity for MMP-3 and -12 should be used to control and maybe to enhance the current results.

In this research we did a double control by including Warmblood horses and DFT samples in the research. Because DFT tissue and aorta tissue has their own properties it might be that the control provided by the DFT samples is not truly reliable. The mean reason for this is the different types of collagen in the tissues. DFT consist of type 1 collagen fibers predominantly which are longtidunal situated.[3,9]
The aorta has another built, the adventitia consist of type 1 collagen prevailingly while the media is built of 30% type 1 collagen and about 70% type 3 collagen.[5,13]

Not all MMP's are even specific and efficient for all types of collagen, for example MMP-3 and -9 do not cleave collagen type 1, while MMP-14 is very efficient in breaking down type 1 collagen.[20]
It is possible that this leads to unequal break down of the different tissues by the MMP's which are present in our samples. Because we did not allocate the present MMP's in our samples we could not preclude the possible effect of this phenomenon.

The results of this research were quite surprisingly. It was expected that the amount of MMP's would be increased in Friesian horses but this research showed the contrary. The concentration of MMP's were increased in affected horses but this might be explained by the location of the sample. It is proven that aortic rupture in the Friesian horse occurs near the ligamentum arteriosum which is located in the first part of the aorta.[30,41]
Because of the cleaning and renewing functions of MMP's in injured tissue the measured increment in affected horses is explicable.[38,42]
Overall the measured concentrations of MMP's were lower in Friesian samples as they were in Warmblood horse samples. When we match this to the fact of the increased prevalence of aortic rupture in Friesian horses it might be suggestive that less MMP activity is a reason for aortic rupture. This theory is based on the important role of MMP's in tissue remodeling. Too much MMP activity could lead to excessive damage but when the MMP activity is too low the turnover of the aortic wall might be inadequate. When the wall tissue is not renewed regularly it is possible that it become stiff and less strong which could be an inducement for aortic rupture.[38,42]

To preclude the uncertainties above more research is necessary. In this research the attention has to be pointed on the total MMP activity in Friesians compared with Warmblood horses in other tissues too. There has to be some work done in determining the specific MMP's present in the used samples as well. The amounts of the separate MMP's has to be defined and for complete results also the MMP-3 and -12 has to be included. The results of our preliminary research might be a good step in figuring out the exact role of MMP's in aortic rupture in the Friesian horse but if we want to be sure more evidence is needed.

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Attachments

	A	B	C	D		A	B	C	D		A	B	C	D		A	B	C	D
1 (U1)	151,345		53,177	97,454	19 (U9)	27,650	87,502	64,895	152,970	28 (U11)	27,201	13,305	24,575	250,377	39 (Edu230)	14,511		47,876	139,510
2 (U2)	28,827	10,624	2,289	117,508	20 (U10)	4,729		12,208	140,779	29 (U12)	84,106	40,004	71,869		40 (Edu979)	4,836		87,734	211,843
3 (U3)	395,139		52,744		21 (NA/8)	62,983	8,422	70,500		30 (NA/11)	39,857		41,647	211,716	41 (Edu989)	34,513		0,869	101,660
4 (U4)	184,585	32,793	7,898	124,298	22 (NA/15)	33,823		24,333	80,351	31 (NA/14)	8,163	19,344	40,353	166,987	42 (Edu990)	99,356		68,017	137,424
5 (U5)	39,189	52,315	36,612	139,179	23 (NA/57)	58,778		2,800	121,374	32 (NA/16)	71,636	67,626	52,536	175,656	43 (Edu1000)	8,830		92,493	193,933
6 (U7)	86,001				24 (NA/62)	62,600	39,733	19,903	58,157	33 (NA/19)	8,780	12,180	9,531	169,928	44 (U13)	7,286	50,651	106,161	
7 (U8)	54,095	13,076	5,721	113,966	25 (NA/18)	14,049	11,133	52,177	73,677	34 (NA/25)	43,331	12,628	206,596	187,712	45 (A/578)	24,446		2,744	108,699
8 (U14)	220,931	43,964	1,350	233,183	26 (NA/28)	44,286	3,750	4,453	137,414	35 (NA/27)	49,042	20,178	33,036	134,976	46 (s-62)				
9 (U15)	10,808	41,655	18,526	120,013	27 (NA/44)	16,118	31,604	3,437	68,149	36 (NA/55)	17,457	3,300	26,528	144,341	47 (s-63)		28,218	32,016	
10 (A/6)			10,627	91,861						37 (NA/63)	106,244	2,137	36,026	112,085	48 (s-64)		18,061	54,575	
11 (A/14)			258,226	225,326						38 (NA/68)		19,621			49 (s-65)		34,853	58,398	
12 (A/20)	74,249		7,816	70,491											50 (s-66)		95,438	14,049	
13 (U6)															51 (s-67)		94,846	17,797	
14 (A21)	120,549	32,970	9,180	108,799											52 (s-68)		35,041	33,021	
15 (Mona)		8,486	13,476	93,210											53 (s-69)		155,124	65,253	
16 (Tomas)	67,698	86,520	14,546	74,361											54 (s-70)		127,340	28,309	
17 (Ditte)	37,612	41,231	30,186	168,074											55 (s-71)		109,159	53,877	
18 (Azaro)	125,513	59,664	83,602	100,278											56 (s-72)		130,719	11,276	
															57 (s-73)		133,628	14,111	
															58 (s-74)		97,820	33,897	
															59 (s-75)		199,918	20,153	
															60 (s-76)		144,951	263,872	
	AF		**A**	AO 1/5											61 (s-77)		61,814	47,544	
	NA/NP		**B**	AO 2/5											62 (s-78)		130,233	16,279	
	NA/P		**C**	AO 3/5											63 (s-79)		54,065	81,995	
	WB		**D**	DFT											64 (s-80)		49,814	25,382	
	not present														65 (s-81)		83,428	78,448	
															66 (s-82)		99,709	20,649	
															67 (s-83)		68,891	127,513	
															68 (s-84)		92,356	20,312	
															69 (s-85)				167,551
															70 (s-86)				139,046
															71 (s-87)				142,761
															72 (s-88)				132,828
															73 (s-89)				136,533
															74 (s-90)				154,801
															75 (s-91)				162,872
															76 (s-92)				324,215
															77 (s-93)				210,771
															78 (s-94)				107,250
															79 (s-95)				246,976
															80 (s-96)				140,631

															81 (s-97)				170,376
															82 (s-98)				214,900
															83 (s-99)				120,646
															84 (s-100)				206,487
															85 (s-101)				130,860
															86 (s-102)				157,115
															87 (s-103)				112,731
															88 (s-104)				135,463
															89 (s-105)				144,958
															90 (s-106)				192,614

Table 3 - Values for fluorescence of all samples