Aortic rupture and aorto-pulmonary fistulation in the Friesian horse

A search for genetic background

Project summary

This project will be part of a larger research project that maps out the phenotypic (clinical) and genotypic appearance of aortic rupture and aorto-pulmonary fistulation within the Friesian breed. Our research group consists of 4 major partners: **The Utrecht University, The Ghent University, The Wageningen University and Wolvega Equine Clinic.** The prevalence of aortic rupture is estimated to be ± 2% within the Friesian breed, which is much higher than the incidence seen in warmblood horses. Unlike warmblood horses, Friesian horses can develop a chronic form of aortic rupture that most often is overlooked by the owner, leading to possible dangerous situations, such as acute death during exercise. **Aortic rupture is always fatal.** Some horses die instantly, others can walk around with this pathology for weeks to months. And even when a veterinarian suspects a patient of having an aorto-pulmonary fistulation, the diagnostic methods are limited. Ante mortem diagnosis is quite often a challenge because the aorta ruptures in Friesian horses occur at another location than that seen in warmblood horses. Post mortem diagnosis requires adaptation of specific standardized cardiac incision techniques during autopsy. This means that the person who performs the autopsy should suspect presence of aortic rupture before start of the autopsy procedure. It learns us that most probably quite some cases are and have been overlooked in the presence and the past.

Over the course of a few years our research group has managed to pool 46 fully illustrated aortic ruptures subjected to complete protocolled autopsy and pre-mortem diagnosis. This has led to the publication of International peer reviewed articles about the disease and presentations at International conferences. In view of the difficulties to recognize and diagnose this pathology, it is important that a lot of clinicians have access to this information.

Epidemiological check of the pedigree of all ruptured cases, going 5 generations backwards has learned us a lot about the way this pathology is inherited. It also has helped us to identify a proper population of Friesian horses that can function as control population for genetic studies. We are currently checking for relatedness between our population of aortic ruptures on one hand and the population of dwarfism and hydrocephaly in horses on the other hand.

This project aims to identify the genomic regions upon which the genes are located that are responsible for aortic rupture and aorto-pulmonary fistulation in the Friesian horse, and Identify strategic regions of base pairs to develop a suitable genetic test that can be used by the studbook to identify carrier horses. This will help to give proper breeding advise, for example which combinations can be created in a safe way?

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Aim of the project is to:

- Identify the genomic regions upon which the genes are located that are responsible for aortic rupture and aorto-pulmonary fistulation in the Friesian horse.
- Identify strategic regions of base pairs to develop a suitable genetic test that can be used by the studbook to identify carrier or susceptible horses
- Sequence the base pare region in order to exactly identify the mutation that is responsible for this genetic disorder

Why is this project needed?

At this moment, most probably Wolvega Equine Clinic in the Netherlands and also to a lesser extend The Equine Clinic of The Utrecht University are the two clinics that receive the largest number of Friesian horses in the world on a yearly basis. When checking for the prevalence of aortic rupture and aorto-pulmonary fistulation within this clinical population, we come to a **prevalence of about 2%**. This means that 2 out of 100 Friesian horses that are admitted to one of both clinics are presented with aortic rupture. Which is likely a lower limit of the true incidence in the population, because on one hand not all horses with aortic rupture are collected by our group or on the other hand not all horses are challenged in their life to express their susceptibility. For comparison: prevalence of neonatal isoerythrolysis, a condition that is taught to veterinary students, in depth, because they are quite likely to encounter this problem in their daily practice, also has a prevalence of 2%[1].

AORTIC RUPTURE HAS A PREVALENCE OF 2% IN THE FRIESIAN BREED. FOR COMPARISON: DWARFISM IN THE FRIESIAN BREED HAS A PREVALENCE OF 0.25[2]%

Because of close collaboration between Wolvega Equine Clinic, the faculty of veterinary medicine of the Utrecht and Ghent University, 46 cases of aortic rupture in the Friesian horse have been collected up until now. The mean age of our cases was 4.4 years old, with a range from 1 to 20 years old[3]. This means that some horses, before the aortic defect became visible, could have had quite **a successful reproductive career**, which is less favorable in case of carrier stallions. This is quite different from other genetic disorders within the Friesian breed, such as dwarfism and hydrocephaly, which clearly are visible at birth or at very early age[2,4,9].

Based upon the research our group already has performed, we know that three forms of aortic rupture can be identified: the **acute form**, the **subacute** and the **chronic form**[3,5]. The acute form entails that these **horses acutely die**, most often in full action, not rarely while being ridden. These are very **dangerous situations**. The chronic form actually had not been identified before our group started to map out anamnesis and background history of all 46 ruptured cases. We know now that Friesian horses actually can walk around with this condition for weeks to months

before they actually die. Again, this can happen in a **very acute and dangerous situation**. We know now that these **chronic cases** often show symptoms like recurrent colic, cough due to subtle pulmonary edema, recurrent nasal bleeding, poor performance, listlessness etc. weeks to months before they die. From **animal welfare** point of view research on the aetiology as well as methods to alleviate this disorder from the population is justified. Obviously it is important to identify these horses in an early stage.

AORTIC RUPTURE IS THE 13TH LEADING CAUSE OF DEATH IN HUMANS

In **human medicine aortic rupture** research, a lot of attention is focused towards collagen and elastin, two very important constituents of the aortic wall[6,7,13]. The Friesian horse research can provide **important strategic information for human research** into aortic rupture[8,10], one of the leading causes of human deaths in the world[11].

Last but not least: Interestingly, histopathological examination of aortic wall more than 40 cm away from the site of rupture, towards the abdomen, shows aberrant collagen build-up of the aortic wall. Collagen is the most important building stone of the aortic wall. In this respect we had the striking discovery, that the same aberrant **collagen build up** is seen in the esophageal wall of Friesian horses suffering from **mega-esophagus** (nonfunctional dilation of the esophagus, leading to recurrent esophageal obstruction). This overlap in collagen aberrant build up can be an indication of the same genetic defect.

Chapter 1: What do we know so far?

1.1 Comparison with human aortic rupture

In humans, aortic rupture is the 13th leading cause of death in the world[11]. Therefore a lot of research is directed towards this fatal pathology. It is important to understand the aortic rupture in human and warmblood horses first, so we can compare them with the aortic rupture in the Friesian horse. There are two types of human aortic rupture:

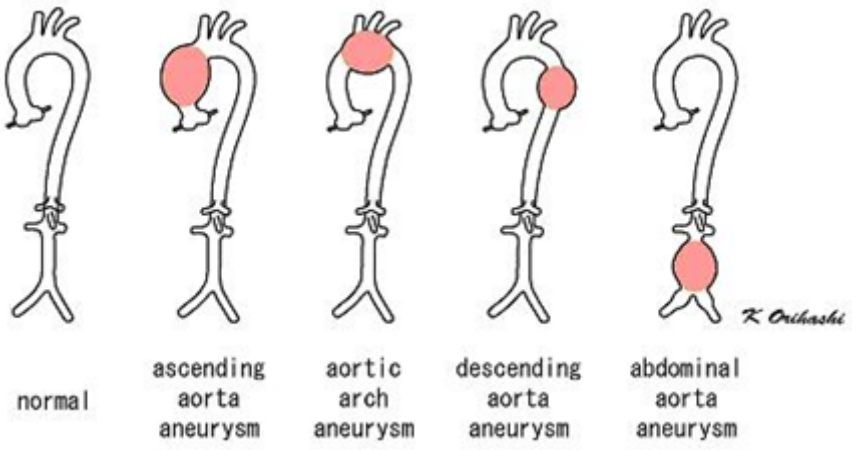


Figure 1: various aortic aneurysms

1.1.1. rupture of the abdominal aorta

This form is encountered most often and responsible for 74% of human aortic rupture cases[11]. This type of aortic rupture is preceded by prior aneurysm formation[6,12] (read: dilation of the aortic vessel in an ampulla form, see figure 1). People develop abdominal aortic rupture due to unhealthy life style, such as smoking, alcohol abuse, fast food, etc[6]. The risk factors for abdominal aortic aneurysm include male gender, smoking history, advanced age, family history, dyslipidemia and hypertension. The disease appears to be a result from interactions between environmental risk factors[7,8,] and genetic predispositions, which exacerbate the normal ageing processes[14,15].

1.1.2. rupture of the thoracic aorta.

This form is responsible for 24% of cases and has a clear genetic background. Typical genetic diseases such as Marfan's Disease, Ehler's Danlos and Luiz Dietz syndrome are associated with this type of aortic rupture[23]. These genetic diseases all have their repercussion on elastin and/or collagen, two important constituents of the aortic wall[6,7,13].

A first reported location for thoracic aortic aneurysm to occur in humans is the sinus of Valsalva[18](figure 2) nearby the base of the heart, a location also often reported in horses[21,22]. Occurrence of congenital aneurysm is more common at this location and is most often caused by weakness at the junction of the aortic media and the annulus fibrosus[18,19]. The structural defect of a congenital sinus of Valsalva aneurysm has been described as a lack of continuity between the aortic media and the aortic annulus. Although the congenital defect is present at birth, the aneurysm usually takes several years to develop as a finger-like extension with perforation frequently occurring at the apex of the aneurysm[20].

Besides the classical aortic aneurysm formation, followed by rupture, scientific reports also describe a rarely occurring but fatal spontaneous rupture of the human thoracic aorta[16,17,18,20]. This rupture occurs in the absence of trauma, infection, iatrogenic injury and without prior aneurysm formation, all factors which normally are the common causes. Patients suffering from spontaneous aortic rupture mostly complain about chest- and back pain and a sense of compression. Furthermore, hoarseness, superior vena cava syndrome, cough and dyspnoea may appear when these pseudoaneurysms(figure 3), resulting from spontaneous aortic rupture, compress the surrounding tissues[16]. Overall the symptoms are very vague and unspecific. In general, spontaneous aortic rupture may involve the aortic root, ascending aorta, aortic arch or descending aorta[18,19,20].

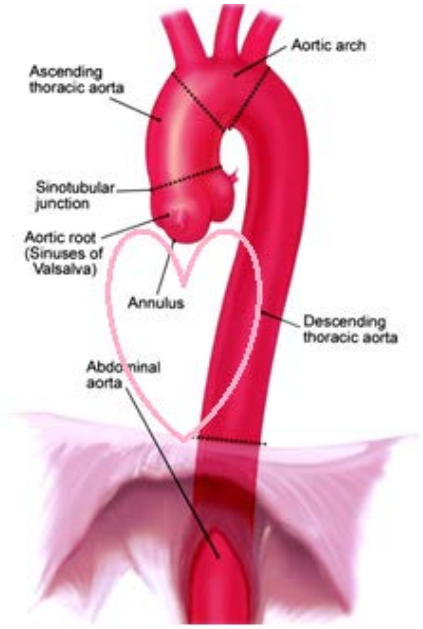


Figure 2. Anatomy of thoracic and proximal abdominal aorta. (©Massachusetts General Hospital Thoracic Aortic Center.)

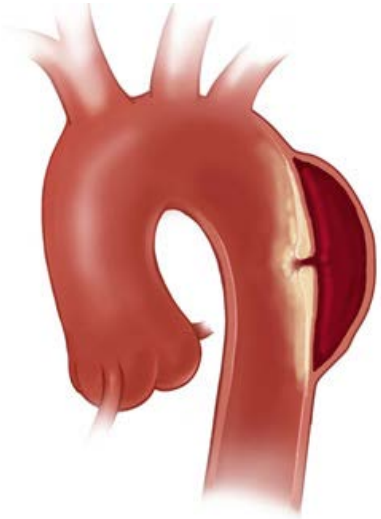


Figure 3: Pseudoaneurysm

1. 2. Aortic rupture in warmblood horses:

Acute aortic rupture is well known in warm blood horses[21,22,24]. We all remember on November 6th 2011 the horrible images of the international show jumping horse Hickstead of Erik Lamaze, who acutely died at an International show jumping competition in Verona, Italy, in the middle of the jumping arena due to aortic rupture.

AORTIC RUPTURE IN WARMBLOOD HORSES OCCURS CLOSE TO THE JUNCTION WITH THE HEART IN CONTRAST TO FRIESIAN HORSES THAT RUPTURE ALWAYS AT A MORE DISTAL LOCATION

Proposed possible causes of aortic rupture in horses are a congenital aneurysm, copper deficiency, long-term degenerative disease with weakening of the aorta, migration of Strongylus vulgaris larvae and extreme high blood pressure.[21,22,24].

Especially the acute death of the somewhat older breeding stallions in full action is well known amongst horse owners[22]. What we see in those cases is that the rupture appears near the sinus of Valsalva, very close to the junction of the aorta with the heart. This means, that when a rupture in the aorta occurs, the pericardial sac around the heart rapidly fills itself with blood. The heart will be compressed en there will be a swift death within seconds to minutes. In a small number of cases the rupture even occurs within the heart. This can result in a connection between the right atrium and left ventricle of the heart. These horses often show signs of colic and an increased ventricular heart rate[27,28].

There are no medical or surgical cures for aortic rupture in horses. If the horse survives, than rest and supportive cardiac medical therapy can help to temporarily reduce the cardiac symptoms and distress. However, the horse will eventually die due to cardiac failure[27,28].

3.3. Aortic rupture in Friesian horses

Over the course of a few years Wolvega Equine Clinic, the faculty of veterinary medicine of the Utrecht and Ghent University, have been confronted with several cases of Friesian horses suffering from aortic rupture, all with fatal outcome. From all cases presented to the clinic with aortic rupture, 75% is from the Friesian breed. This percentage is quite high if you imagine that the other 25% includes all non-Friesian breeds and 9% of all breeds presented to the clinic is a Friesian. This indicates that aortic rupture has a much higher incidence in the Friesian breed when compared with warm blood horses[3].

AORTIC RUPTURE HAS A MUCH HIGHER INCIDENCE IN THE FRIESIAN BREED WHEN COMPARED WITH WARMBLOOD HORSES

Recently our research group has reported that the rupture in Friesian horses occurs at a very specific location, which differs from the location reported in warmblood horses[3,25,26,27]. In all Friesian horses aortic rupture occurs further downstream the course of the aorta when compared to warmblood horses**. In Friesians rupture always occurs several centimeters away from the heart, close to the ligamentum arteriosum at the level of the aortic arch[3,5].** These

aortic ruptures seems to occur in the absence of trauma, infectious disease or iatrogenic trauma. The specific location for the Friesian breed suggests a genetic basis for this disease within the Friesian breed.

Current findings at postmortem examination show that there are **3 different forms of aortic rupture** to be discerned within the Friesian breed: **the acute, the subacute and the chronic form**[3].

In the acute form the aorta ruptures into the thoracic cavity leading to acute hemorthorax (pooling of blood into the thoracic cavity) and subsequent death within minutes. Horses suffering from this acute form, showed signs of acute dead during rest or physical strain, with no preceding symptoms.

In the subacute form horses show more subtle signs of illness, weeks to even months before they are presented with signs of acute cardiac failure. These horses are able to encapsulate the aortic rupture before it will burst. They create a cuff like bandage around the rupture, filled with blood clots, comparable to an isolation sleeve around a water pipe. Every once in a while more blood will flow into this cuff and the horses heart encounters problems with pumping blood out of the heart into the aorta that obviously is partly squeezed at its origin due to the presence of this cuff like structure. Noteworthy: sometimes this cuff is more than one meter long! Quite often this obviously very dangerous situation is overlooked by the horse owner, since these horses only show subtle signs of illness. Then, one day, the rupture breaks through into the pulmonary artery, that crosses underneath the aorta. At that point acute cardiac failure will evolve together with pulmonary oedema, since the aorta will pump through the fistulation a load of extra blood into the pulmonary artery, right into the lungs, with every heartbeat.

ALSO SUBACUTE TO CHRONIC CASES TEND TO DIE IN FULL ACTION, LEADING TO VERY DANGEROUS SITUATIONS

3.3.1 Clinical signs of aortic rupture in Friesian horses; a guide line for equine vets[3]

Our research demonstrates that accurate ante mortem diagnosis of this condition is quite a challenge at present. Case history and specific clinical signs may give helpful indications, however definitive ante-mortem diagnosis requires specialized experience with this disease..

Case history highlights for ruptured cases

Obviously equine vets are most often confronted with subacute to chronic cases. In view of the mean age of rupture in Friesian horses, one can expect that environmental factors are important as well for developing aortic rupture in Friesian horses. Quite often anamnesis learns us that symptoms became clear a few months after start riding these horses. Owners tend to mention that they didn't notice any abnormal behavior in the weeks or months prior to start riding these horses. So apparently applying physical strain is often the start of all problems in predisposed Friesian horses. Often, ruptured young horses are presented to veterinary clinics soon after being broken, with the complaint of listlessness and poor performance. An explanation for this could be found in the fact that when a body is exposed to physical strain the heart rate will go up and blood pressure will increase. Both, the increased heart rate and the blood pressure have a

negative impact on the aortic wall. This indicates that environmental factors, besides genetic factors alone, also have their impact on developing an aortic rupture. Something which is well described in human medicine. One or more of following signs are often reported by the horse owner:

Signs reported by the horse owner
Colic
Loss of appetite
Restlessness
Depression
Poor performance
Coughing in the days or weeks prior to the fatal cardiac failure
Noose bleeding
Peripheral oedema, which can wax and wean
Fever(≥38.5°C)
Increased heartbeat at rest
Varying lameness typically switching from one leg to the other

In all cases suffering from recurrent colic, rectal examination reveals no abnormalities such as impaction or displacement of the intestine.

> ***ONSET OF PROBLEMS RIGHT AFTER START UP OF RIDING YOUNG HORSES LEARNS US THAT DEMAND OF PHYSICAL EFFORT IN PREDISPOSED FRIESIAN HORSES CLEARLY IS A STIMULATING FACTOR***

Clinical examination; highlights

One of following clinical signs can be encountered:
Increased rectal temperature (>38.5°C)
Peripheral oedema
Increased respiratory rate (>14 breaths/min)
Increased hyperkinetic arterial pulse rate (>40 beats/min)
Carotid hammer pulse
Pronounced jugular pulsations
Pale mucous membranes
Cardiac arrhythmias
Increased heart rate and presence of heart murmurs, most often diastolic on the left side

Final diagnosis requires specialized experience with this disease. Specific echocardiographic windows need to be viewed, other than the cross sections that are usually made during cardiac

ultrasound. In more heavy horses triceps musculature will impede proper transthoracic ultrasound viewing. In these cases trans-esophageal ultrasound can be used. This technique was developed by our research group specifically for this purpose. Our group is also working on intra-cardiac ultrasound. Finally heart catheterization can aid in making the diagnosis.

ANTE-MORTEM DIAGNOSIS IS A REAL CHALLENGE. MANY CASES SHOW NO GROSS PATHOLOGICAL ABNORMALITIES AT THE LEVEL OF THE HEART WHEN OPENING THE THORACAL CAVITY

Gross post mortem examination; highlights in ruptured cases

It should also be realized that this aortic disease requires a specific approach of the post-mortem examination as well, especially with respect to the cardiac incisions. These should be made in such a manner that the diagnosis cannot be missed. Classical cardiac autopsy incisions that are taught to veterinarians will section the ruptured area making a definitive post-mortem diagnosis impossible. Therefore, in case veterinarians suspect presence of aortic rupture or aorto-pulmonary fistulation, an adapted cardiac incision protocol needs to be applied: after in situ examination and opening of the pericardial sac, the thoracic aorta at the diaphragm should be dissected. The heart, thoracic aorta, some lung tissue that is left attached and the undamaged pulmonary artery are removed from the thoracic cavity. The left ventricle (up to the aortic valves) and atrium are opened by cutting from the apex following the caudal border of the left ventricle. Then the right ventricle and atrium are opened with a V-shaped incision with the apex of the heart forming the tip of the V. The truncus pulmonalis is then cut open and, when present, the edges of a pulmonary rupture can be observed. Next the dorsal side of the aorta is incised from the thoracic side towards the heart base. By cutting through the cuff of blood surrounding the aorta and transecting the aortic tear, the scar of the ligamentum arteriosum will be transected. In this area a small probe is needed to visualize the aorto-pulmonary fistula, if present.

PROPER POST MORTEM DIAGNOSIS REQUIRES AN ADAPTED CARDIAC INCISION PROTOCOL. CLASSICAL INCISION PROTOCOLS WILL CROSS THE LOCATION OF THE RUPTURE, IMPEDING CORRECT DIAGNOSIS.

Important to realize is the fact that in many cases no macroscopic abnormality is visible at the level of the heart when opening the thoracic cavity. In other words: proper incision of the heart is necessary to visualize the fistulation and the rupture. Most probably a lot of cases have been overlooked in the past because of this. Pictures from the ruptured sides are added in the appendix.

Chapter 2: Epidemiological study of pedigree of ruptured Friesian horses

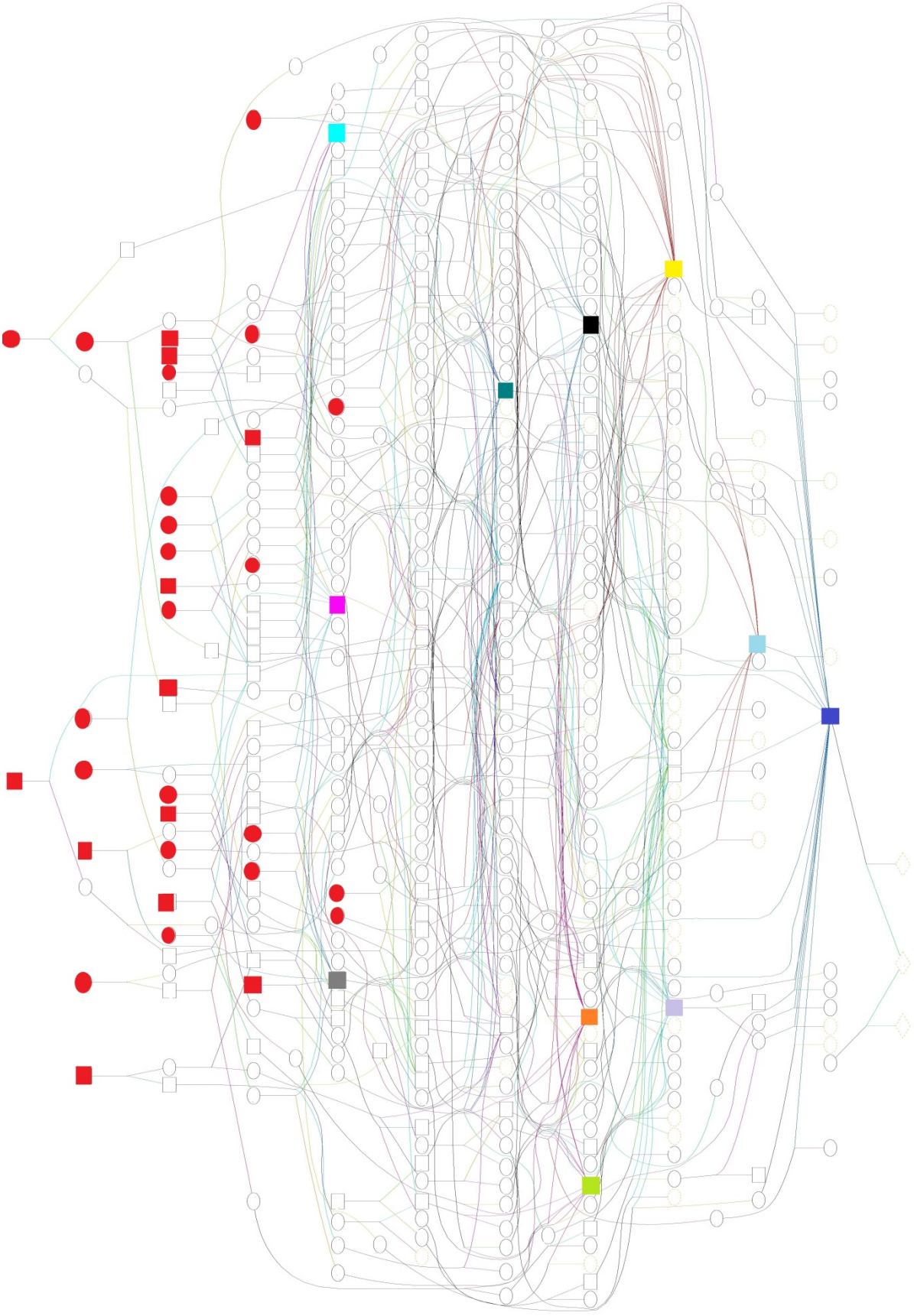
To see if there is a correlation between the genetic background and aortic rupture in Friesian horses, we compared the pedigree of 35 ruptured cases in cooperation with the Wageningen University. Data from Friesians with a missing or unclear identity number where not included in the study. Using the information provided by the Friesian studbook we managed to compare the pedigrees for each horse up until respectively over 3 and 5 generations. There were 21 mares, 11 stallions and 3 geldings included with a mean age of 4.4 years.

At first we created a pedigree for over 5 generation. This pedigree was not really clear as there were so many horses involved, the figure became blur. Than we decided to make a pedigree for over 3 generations but it did not show enough background. Studying the two pedigrees we noticed one horse in particular that had a big impact on our pedigree. We decided to make his pedigree and the outcome is shown in figure and table 4.

We not only compared the pedigrees of the ruptured cases with each other, but we also compared them as a group with other Friesian horses of the same generation (1999-2009, 60.000 in total). Because the Friesian horse studbook is a closed studbook with a small genetic base, it is not a surprise that every horse is somehow related to another[29]. Therefore also an effort was made to check whether horses within the ruptured group were more related to each other than the relatedness seen within the generation group. The importance here was to see if our group of cases had a higher relatedness than their peer generation in general. Results of that analysis showed that there was indeed a higher relatedness in the ruptured case group than the generation group, clearly supporting a hereditary genetic background for this disease.

A GENETIC BACKGROUND FOR AORTIC RUPTURE HAS BEEN DEMONSTRATED BY A HIGHER RELATEDNESS SEEN AMONG THE RUPTURED CASES

We also looked at which founders were represented in the pedigree of the ruptured case group. Again, representation of these founder stallions was compared with their representation within the generation group. We calculated the total and the marginal contribution of every horse and results showed that some founders that where listed high in genetic contribution to the ruptured case groups where not that important for their peer generation group. On the other hand, some horses which had a big contribution in the generation group were not that much represented within the ruptured case group. Again this finding clearly supports a hereditary genetic background for this disease.



Figuur 4

Table of figure 4

	Cases of aortic rupture. Squire = stallion, oval = mare
	Stallion who has a big contribution in the aortic rupture cases and in the generation control group
	Stallion who has a big contribution in the aortic rupture cases, but did not contribute that much in the control generation group.
	Stallion who has a big contribution in the aortic rupture cases, but contributes less in the control generation group.
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	Stallion who did not have a big contribution in the cases and the control group
	Stallion who has a big contribution in the aortic rupture cases and in the generation control group
	Stallion who has a big contribution in the aortic rupture cases, but did not contribute that much in the control generation group.

Chapter 3: Project plan

THE AIM OF THE PROJECT IS TO IDENTIFY GENOMIC REGIONS RESPONSIBLE FOR AORTIC RUPTURE IN FRIESIAN HORSES AND TO SEQUENCE THIS REGION IN ORDER TO IDENTIFY THE RESPONSIBLE FUNCTIONAL GENES

3.1. What is a genome-wide association study?

In genetic epidemiology, a genome-wide association study (GWA study, or GWAS), also known as whole genome association study (WGA study, or WGAS), is an examination of many common genetic variants in different individuals to see if any variant is associated with a trait. GWAS typically focus on associations between single-nucleotide polymorphisms (SNPs) and traits like major diseases, in our case aortic rupture. A SNP can be explained as a part of the genome where at least 1% of the population will show a genetic variation. Every population has a various number of SNPs.(figure 5)

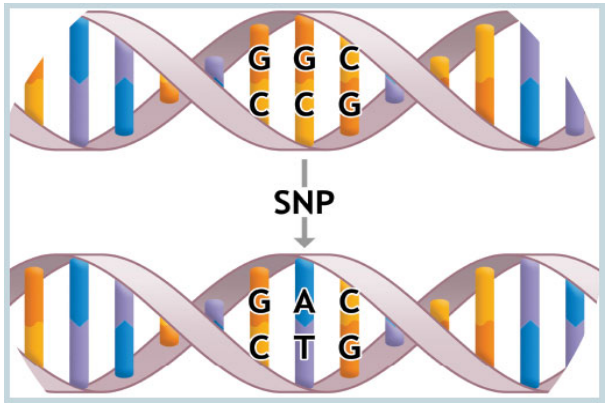

figure 5: A variation in a part of the genome

In case of diseases, GWAS studies normally compare the DNA of two groups of participants: horses with the disease (cases) and similar horses without (controls). From each horse a sample of DNA is retrieved, from which 70.000 genetic variants are read using SNP arrays (an array of almost 800.000 SNPs will soon be available to test horses) . If one type of the variant (one allele) is more frequent in horses with the disease, the SNP is said to be "associated" with the disease. The associated SNPs are then considered to mark a region of the equine genome which influences the risk of disease. In contrast to methods which specifically test one or a few genetic regions, the GWA studies investigate the entire genome. The approach is therefore said to be non-candidate-driven in contrast to gene-specific candidate-driven studies. GWA studies identify SNPs and other variants in DNA which are associated with a disease, but cannot on their own specify which genes are causal. For that purpose, proper sequencing of the targeted region is necessary.

RECENTLY OUR RESEARCH GROUP HAS DEVELOPED A GENETIC TEST FOR IDENTIFYING CARRIER STATUS IN FRIESIAN HORSES FOR TWO IMPORTANT GENETIC DISEASES: DWAFISM AND HYDROCEPHALY

Recently, a draft sequence of the horse genome was completed[30]. This, together with advances in both technology and methodology of human genetic disease analysis studies has led to realization of many GWAS studies in the equine species[2,31,32,33,34]. Recently our research group developed a genetic test for identification of carrier Friesian horses for two important genetic disorders: dwarfism and hydrocephaly[2,4]. Specifically, the advent of high-density single nucleotide polymorphism (SNP) based genotyping arrays and the growth in knowledge of the haplotype structure of the mammalian genome has led to rapid development of GWAS for disease gene mapping.

3.2 Is it done before in the Friesian horse?

Yes indeed. Our recent study reports on the successful identification of the genetic region responsible for dwarfism and hydrocephaly in the Friesian breed. Our study also demonstrates that association-based mapping still can be effective for the detection of genetic disorders, using a small number of individuals. Indeed, in that study we managed to localize the corresponding gene region by using only ten selected Friesian dwarf horses and ten normal Friesian horses (controls).

Important to notice is that an overlap can be seen between the founders identified during the epidemiological pedigree study for hydrocephaly on one hand and aortic rupture on the other hand. Therefore it is important to check for possible genetic association between both diseases and to really identify the functional gene responsible for both traits. **This is only possible when the GWAS study is followed by sequencing.**

3.3 Materials and methods

3.3.1. Horses

A group of 41 ruptured Friesian horses will be included in this study. Proper DNA material is available of all these horses. Based upon epidemiological pedigree check a control group was created, suitable for the GWAS study. DNA isolation can be verified from heparinized blood samples or frozen pathological samples, using QIAamp DNA blood maxi kit from Qiagen according to manufacturer's instructions. DNA samples can be quantified using Quant-iTPicoGreen dsDNA kits. The Quant-iT PicoGreen dsDNA Assay from Molecular Probes (Invitrogen) is more specific for DNA and is about 1000 times more sensitive than traditional absorbance methods.

3.3.2. Genotyping chip

Samples can be genotyped by using EquineSNP50 Genotyping BeadChips (Illumina). This array contains approximately 54 000 SNPs ascertained from the EQUCAB2 SNP database of the horse genome (http://www.broadinstitute.org/mammals/horse) and has an average spacing of 43.2 kb between adjacent variants. A SNP array of a much higher density (777.000 SNPs) is expected to

become available very soon. If available then we will use that one in this study. Genotyping can be performed on an Illumina Bead Station according to the manufacturers recommended protocol. To ensure an adequate number of samples for genotype cluster seeding, we can duplicate our samples for QC purposes and see how many percentage concordance between the duplicate pairs is present. The results of GWAS gives insight in the most optimal selection strategies to reduce or remove the disorder from the population. The results also points out the prospects of developing a DNA-test that is sufficiently reliable to contribute to a reduction of the disorder in the population.

3.3.3. Detect genes and haplotypes related to aortic rupture by sequencing

A few case and control horses selected from the GWAS-study will be sequenced. Depending on the results of the GWAS-study, one or several regions of interest will be sequenced. The sequences of cases and controls will be aligned followed by the identification of variation. As a major gene is expected, the sequencing of only a few animals is sufficient to detect causal variants. If the GWAS-study reveals only one region of interest then sequencing of that specific region with high coverage will be performed to detect the functional gene and the causal mutation. Especially in view of the overlap seen in collagen aberrant build up between aortic rupture and mega esophagus, it is imperative to identify the exact functional gene to see if the overlap can be genetically confirmed by a common gene underlying both disorders.

Regions of interest can be amplified by polymerase chain reactions using platinum Tag DNA polymerase kits (Invitrogen). The method can be performed by Sequenom using iPlex chemistry on the MassArray platform. The results will be the base of a genetic test with accuracy of 100%, as the test is based on the functional mutation or on haplotypes highly related to it.

3.4 Cost calculation

Our proposed budget is as follows:

Expenditure	costs	Grant assistance
DNA isolation	**400 Euro**	
Genotyping	**20 000 Euro**	**30 000 Euro**
Sequencing	**15 000 Euro**	**50 000 Euro**

Conclusion

Who:

- Our research group consists of 4 major partners which have many years of experience with research in the Friesian breed in cooperation with the Royal Friesian Studbook: The Utrecht University, The Ghent University, The Wageningen University and Wolvega Equine Clinic.

- The project will be part of a larger research project that maps out the clinical and genotypic appearance of aortic rupture and aorto-pulmonary fistulation within the Friesian breed.

Why:

- Aortic rupture is always fatal and can lead to very dangerous situations.

- The prevalence of aortic rupture is estimated to be ± 2% within the Friesian breed, which is much higher than the incidence seen in warmblood horses.

- There are 3 different forms of aortic rupture to be discerned within the Friesian breed: the acute, the subacute and the chronic form.

- Aortic rupture in Friesian horses occurs at a completely different location when compared with other horse breeds making actual pre-mortem diagnostic imaging a difficult task.

- Horses tend to rupture in a life time stadium where they have been able to have had a reproductive career, in contrast to two other genetic disorders: dwarfism and hydrocephaly

- A pedigree study showed clear evidence for a genetic background of the disease.

- A suitable genetic test is necessary for to identify carrier or susceptible horses enabling to select effectively against the disorder and avoid mating of susceptible parents.

- Knowing which gene causes aortic rupture in the Friesian horse, might help to understand other disorders, as aortic rupture in humans and mega-esophagus in the Friesian horse, as well.

How:

- Identify the SNP regions responsible for aortic rupture in Friesian horse by performing a GWAS

- Identify the mutation that is responsible for this genetic disorder, by sequencing the SNP regions

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