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Genetic factors in feline heart and eye disease

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List of Abbreviations

ACD	Assembly Competence Domain
ACMG	American College of Medical Genetics
ACTC1	Actinine 1
ACVIM	American College of Veterinary Internal Medicine
ALMS1	ALMS1 centrosome and basal body associated protein
cDNA	Complementary DNA
CI	Confidence Interval
DMD	Dystrophin
FHV-1	Feline Herpesvirus I
gDNA	Genomic DNA
GWAS	Genome-Wide Association Study
HCM	Hypertrophic Cardiomyopathy
LOF	Loss Of Function
MSM	Myosin Storage Myopathy
MYBPC3	Myosin Binding Protein C3
MYH6	Myosin Heavy Chain 6
MYH7	Myosin Heavy Chain 7
MYL2	Myosin Light Chain 2
MYL3	Myosin Light Chain 3
NT-proBNP	N-Terminal pro B-type Natriuretic Peptide
OR	Odds Ratio
OMIA	Online Mendelian Inheritance in Animals
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
TNNTI3	Troponin I 3
TNNT2	Troponin T 2
TPM1	Tropomyosin 1
TTN	Titin

1.Introduction

1.1 The origin of cats

Domestic cats belong to the Felidae, a family of the order Carnivora. This family consists of 41 species of predators that range in body size from 1 to 300 kg.[1,2] Shared characteristics of the Felidae are large eyes, ears and canines, short jaws, retractable claws (in most species) and a combination of powerful hind legs and flexible front legs.[1] With a few exceptions, they are solitary predators that require a large territory to catch sufficient prey. Due to human destruction and partition of their habitats, hunting and persecution by humans who view them as a threat to their livestock or themselves, most species of the Felidae family are highly threatened or even at the brink of extinction.[1]

The obvious exemption to this regrettable trend is the domestic cat, *Felis catus*. This is one of the seven species of the genus Felis, a genus of small cats that are spread throughout Europe, Africa and Asia.[2] Modern-day domestic cats are most closely related to the wild species *Felis lybica lybica*, a subspecies that occurs in north Africa and south-west Asia.[2,3] This corresponds to the archaeological evidence of the first co-habitation of cats and humans in the eastern Mediterranean. Interaction between cats and humans in this region is the result of the development of agriculture.

Agriculture was first practiced around 10 000 BC, when it was only a minor activity among other methods of food production, such as hunting, gathering and fishing. Over the following millennia, human population growth, climate change and depletion of natural resources forced more and more humans to fully commit to farming.[4,5] The abundance of food in the fields and storages of these farmers attracted small animals such as mice, rats and sparrows, who adapted to life in and around human settlements. In turn, these concentrations of prey animals attracted cats.[6]

The first evidence of human-feline interaction are the remains of a cat buried in a human cemetery around 7500 BC.[7] Other early cat remains found in human settlements stem from 6700 BC in Palestine[8] and 3300 BC in the Chinese province of Shaanxi.[9] However, this commensalism is not the same as domestication, where humans actively control the survival

and reproduction of cats and change the genetic makeup of a population by artificial selection.[10] Furthermore, commensalism does not necessarily lead to domestication, as many animals, such as sparrows and raccoons, have adopted a commensal existence without ever being domesticated.[11]

Cats were nevertheless domesticated, and convincing evidence for the domestication of cats appears in archaeological remains from the Egyptian Middle Kingdom (2040 - 1782 BC).[8] The cats were kept and bred in temple catteries for use as sacrificial animals in religious rituals. It is likely that different felid species were tamed and kept for this purpose, but that only *Felis lybica* successfully reproduced in captivity and was therefore the only cat species to be domesticated.[12] All modern domestic cats seem to be descendants of the cats that were domesticated in Egypt.[8,12] These were dispersed all over the world by human traders, conquerors and migrants during the following millennia.[12] The main routes of migration were over the Mediterranean to Europe and over land to Asia, and contemporary cats can be divided into genetically distinguishable Eastern and Western populations.[13]

Breed formation is a relatively recent development in cats and not as extensive as in dogs. The International Cat Association currently recognizes 73. Some of these breeds are derived from regional populations that had developed their own characteristics before recognition as a breed. For example, the Maine coon and Turkish angora breeds are longhaired variants of local cat populations.[14,15] Most breeds were developed in the last 50 years from one or a few cats with a remarkable trait, such as baldness, folded ears, or a specific coat structure or pattern.[15] Breeds can also result from hybridisation. This can be a hybridisation of existing domestic cat breeds, such as the Abyssinian, American shorthair and Siamese to form the ocicat, or a hybridisation of domestic cats with other cat species, such as with the Asian leopard cat (*Prionailurus bengalensis*) to form the Bengal breed.[16] The reproduction of these purebred cats is artificially restricted to members of the same or a few related breeds, but in contrast to other domestic species, there is no selection for traits related to food production or work performance. Despite the popularity of purebred cats, the vast majority of the cats are domestic shorthairs or longhairs that do not belong to any recognized breed.[17]

1.2 The feline genome

A genome is the complete genetic makeup of an organism. Together with the environment in which the organism lives, it determines the phenotype. This phenotype encompasses all observable characteristics of an organism, ranging from anatomy and physiology to behaviour and life cycle. The genome of eukaryote organisms is composed of DNA, that is organised into several large molecules, the chromosomes. Diploid organisms, including all domestic mammals and humans, have two sets of chromosomes, one inherited from their mother and one from their father. During meiosis, chromosomal regions are exchanged between each pair of chromosomes in the process of crossing-over. One mixed chromosome of each pair is then randomly allocated to the resulting gamete. Fertilization with another gamete then gives a new, unique and complete diploid genome.

Genes are the parts of the genome that form the template for an RNA molecule during the process of transcription. This starts with copying the full sequence of the gene, after which some parts of the RNA (the introns) are excised out and the remaining parts, the exons, are joined together to form the final transcript. In the case of protein-coding genes, this RNA transcript serves as a template for a protein, while in non-coding genes, the transcript is the final product.

The (haploid) genome of a cat is approximately 2.5 billion base pairs long. Cats have 18 pairs of autosomes and one pair of sex chromosomes (XX or XY). In contrast to the sequential numbering in most other species, the feline autosomes are named on the basis of their size and the position of their centromere. The three large submetacentric chromosomes are named A1 to A3, four large subtelocentric B1 to B4, two large metacentric C1 and C2, four small subtelocentric D1 to D4, three small metacentric E1 to E3 and two small acrocentric F1 and F2 (Figure 1.1).[18] The cat genome, as presented in the Ensembl database, is estimated to contain almost 20 000 protein-coding genes and more than 9 000 non-coding genes.

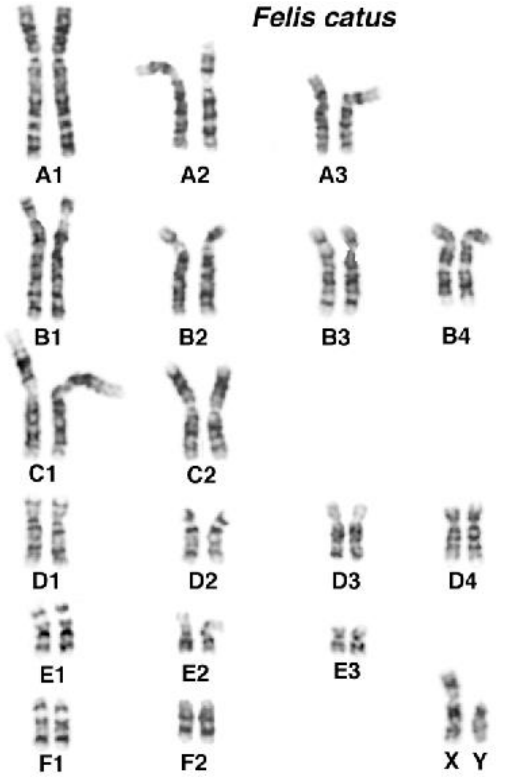

Figure 1.1 Chromosomes of the domestic cat.[19]

Genomic variation

Every time a cell divides, the billions of nucleotides in the genome are copied by DNA polymerases. The high specificity of these enzymes and their build-in proofreading mechanism, as well as mismatch repair by other enzymes, ensure that this replication process has an extremely high accuracy.[20] The error rate of this process is estimated to be as low as 1 error in every 10^{10} copied bases.[21,22] Nevertheless, as the entire genome of germline cells is copied at least a few dozen times every generation,[23] it is inevitable that some errors occur. These errors are the main ultimate source of genetic variation between individuals. In addition, DNA damage in germline cells due to exogenic factors (such as mutagenic chemicals) and endogenic factors (such as reactive metabolic products), can result in genetic variation when it is incorrectly repaired.[24]

The resulting genetic variation comes in several forms. The most commonly found form is substitution, where a single base has been replaced by a different single base. The other major classes are insertions and deletions, where one or more nucleotides are inserted into or deleted from, respectively, a DNA sequence. Furthermore, DNA sequences may be inversed, duplicated or moved to a different location in the genome.[25]

The effect of these variants depends on their position in the genome. Some portions of the genome are located outside genes and not involved in transcriptional activity and variation in these regions does not affect the phenotype. Approximately 8% of the human genome is involved in the regulation of transcription[26] and variants in these regions can affect gene expression. Genetic variants within gene regions themselves may have no impact on the protein sequence, either as intronic variants or as exonic variants that code for the same codon. These variants may nevertheless affect the phenotype, for example by affecting the stability or the splicing of the mRNA. As a final category, variants in coding regions can change the protein's amino acid sequence if they code for a different codon or stop codon or cause a frameshift.

The phenotypical effect of a variant may range from virtually imperceptible to dramatically large. In the context of disease, these phenotypical effects are usually classified as monogenic diseases or complex diseases. As the name implies, monogenic diseases are caused by an alteration of a single gene, usually by a single disease-causing variant. However, the disease phenotype is still influenced by other genetic variants (modifier variants) and the environment. As a result, the disease can be markedly different in individuals affected by the same disease-causing variant (variable expression) or may even be absent in individuals who are genetically predisposed (incomplete penetrance).[27] Complex diseases are caused by a combination of multiple genetic variants and environmental factors. In some diseases, the genetic component seems to consist of many different genes with small effects, while in other cases, some risk variants have a larger effect than others.[28]

Disease-causing variants

A common mechanism by which variants can cause a monogenic disease is the loss-of-function of a protein. Due to a disruption of the process of transcription, splicing or translation, or a change in a functionally important part of the amino acid sequence, a gene may no longer produce a (functional) protein. If an animal inherits such a variant from one parent, this usually has no notable impact on the phenotype, because the gene inherited from the other parent

can compensate for this loss. The effect of the variant will only become apparent if the same variant, or another variant with a similar effect on the same gene, is inherited from the other parent. The phenotype associated with this variant is then said to follow a recessive pattern of inheritance. Recessive disease-causing variants are very common and it is estimated that every eukaryote animal carries one to two recessive lethal variants.[29]

In some cases, only one copy of a variant is sufficient to cause a major change in the phenotype. This results in a dominant pattern of inheritance, which can be explained by three different mechanisms. The first is haploinsufficiency, which involves loss-of-function similar to that in recessive variants. However, in these cases, the functional gene inherited from the other parent is not sufficient to compensate for this loss. The second mechanism is gain-of-function, where the expression or activity of the resulting protein is increased. The third mechanism is a dominant negative effect, where the protein retains some, but not all, of the different activities that it needs for full functionality. For example, the protein may still interact with both itself and its functional version to form homodimers, but any homodimer containing this protein is non-functional.[30]

When located on an autosome, individuals can inherit a variant from either parent, regardless of their sex. For a variant with a dominant effect, inheriting it from one parent is sufficient to cause disease, while recessive variants will only cause disease when inherited from both parents. If the variant is located on the X chromosome, females can inherit it from both parents, while males can only inherit it from their mother. With only one X chromosome, males are more vulnerable to disease caused by a deleterious variant (Figure 1.2). Females have two X chromosomes, one of which is inactivated in each cell. Although females are often not or only mildly affected by X-linked diseases, some X-linked diseases have an intermediate penetrance in females that does not fit the concept of dominant and recessive.[31] Genetic variation on the Y chromosome is only present in males and will always be transmitted from father to son, but generally has little phenotypical impact because of the small number of genes on this chromosome. The mitochondria, the energy-producing organelles of cells, have their own DNA that is only transmitted by the mother and can contain disease-causing variants.

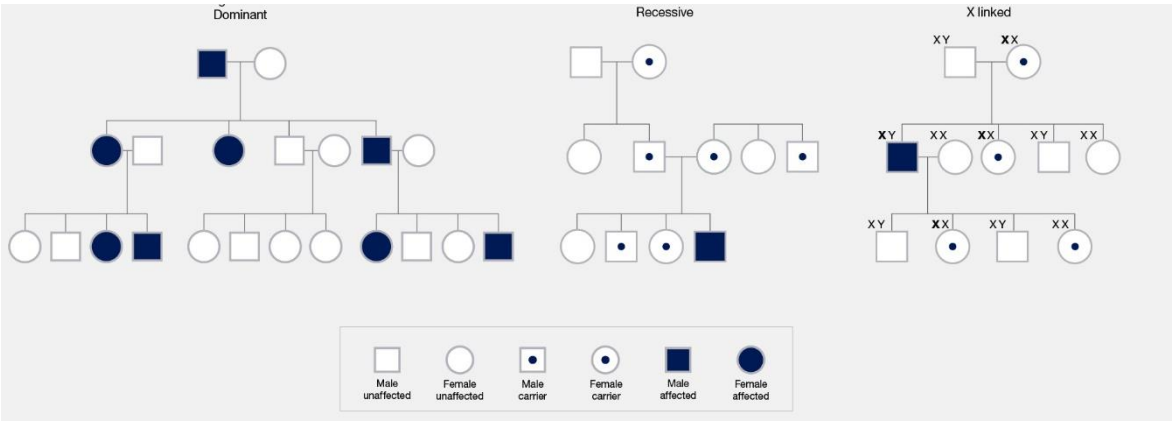

Figure 1.2 Inheritance patterns of monogenic traits or diseases. Source: National Human Genome Research Institute.

Effects of domestication

Human intervention in the reproduction of domestic animals has a strong impact on their genomes. On the one hand, humans restrict the number of animals that reproduce, the effective population size, and thereby reduce the genetic diversity of the population. On the other hand, they select animals with desirable traits for reproduction, thereby manipulating the frequency of variants.

The effect of domestication on genetic diversity is classically presented as two historical population bottlenecks, where the population size is reduced dramatically before it increases again. The first bottleneck would have taken place at the moment of domestication and the second at the moment of breed formation. Breed formation seems to have the greatest impact, whereas the importance of the first bottleneck seems negligible in cats[6] and is recently also disputed for other species.[32]

As population bottlenecks only contain a small subset of a population, genetic variants that were unique to the excluded portion of the population are lost. In addition, chance will have a much greater impact on the allele frequencies if the number of reproducing individuals is small, resulting in the loss of variants due to genetic drift.[33] With 9.6 million single nucleotide variants in an average individual, cats show much more genetic variation than other domestic species.[34] Their number of variants is also twice as large as that in humans, who are thought to have experienced at least one bottleneck in their recent evolutionary history.[35]

Although cats in general show a large amount of genetic variation, there are large differences between breeds. Genetic diversity is generally lower in Eastern than in Western cats. Some breeds with small closed populations, such as the Burmese, Chartreux and Korat, have low levels of heterozygosity, indicating limited genetic diversity. Other breeds, such as the outbred Munchkin or the only recently established Siberian, have levels of heterozygosity comparable to domestic shorthair cats.[13]

Selection in purebred cats usually focusses on the one or few characteristics that define a breed. Compared to other species that have undergone extensive selection on complex trait, relatively few genomic regions show traces of selection in cats.[16] Nevertheless, potentially harmful breeding practices do occur in cat breeding. This may include the excessive use of one or a few sires in breeding (the popular sire effect) or mating of related individuals (inbreeding).[36,37] Such practices can increase the frequency of a harmful allele and/or the probability that an animal inherits two copies of a harmful recessive allele.

1.3 Genetic diseases in cats

Breed-specific diseases are a major concern in veterinary medicine. The problem is most prominent in dogs, but is also recognized in cats. These diseases can be divided into two major groups: inherited diseases that spread as the unintentional result of the population structure of these breeds and extreme phenotypes that result from deliberate selection.[38] Diseases and traits of all animals other than humans, mice, rats and zebrafish are catalogued in the OMIA (Online Mendelian Inheritance in Animals) database, with information on their inheritance and variants involved, if known.[39] The variants responsible for several common monogenic diseases have been identified in cats,[40] most notably a widespread variant that causes autosomal dominant polycystic kidney disease in Persian cats.[41] An example of a deliberately selected phenotype with negative consequences in cats is the autosomal dominant folded ear phenotype in Scottish fold cats, caused by a variant that can also cause osteochondrodysplasia in heterozygotes.[42,43]

In this thesis, two feline diseases will be investigated for which less is known about the genetic background. Hypertrophic cardiomyopathy is a widespread disease in both purebred and mixed breed cats for which a few causal variants have already been identified, but the cause of most cases remains unknown. Corneal sequestrum is a disease in the Persian and related breeds that may be related to the facial morphology, but several hypotheses about its aetiology exist.

Hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is defined as a thickening of the left ventricular myocardium that cannot be explained by abnormal loading conditions or systemic disease (Figure 1.3).[44] It is the most common heart disease in cats.[45] The prevalence in the general population is estimated to be approximately 15%.[46,47] Males are more often affected than females and the risk increases with age.[47] The median age of diagnosis is 6.5 years.[48] The Maine coon,[49] ragdoll[50,51] and sphynx[52] were marked as predisposed breeds because of a remarkably high prevalence or severe clinical course, although this may by now have been reduced by selection of breeding animals.

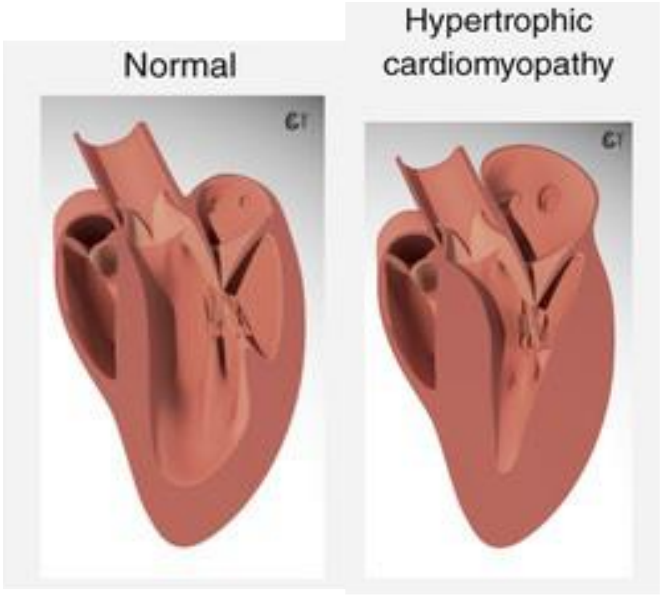

Figure 1.3 A normal heart compared to a heart affected by hypertrophic cardiomyopathy.[53]

The increased stiffness of a hypertrophic ventricular wall hampers relaxation during the diastole. Due to this diastolic dysfunction, a higher pressure in the atrium is required for filling of the ventricle. The elevated pressure causes the atrium to dilate and is communicated back to the pulmonary veins. Increased hydrostatic pressure in these veins can lead to the leakage of fluid into the lung, creating pulmonary edema and potentially life-threatening congestive heart failure.[54] The enlarged left atrium also predisposes for the formation of thrombi. If (a part of) such a thrombus enters the systemic circulation, it will obstruct smaller blood vessels. The most typical result is an arterial thrombo-embolism of the hind limbs, an extremely painful and debilitating condition that often leads to death or euthanasia.[55] The remodelling of the ventricular wall can also lead to ventricular arrhythmias when electrical impulse conduction is disturbed e.g. by fibrosis.[56] When this manifests as ventricular fibrillation, without immediate intervention, it leads to sudden cardiac death.

Congestive heart failure, arterial thrombo-embolism and sudden cardiac death are all acute complications of advanced HCM. As long as these do not occur, affected cats generally show no clinical signs. Some patients remain subclinical for their entire life and ultimately die of other causes. On auscultation, a heart murmur can be heard in 31%[46] to 96%[57] and a gallop sound in 33%[58] of the HCM-affected cats, but their presence is not specific for HCM, and neither can HCM be excluded on the basis of their absence.[54] Similarly, thoracic radiographs or electrocardiograms may be unremarkable or show nonspecific abnormalities in cats with HCM. Sensitive assays measuring the biomarker cardiac troponin I and NT-proBNP perform well in differentiating between healthy cats and cats with subclinical HCM, but echocardiography is still warranted to confirm the diagnosis and exclude other causes of myocardial injury.[59-61]

Echocardiography is the current ‘gold standard’ and the method of choice for diagnosing HCM in veterinary medicine. The main parameter is the end-diastolic thickness of the left ventricular wall, although the optimal cut-off is still debatable. In general, a thickness ≤5 mm is considered normal, ≥6 mm indicates ventricular hypertrophy and measurements in between must be interpreted in the context of other findings or classified as equivocal.[62] Furthermore, there are bodyweight-based and even breed-specific echocardiographic reference intervals for left

ventricular wall thickness to aid the diagnosis of left ventricular hypertrophy.[63-65] However, ventricular hypertrophy is not necessarily HCM. The hypertrophy may be transient[66] or may be the result of other diseases. In cats, hyperthyroidism and systemic hypertension are the most common causes of secondary hypertrophy. That is why the term 'HCM phenotype' is preferentially used when describing the echocardiographic finding of a thickened left heart, and why efforts must be made to exclude secondary causes before diagnosing genetic HCM.[62]

On post mortem examination, the myocardium is contracted, which makes the limits for echocardiographic measurement invalid.[67] Hearts affected by HCM have a thickened, stiff ventricular wall, decreased ventricular lumen and possibly ventricular fibrosis, enlarged atria and atrial thrombi.[68] A heart weighing 20 gram or more may be considered hypertrophic,[69] although there is considerable variation in healthy heart weights and therefore some overlap in healthy and affected hearts for this criterion.[70] The histopathology of HCM is characterized by disarray and hypertrophy of the cardiomyocytes and interstitial fibrosis.[68]

There is currently no treatment that can reverse the pathological remodelling of the heart in feline HCM, but complications can be treated or prevented. The beta blocker atenolol is often administered to subclinical HCM patients, but there is no evidence for a beneficial effect.[71] In cats with an enlarged left atrium, the risk of thrombus formation can be attenuated with a platelet inhibitor such as clopidogrel. In case an arterial thrombo-embolism develops, the prognosis is generally poor, although recovery may be possible with intensive care in the acute phase and long revalidation.[72] Cats that develop congestive heart failure are primarily treated with diuretics, but less than 30% survives for more than 2 months.[48]

Genetics of HCM

HCM also occurs in humans and feline and human HCM are highly similar.[72] Human HCM was first reported to be a familial disease with an autosomal dominant pattern of inheritance in 1960.[74] A clear familial disease is observed in approximately 60% of the human HCM cases.[75] Several genes have been associated with human HCM. Most notably are the eight sarcomeric genes *ACTC1*, *MYBPC3*, *MYH7*, *MYL2*, *MYL3*, *TNNTI3*, *TNNT2* and *TPM1*, of which *MYBPC3*

and *MYH7* contain the majority of the HCM-causing variants.[75,76] Other genes have been implicated in HCM as well, but strong evidence is often lacking[77,78] and the clinical utility of screening is limited for most of these genes.[79] More than 1 500 different variants, most of them unique to a single family, have been identified as HCM-causing.[80] This makes human HCM a disease with extreme genetic heterogeneity.

In cats, familial HCM has been reported in American shorthairs,[81] British shorthairs,[57] domestic shorthairs,[82-84] Maine coons,[67] Norwegian forest cats,[85] Persians[86] and sphynx cats.[52,88] Six variants have been described as causes of HCM in cats. Three of these are located in *MYBPC3*. *MYBPC3* encodes the cardiac myosin binding protein C. The protein interacts with myosin, actin and titin and plays a role in the regulation of sarcomeric contractility.[88] Both missense and truncating variants in *MYBPC3* have been associated with HCM in humans.[74,75] The first variant was found in a research colony where HCM-affected Maine coons were bred.[89,90] It is a G to C substitution at position 91 of the gene's coding region (*MYBPC3*:c.91G>C), leading to a substitution of alanine by threonine at position 31 of the protein. The variant's estimated allele frequency in Maine coon cats is 18%,[91] while it is virtually absent in other breeds.[90,91] HCM is also common in homozygous wild type Maine coons, so not all cases of HCM in this breed can be explained by this variant.[92-96] The clinical significance of the variant has even been questioned,[94] but epidemiological studies have shown that homozygosity for this variant strongly increases the risk of HCM.[96] The variant alters the structure of the resulting protein, but does not lead to lower concentrations or aberrant sarcomere incorporation of the protein.[97] The exact mechanism of disease for this variant remains unknown.

The second variant in *MYBPC3* was identified in HCM-affected, client-owned ragdoll cats.[98] It changes a single base from C to T at coding position 2455 (c.2455C>T) of *MYBPC3*, resulting in a change from arginine to tryptophan at the 819th amino acid residue of the corresponding protein. The variant has an estimated allele frequency of 14%[99] and was not found in other breeds.[96] One study has shown an association with HCM that is just statistically significant[100] and the variant is also associated with a risk of sudden death, increased wall thickness and

increased concentrations of cardiac biomarkers.[100-102] HCM-affected ragdolls without this variant have also been described.[100] An orthologous variant in humans seems to cause HCM with an autosomal recessive pattern of inheritance, based on a single family.[103]

A third variant in the same gene, *MYBPC3*:c.220G>A, has been suggested as a cause of HCM in Maine coons in a conference abstract, but the study was never published and the variant's causal role has never been proven.[94,96,104] A fourth variant, in the *MYH7* gene, is presented in chapter 3 of this thesis. A fifth variant, in *TNNT2*, was published in 2020 and is further investigated in chapter 4. Finally, a sixth variant was published in 2021 and is located in the gene *ALMS1*, a gene that has not been associated with HCM before. This variant is discussed in the general discussion in chapter 6.

Corneal sequestrum

The second feline disorder that is investigated in this dissertation is corneal sequestrum. A corneal sequestrum is a local degeneration and brown to black discoloration of the cornea. The prevalence is estimated to be 2.4% on the basis of a population of cats presented to a veterinary teaching hospital.[105] Male and female cats are affected equally. The age of onset ranges from 3 months to 17 years, but most often lies between 2 and 7 years.[106] Although corneal sequestrum can develop in any breed, there is a marked breed predisposition in Persian, exotic or Himalayan cats. Other breeds suggested to be predisposed are the Siamese, Birman and Burmese.[107,108]

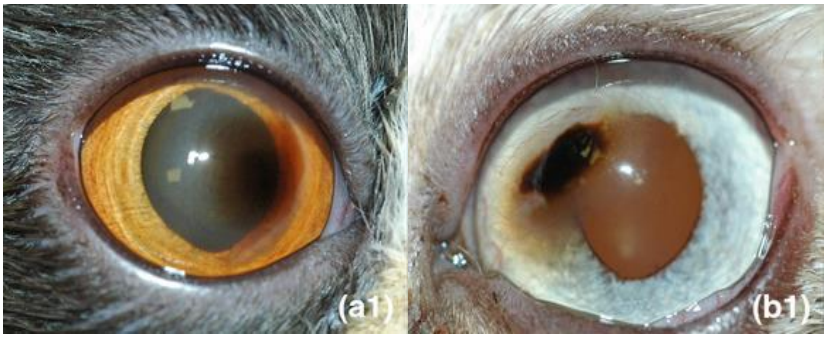

Figure 1.4 Feline eyes affected by a corneal sequestrum.[109]

A corneal sequestrum is characterized by necrosis of the anterior corneal stroma, which generally involves the epithelium when discoloration occurs.[110] The lesion can extent to the deeper layers of the corneal stroma and may in some instances lead to corneal perforation.[109,111] Multiple hypotheses about the cause of corneal sequestra have been proposed. Most revolve around trauma or chronic irritation of the cornea. Corneal trauma can be accidental or iatrogenic.[112,113] Chronic irritation may be caused by infection, most notably feline herpesvirus I.[111] Corneal irritation may be particularly common in brachycephalic cats, where trichiasis, entropion and lagophthalmos occur frequently.[111,113,114] Other possible causes of corneal sequestrum are ulcerative keratitis or abnormal tear quality or quantity.[111,113,115,116] An autosomal recessive pattern of inheritance has been suggested,[117] but no pedigree analysis or other genetic findings have been published to support this notion.

Equivalents for primary corneal sequestration appear to be rare to non-existent in other species. The condition is not known in human ophthalmology and the few described cases in dogs and horses could be attributed to an underlying disease.[118-120] A similar, but milder condition in horses seems to be a primary disease, but only three cases have been reported.[121] As such, other species cannot provide extra information about this condition.

Some patients show signs of severe pain, such as blepharospasm and epiphora, while other patients show no pain at all.[122,123] The appearance of the sequestrum can vary from a diffuse amber haze in early stages to a well-defined black plaque and it may be surrounded by corneal oedema or vascularization. Most cats are unilaterally affected, but bilateral sequestration is not uncommon and occurs mainly in cats from brachycephalic breeds.[111]

Surgical treatment is indicated to relieve the cat from its discomfort, to prevent expansion of the lesion to deeper layers of the cornea and to restore vision. This starts with keratectomy to remove the sequestrum, which is often followed by a reconstructive technique to protect the lesion.[113] The treatment is successful in most cases, although it may result in corneal opacity and recurrence is seen in 11-20% of the cases.[111,123]

1.4 Identifying genetic causes of disease

Candidate gene studies

The enormous size of a mammalian genome makes the identification of disease-associated variants a herculean task. Genetic studies therefore usually involve some way of confining the genomic region(s) under investigation. One straightforward method is to focus on a gene whose function can be linked to specific characteristics of the disease. Human diseases with straightforward candidate genes, such as hemoglobinopathies, were the first to be linked to a causative variant.[124] Similarly, the first causative variant in domestic animals was found in the thyroglobulin gene, an obvious candidate for goitre in cattle.[125] Remarkably, this was the first association of this gene with disease in any species and variants in the orthologous human gene were only later linked to a similar disease.[126] Some early discoveries in feline genetics, such as the causative variants for haemophilia B[127] or HCM, [89] were based on candidate genes from human diseases.

However, the candidate gene approach has major limitations. Historically, knowledge of the pathogenesis of a genetic disease was often insufficient to have a clear candidate, and most disease genes identified in humans by other methods had never been considered a candidate gene.[128] On the other hand, well-studied diseases that are genetically heterogeneous can have an overwhelming number of candidate genes. For example, 111 genes are included in genetic testing for human dilated cardiomyopathy, including the largest human gene (DMD, encoding Dystrophin) and the gene encoding the largest protein (TTN, encoding Titin).[129]

Genome-wide association studies

One of the laws deduced from Mendel's first descriptions of genetic inheritance is that genes segregate independently. However, this is only fully true for genes that are located on different chromosomes. Alleles on the same chromosome are inherited together, unless crossing over occurs between the genes during meiosis. As the probability of crossing over is related to the distance between two genes, alleles are more likely to be inherited together if they are located

close together on the same chromosome. This association of alleles at different loci is termed linkage.[130]

Linkage can be exploited in the search for disease-causing variants, as a locus that is in linkage disequilibrium with such a variant, will also be associated with the corresponding phenotype. Linkage analysis is a method that traces genotypic markers across generations to identify markers that are associated with a phenotype. This method, usually followed by analysis of candidate genes in the associated region, allowed to increase the number of human diseases linked to a specific gene from 100 to over 2 000.[128]

When thousands of common variants in a species are known and can be genotyped by means of arrays (see below), genomic mapping can be advanced to the population level in the form of a genome-wide association study (GWAS). The most common GWAS design compares the allele frequencies in a group of affected animals to those in a healthy control group. If this yields a statistically significant difference (after correction for multiple testing) for one or several neighbouring variants, this is a strong indication that the disease-causing variant is located in this region. This technique has been applied extensively in veterinary medicine to localize causative variants, with several examples of feline Mendelian traits[131,132] and diseases.[133,134]

In human medicine, GWASs are currently mostly used for studying complex diseases. Since the effects of single variants in these diseases are generally much smaller than in Mendelian diseases, much larger sample sizes are required to detect statistically significant associations.[135] Recently, GWASs of feline complex diseases have also been reported[136,137] and significant genetic associations have been reported for hyperthyroidism and diabetes mellitus.[138]

Complex disease modelling

Complex diseases can be analysed with models developed in the field of quantitative genetics. These models assume that the dichotomous phenotype is the result of a continuous variable that does or does not cross a threshold value.[139] The population variance for this underlying variable can be divided into an environmental and a genetic component, the latter of which can

be further subdivided into additive, dominance and epistatic variance. The additive variance is the component with the most practical relevance, as this determines the response to selection. The relative importance of the additive variance compared to all other components is commonly expressed in the form of heritability, defined as the ratio of the additive variance over the total phenotypic variance.[139]

Heritability is a notoriously difficult concept to interpret. As it is a ratio of variances, it is only applicable at the population level and dependent on the genetic composition of that population, as well as the environmental circumstances that were present in the analysed population.[140,141] More importantly, this statistical representation cannot directly be translated into a biological interpretation where the causes of a disease can be neatly separated into the mutually independent categories of genetics and environment. Instead, all biological traits result from complex interactions between genetic and environmental factors.[141]

Technologies for genotyping

Studies that aim to identify a disease-causing variant are dependent on technologies that can provide detailed information about a DNA sequence. One technique that is an essential part of many genetic research methods is the polymerase chain reaction (PCR). PCR allows making billions of copies of a specific DNA fragment from a minuscule amount of input DNA. The reaction consists of the cyclic repetition of three steps, of which the first is the denaturation of DNA at a temperature around 95 °C. During the second step, a DNA oligonucleotide (primer) anneals to each strand at the 5'-end of a target fragment. During the third step, a polymerase derived from the bacterium *Thermus aquaticus* produces a new copy of the fragment by elongating the primers in the 3' direction. This cycle is typically repeated at least thirty times and theoretically doubles the number of copies each cycle. In practice, a large number of copies inhibits the reaction and the exponential increase is slowed down to a plateau.

PCR products can be visualized by gel electrophoresis and the fragment size can be estimated by comparing the fragments to commercially available ladders of fragments with known lengths. This enables the detection of insertions or deletions that are large enough to cause a

visible difference between DNA fragments, but gives no information about the more common substitutions. The detection of such variants requires either a modification of the PCR process or subsequent manipulations of the PCR product that can bring this type of genetic variation to light.

A popular modification of the PCR reaction that can detect substitutions is the probe-based qPCR assay. This assay utilizes an oligonucleotide probe that anneals downstream to one of the primers and contains a fluorophore and a quencher. During the elongation step, the exonuclease activity of the Taq enzyme ensures that the probe is digested and the fluorophore and quencher are released. At the end of each PCR cycle, the reaction mixture is exposed to light that brings the fluorophores into a state of excitation. The energy of fluorophores that are still attached to a probe will dissipate to the quencher, while the released fluorophores will emit their energy in the form of visible light. The intensity of the emitted light is therefore proportional to the number of copies of the fragment. With allele-specific probes that each have their own fluorophore, a probe-based qPCR assay can be used to genotype an animal for a specific substitution variant.

PCR can also be the first step in a genotyping method that applies other techniques to the PCR product. Two of these techniques, restriction enzyme digestion and Sanger sequencing, will be discussed here.

Restriction enzymes are bacterial enzymes that “restrict” the activity of bacteriophages by cutting the DNA helices of these viruses.[142] Each of these endonucleases recognizes its target by a specific, though sometimes degenerate, nucleotide sequence and cuts at a specific distance from this recognition site. To avoid falling victim to their own defences, bacteria modify the nucleotides in recognition sites in their own genome, for example by DNA methylation.[143]

Restriction enzymes are also useful for the detection of known DNA variants. If the DNA sequence containing a variant is a recognition site of a restriction enzyme and the enzyme recognizes one, but not the other allele, the genotype of an animal can be deduced from the fragments into which the DNA is cut. This genotyping technique is called restriction fragment length polymorphism (RFLP). Thousands of restriction enzymes have been identified and more

than 300 of these are commercially available,[144] giving geneticists a plethora of tools to design an RFLP assay for genotyping.

Sanger sequencing is somewhat akin to PCR, but with two crucial modifications. First, only one primer is used instead of a pair of primers. This ensures the copies made during the sequencing reaction all originate from the same DNA strand. Second, and most importantly, the standard nucleotides provided for the polymerase are mixed with dideoxynucleotides. These dideoxynucleotides not only lack a hydroxyl group at the 2'-position, like all DNA nucleotides, but also at the 3'-position, and are labeled with a fluorophore specific to their base. Without the 3' hydroxyl group, the polymerase can no further elongate the DNA fragment. As the dideoxynucleotides are built in randomly, a mix of DNA fragments with different lengths is produced, with each fragment ending in a labelled nucleotide.

When these fragments are separated by electrophoresis and the fluorophores of their last nucleotides are read out, the nucleotide sequence of the DNA fragment can be reconstructed. Probe-based qPCR and RFLP assays can only be efficiently employed to screen for variants that are already known. They can be efficiently used to determine an allele frequency in a population or to screen breeding animals for a variant. Sanger sequencing, in contrast, requires no more than the means to amplify a sequence by PCR or cloning. This technique can therefore also be used for the discovery of new genetic variants and is viewed as the gold standard for validating variants found by massive parallel sequencing (discussed below).

Arrays

DNA arrays are designed to genotype a large number of known variants simultaneously, which makes them the ideal tools for performing GWAS. They consist of single-strand oligonucleotide probes fixed on the solid surface of a DNA chip. When a sample of fragmented and denatured DNA is brought on this chip, these probes will bind fragments that contain a complementary DNA sequence. On arrays that use the technology developed by Illumina, Inc. (San Diego, CA), each probe ends one base upstream of the site of a variant. After hybridization with the sample, the probe is extended with a single fluorophore-labelled nucleotide that is

complementary to the variant. At that moment, the allele of the fragment can be inferred from the light signal.[145] More recent arrays by Affimetrix, Inc. (Santa Clara, CA) the second major producer of DNA arrays, use a similar technique with fixed probes complementary to the sequence flanking the variant and dissolved allele specific probes that contain a binding site for a dye.[146]

Although DNA arrays by themselves cannot identify novel variants, they have proven to be a versatile tool for the localization of trait- or disease-associated loci via genome-wide association studies. Several dozen DNA arrays designed for various clinical applications or specific ethnicities are available for humans, containing up to 4.1 million variants.[147] Custom arrays allow to genotype any organism with a good reference sequence, but validated array designs are available for commonly investigated animals[148,149] and plants.[150] Compared to arrays for other species, e.g. cattle,[151] chicken[152] and horses,[153] that contain over half a million variants, the current feline 63K array is more limited with 62 897 variants.[154] A recent study utilized a custom array with 297 034 reliably genotyped variants.[138]

Massive parallel sequencing

Massive parallel sequencing, also called second generation sequencing, encompasses a group of technologies which can be used to sequence an entire genome. These techniques, of which the Illumina technology is the most prominent, start with the fragmentation of the target DNA. The ends of the fragments are then repaired and ligated with adapters.[155] Before the sequencing itself, the fragments can be amplified by PCR to create more input DNA, but PCR-free techniques have also been developed to avoid PCR-related sequencing artefacts.

The fragments are brought on a flow cell, where their adapters will anneal to fixed primers and the fragments are copied to create clusters. The fragments are then sequenced in parallel by sequencing by synthesis. On Illumina platforms, each newly incorporated nucleotide carries a fluorophore and a blockage group that prevents further extension of the DNA strand. After reading out the fluorescent signal, both the fluorophore and the blocker are removed and the process is repeated for the next nucleotide in the strand. Platforms by Ion Torrent, Inc. (Gilford,

NH) use a different approach and only add one type of nucleotide at a time. The incorporation of the presented nucleotide is detected on the basis of H^+ release and the resulting pH change.[156] The maximum length of the reads produced by these sequencing platforms ranges from 150 to 600 bp.[157]

Other technologies, also called third generation sequencing, have been developed to provide reads of much greater lengths, despite a higher error rate than previous technologies. The single-molecule real-time sequencing technology by Pacific Biosciences of California, Inc. (Menlo Park, CA) uses a fixed polymerase enzyme that synthesizes a complementary sequence with fluorescently labelled nucleotides. The fluorophore is cleaved of each base that is incorporated in the sequence and its signal is then recorded, allowing read lengths of up to 30 kb. The sequencing technology by Oxford Nanopore Technologies, Ltd. (Oxford, UK) brings a single DNA strand through a nanopore channel in a membrane. This causes disturbances in an electric current running through the membrane, from which the DNA sequence can be deduced.[157]

Variant interpretation

Since the advent of massive parallel sequencing technology, medical researchers have been confronted with the full amount of genetic diversity of their patients. Among the many variants in any given mammalian genome, there are always some for which a seemingly convincing argument for pathogenicity can be constructed.[158] Simultaneously, sample sizes have shrunk as large families no longer are imperative for a genetic analysis. In feline medicine, where locating the patient's family members can be challenging, it is not uncommon that a disease-causing variant is reported on the basis of a single patient.[159-163] In such cases, it is crucial that sufficient evidence is provided to support the claim. Detailed guidelines exist in human medicine on the types of evidence and their relative strengths. The most influential of these are the guidelines developed by the American College of Medical Genetics and Genomics (ACMG) in association with the Association for Molecular Pathology.[164] These guidelines are

set as general rules for monogenic diseases, but are open for adaptation for specific diseases. With some caveats, the principles from these guidelines are also applicable to feline genetics. The ACMG guidelines are meant to classify variants into five categories: benign, likely benign, unknown significance, likely pathogenic and pathogenic. Variant of unknown significance is the default category, in which all variants are placed if there is no sufficient evidence to classify them otherwise or if different lines of evidence are contradictory.[164] The term “likely” is supposed to indicate at least 90% certainty, although non-quantitative nature of most evidence means that this level of certainty can only be roughly estimated.[164,165] The guidelines distinguish four levels of evidence: supporting, moderate, strong and very strong.[164]

The majority of the criteria for classifying variants depend on the gene that is affected by a candidate variant. Researchers investigating a variant in a gene that has not been previously associated with the disease under investigation face two challenges at once: proving the involvement of the gene in the disease and proving the pathogenicity of one (or more) variant(s) in this gene.[158] In line with classical mapping studies, evidence for the involvement of a gene is primarily statistical in nature.[77,158] If enough samples are available, this can be based on pedigree segregation or population risk ratios. In other cases, a formal null model may be specified, such as the probability of finding multiple *de novo* variants in the same gene in a certain cohort of patients. In addition to statistical evidence, the involvement of a gene can be supported by a link between the biological function of the gene and the disease process or by reports of a similar disease phenotype in experimental animals in which the gene was knocked out.[158]

The ACMG guidelines are only applicable once the involvement of the gene containing the variant under investigation is proven. The gene itself may provide some evidence if it is linked to a very specific phenotype. The gene can also determine what kind of variants might be considered pathogenic. A missense variant is more likely to be pathogenic in a gene or critical gene region in which missense variants are rarely benign, but less likely in a gene in which all known pathogenic variants are truncating variants.[164] In a proposed adaptation for classifying

variants in HCM patients, the evidence for pathogenicity of a variant increases with the statistical association of its gene region with HCM.[78]

The nature of the variant is the most influential factor. Loss of gene function caused by a truncating variant is considered very strong evidence for pathogenicity. Missense variants are more likely to be classified as pathogenic if they cause the same or a similar amino acid change as a known disease-causing variant, while silent variants point more towards a classification as benign. If no such data are available for a missense variant, computational predictions or, more convincingly, results from functional laboratory studies can provide evidence for or against pathogenicity.[164]

Computational tools are an easy way to assess the effect of an amino acid substitution or the effects of a variant on splicing and mRNA stability. Examples of popular tools to do the former are SIFT[166] and PolyPhen-2.[167] These tools estimate the impact of a substitution on the basis of several parameters, such as evolutionary conservation, functional protein annotation and the physicochemical properties of the amino acids. They have high false-positive rates: on average, both SIFT and PolyPhen-2 label more than 100 variants as pathogenic in a human genome.[168] These predictions should therefore be interpreted with caution: multiple programs should be used and if their predictions do not contradict, this counts only as supporting evidence. Predictions of aberrant mRNA splicing, which can have a dramatic impact on protein structure, must be confirmed by sequencing the mRNA.[164]

In addition to molecular data, family and population data can support the classification of a variant as pathogenic or benign. Co-segregation of a variant with a disease is only counted as strong evidence when found in multiple families, as it can be explained by linkage disequilibrium in a single family. With population data rather than family data, a significant odds ratio or relative risk > 5 is also considered strong evidence for pathogenicity. On the other hand, lack of co-segregation or finding the variant in a healthy person when early and full penetrance is expected, is strong evidence for a benign interpretation. If the allele frequency of a variant is higher than expected for the disease prevalence in the general population, this too strongly supports a benign classification. Furthermore, an allele frequency higher than

expected on the basis of the disease prevalence is strong evidence for a benign interpretation and variants with a frequency >5% in the general population are always considered benign in humans.[164]

1.5 Genetics in veterinary practice

Veterinary genetics can be part of the diagnostic process. When a differential diagnosis includes a monogenic disease for which a causative variant is known, the presence of this variant can easily be confirmed or disproven. However, a different variant causing the same disease cannot be excluded on the basis of a DNA test. To detect previously unknown disease-causing variants, whole exome or whole genome sequencing can be applied.[169] If a causative variant can be identified successfully, family members that are potentially at risk can be tested for this same variant.

Another important application of veterinary genetics, lies in health screening of potential breeding animals. Belgian law obliges breeders to take the health of the offspring into account when selecting breeding animals, but does not require any health screenings. Some breeding societies demand specific screening tests for breeding animals. For example, DNA testing for four recessive diseases (glycogen storage disease type IV, retinal degeneration II and gangliosidosis GM1 and GM2) in the relevant breeds is mandatory for breeders associated with the Fédération Internationale Féline (FIFe) and its Belgian member society, Felis Belgica. Other screenings, including DNA tests and echocardiographic screening for HCM, are recommended, but not mandatory. Other Flemish cat associations do not post screening obligations on their web sites. Screening for HCM is thus voluntary in Belgium. Castration is advised for HCM-affected cats.

Until recently, the objective of genetic testing was considered to be the elimination of disease-causing variants from the animal population. However, these variants often have a very high allele frequency in the small and closed population of a breed. Excluding all animals that carry a common variant from breeding can substantially decrease the (often already low) effective population size and genetic variation within a breed. Stringent selection thereby induces the

accumulation of other deleterious variants with potentially more negative than positive consequences for the breed.[170]

An alternative, more feasible aim is to minimize the risk of disease among the offspring. For autosomal dominant diseases or X-linked diseases that can manifest in heterozygous females, this implies no fundamental change in selection practice, as all carriers have a risk of affected offspring. In recessive diseases however, the situation is entirely different. As long as they are mated with a homozygous wild type animal, carriers and even affected homozygotes have no risk of affected offspring. This breeding strategy can therefore vastly expand the effective population size of a breed with a highly prevalent recessive disease.[171] An important condition for the use of affected animals, especially female animals, in breeding is that the breeding should not affect their welfare or that of their offspring. For X-linked recessive diseases, it is safe to use hemizygous males for breeding with homozygous wild type females, as they cannot pass on the causative variant to their sons. Here the welfare of these sires is also a point of concern, as hemizygotes all have a risk of being affected.

A complicating factor in developing a breeding strategy is that the mode of inheritance is not always clear. The HCM-causing *MYBPC3* variant in Maine coons for example, was originally proposed to have an autosomal dominant mode of inheritance.[67,89] However, when comparing both genotypes to homozygous wild type cats, homozygotes had a significantly increased odds ratio for HCM and heterozygotes did not which was interpreted as a decreased penetrance.[96] The disease might therefore be approached less stringently, avoiding only matings that can give homozygous offspring.[101] As the variant has an allele frequency of 18%, the stringency of the selection can strongly decrease the genetic variation of the breed.

Selection without DNA tests

For many genetic diseases in domestic animals, no DNA test is available. This can be the case in monogenic diseases, when no genetic study has yet successfully identified the causative variant. It is also the case for multifactorial diseases, where a single variant, as detected in a

classic DNA test, has only very little effect on the phenotype. In these cases, the selection is mostly based on the phenotype of the animals.

The most straightforward form of selection on the basis of phenotype classifies potential breeding animals as either healthy or affected and only retains the healthy animals for breeding. This is common practice in selection against HCM, where animals are screened via echocardiography and affected or equivocal animals are excluded from breeding.[172] In case of a monogenic disease, any affected animal is considered a carrier of a disease-causing variant and differences in disease severity can be attributed to other genetic or environmental factors. Therefore, all animals showing signs of the disease should be excluded from breeding.[171] It is not known how effective this selection strategy is in reducing the prevalence of HCM.[172]

When compared to genetic testing for HCM, echocardiography has some disadvantages. HCM is not apparent at birth and only develops later in life, with an average age of diagnosis of 6 years in clinical practice.[54] Carriers of HCM-causing variants may therefore have no detectable left ventricular hypertrophy in their first years and only be identified when they already have offspring. If HCM-causing variants in cats have a variable penetrance similar to those in humans, such carriers may even remain healthy throughout their lives, but nevertheless put their offspring at risk.

Reliable echocardiographic examination also requires considerable skill and is therefore best done by a specialist or experienced practitioner. This makes echocardiography generally more expensive than DNA testing. The cost is further increased by follow-up examinations to detect the development of hypertrophy at a later age.[172] Even when multiple echocardiographic examinations are performed by a specialist, the result may remain equivocal, as the distinction between physiological variation and mild pathological cannot always be made.[62] In addition, the detection of cardiac hypertrophy should be complemented with further diagnostic testing to distinguish secondary hypertrophy from primary HCM.

For a complex trait, the efficiency of selection increases with the heritability of the trait and with the difference between the population mean for that trait and the mean of the animals selected for breeding.[139.] Unfortunately, when only a binary phenotype of an animal can be observed,

very little is known about the value of the presumed underlying continuous variable.[139] The efficiency of selection can be improved by calculating the estimated breeding value of an animal on the basis of its phenotype and that of its relatives. The most efficient method is genomic selection, where the breeding value is predicted on the basis of variants on a genome-wide array.[174]

The possible effectiveness of phenotypical selection was demonstrated by a decrease in myxomatous mitral valve disease prevalence after ten years of excluding dogs with signs of the disease on auscultation or echocardiography.[175] A similar study found a much smaller effect, but this might be because only auscultation was used for phenotyping and participation in the breeding program was not mandatory.[176] Another study found no effect, but this study was also limited to auscultation and had a much smaller in sample size and duration.[177]

DNA tests have several advantages over phenotypical screening methods. For a recessive disorder, they can distinguish carriers from homozygous wild type animals, allowing optimal breeding decisions. By testing directly on the level of the DNA, they also circumvent the problems of incomplete or age-dependent penetrance.[178] They are also generally cheaper than phenotypical screening tests. By facilitating more DNA tests, genetic investigation of monogenic disorders has a strong potential to help improve the health and welfare of pets.

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2. Aims of this thesis

The knowledge of feline genetics is expanding at an increasing rate, as witnessed by the rising number of feline genetic variants that are associated with a trait or disease. This dissertation is meant to contribute to this growing body of knowledge and its application in animal breeding. Its aims are twofold: (i) to provide information for veterinary geneticists to advance the knowledge of feline medical genetics and (ii) to provide tools for cat breeders to reduce the incidence of disorders with a genetical component, without compromising genetic diversity. It focuses on two feline diseases, hypertrophic cardiomyopathy (HCM) and corneal sequestration.

Based on the similarity between feline and human HCM, feline HCM may also be caused by variants in sarcomeric genes, most notably *MYBPC3* and *MYH7*. The aim of the study described in chapter three was to identify HCM-causing variants by sequencing cardiac cDNA of these two genes from nine cats that had been diagnosed with HCM.

In chapter four, a variant in the sarcomeric gene *TNNT2* is investigated. This variant was proposed earlier as a cause of HCM, but with little evidence. The aims of this chapter were to: (i) to estimate the variant's allele frequency; (ii) to determine its association with HCM; (iii) to investigate its molecular effects on the mRNA level and (iv) to classify the variant according to the ACMG guidelines.

The fifth chapter deals with corneal sequestration. As this disease has no equivalent in other species and its genetic aspects have not been examined before, it is not known what the role of genetics in this disease is. The aims of this study ~~was~~ were: (i) to determine the effect of the parents on the risk of developing corneal sequestrum; (ii) to estimate the heritability of this disease and (iii) to identify genomic regions associated with the disease.

The aims of chapter six, the general discussion of the thesis, were to discuss the implications of the findings in this thesis: (i) in relation to other recent scientific developments (ii) for future scientific research and (iii) for cat breeding.

3. A feline orthologue of the human *MYH7* c.5647G>A (p.(Glu1883Lys)) variant causes hypertrophic cardiomyopathy in a domestic shorthair cat

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3.1 Abstract

Hypertrophic cardiomyopathy (HCM) is the most common inherited human heart disease. The same disease has a high prevalence in cats, where it is also suspected to be inherited. More than 1 500 variants in *MYBPC3*, *MYH7* and other sarcomeric genes are associated with human HCM, while in cats, only two causative variants in *MYBPC3* are currently known. Here, we describe an adult domestic shorthair cat with arterial thrombo-embolism and heart failure that was diagnosed with HCM on necropsy. Sequencing of the coding regions of *MYBPC3* and *MYH7* revealed 21 variants, of which the *MYH7* c.5647G>A (p.(Glu1883Lys)) variant was further analysed, because its orthologous variant had already been reported in a human patient with HCM, but with limited causal evidence. This variant affects the highly conserved assembly competence domain, is predicted *in silico* to be damaging and was found only once in population databases. Recently, functional studies have confirmed its predicted damaging effect and a paralogous variant in *MYH6* has been associated with cardiac disease in humans as well. This report of an orthologous variant in a cat with HCM and its absence in 200 additional cats provides further evidence for its disease-causing nature. As the first report of feline HCM caused by a variant in *MYH7*, this study also emphasizes this gene as a candidate gene for future studies in cats and highlights the similarity between human and feline HCM.

3.2 Introduction

Hypertrophic cardiomyopathy (HCM; Phenotype MIM number: 192600) is the most common inherited human heart disease, affecting at least 1 in 500 people.[1] It is characterized by concentric hypertrophy of the left ventricle that cannot be attributed to secondary causes.[2] The clinical expression is widely variable, ranging from longevity without symptoms to congestive heart failure or sudden death.[2] More than 1 500 variants are associated with human HCM, most of them limited to a single patient family.[3] They mainly reside in sarcomeric genes, of which *MYBPC3* and *MYH7* are the most important, containing 70% of the variants.[1]

MYBPC3 (human geneID: 4607) consists of 33 exons and encodes the cardiac myosin binding protein C of 1 274 amino acids. The protein seems to play a key role in the regulation of

sarcomeric contractility and may also influence sarcomere structure.[4,5] Known causative variants in *MYBPC3* are either missense or truncating variants.[6] *MYH7* (human geneID: 4625) comprises 40 exons and encodes myosin-7 of 1 935 amino acids, the dominant myosin heavy chain protein in cardiac and type I skeletal muscle fibres.[7,8] This protein generates contractile force through hydrolyzation of ATP and interactions with actin at the N-terminal head and channels this force through its C-terminal tail to the cytoskeleton.[8] HCM-causing variants in *MYH7* are principally missense variants.[6] Missense variants in *MYH7* have also been associated with dilated cardiomyopathy, left ventricular non-compaction and skeletal muscle diseases.[2,9] One such skeletal muscle disease is myosin storage myopathy (MSM), characterized by slowly progressive muscle weakness and hyaline bodies in type I muscle fibres, that is caused by variants in exons 37-40 (exons are numbered like in NG_007884.1).[9]

HCM (OMIA 000515-9685) is the most common heart disease in cats, affecting almost 15% of the feline population.[10] HCM is often associated with certain breeds, for example the Maine coon, ragdoll and British shorthair breeds.[11] Nevertheless, most patients are non-pedigree domestic shorthairs.[12] Feline HCM is considered the best spontaneous animal model for human HCM for its similarity in morphology, histopathology and clinical course.[13,14] A genetic aetiology is suspected in several breeds as well as in domestic shorthairs.[12] However, only two causative variants in cats have yet been identified in *MYBPC3*, XM_019812396.1:c.91G>C in Maine coons[15] and c.2455C>T in ragdolls.[11] An orthologue of the latter variant also causes HCM in humans.[16]

The aim of this study was to identify the causative variant in cats affected with HCM. Based on the hypothesis that human and feline HCM have a similar genetic cause, we sequenced the coding regions of *MYBPC3* (geneID: 101094698) and *MYH7* (geneID: 101096736). In this report, we describe the case in which a causal variant was identified. The results from eight other cases that were sequenced, but where no causal variant was found, are described in Supplementary Table 6.

3.3 Materials and methods

Clinical examination

A male castrated domestic shorthair, estimated to be six years old, was presented to the emergency department of Ghent University's small animal clinic for acute paraplegia, pain and severe dyspnoea with cyanosis. A physical examination and thoracic and cardiac focused assessment sonography scan were performed.[17] Due to the severe clinical signs and poor prognosis, the cat was euthanized.

Pathological examination

A complete necropsy was performed, including examination and weighing of the heart, and the heart was frozen at -80°C thereafter. A transverse slice of frozen ventricular tissue was fixated in 4% buffered formaldehyde, paraffin embedded and routinely processed for histopathological examination. Five µm sections were stained with haematoxylin-eosin, Von Giesson, Masson's Trichrome, Prussian blue and Alcian Blue (pH 2.5). Immunohistochemical stainings included elastin (Novocastra lyophilized monoclonal mouse antibody elastin, Cat No. NCL-ELASTIN, Leica biosystems, Newcastle, United Kingdom) and smooth muscle actin (monoclonal mouse anti-human smooth muscle actin clone 1A4, Cat No. M085101-2, Dako, Glostrup, Denmark).

Genetic analysis

Genomic DNA was isolated from 100 mg frozen heart tissue from the case by proteinase K digestion and subsequent phenol/chloroform extraction and ethanol precipitation.[18] The purity and quantity of the DNA were determined with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The gDNA was first tested for the two known feline HCM-causing *MYBPC3* c.91G>C and c.2455C>T variants using an in-house developed probe-based qPCR assay.

Total RNA was isolated from 100 mg frozen heart tissue from the case using the Aurum Total RNA Fatty and Fibrous Tissue Kit, including an on-column DNase digest (Bio-Rad Laboratories, Hercules, CA, USA). The purity and quantity of the RNA were determined with the NanoDrop ND-1000 spectrophotometer, the integrity was assessed by evaluating the

28S/18S rRNA bands on agarose gel and possible DNA contamination was detected with minus-RT-PCR using an in-house developed UBC integrity assay.[19] Reverse transcription was performed on 1 µg high-quality, DNA-free RNA using the ImProm-II Reverse Transcription System (Promega Corporation, Madison, WI, USA) with oligo(dT) and random hexamer primers. Complementary DNA was 10 times diluted with water and 2 µl was used as a template for PCR. The integrity and amplifiability of the cDNA were tested with the UBC integrity assay. Primer pairs were designed to generate amplicons covering the complete coding regions of *MYBPC3* (Acc. No.: XM_019812396.1) and *MYH7* (Acc. No.: XM_006932746.4) on cDNA using the NCBI Primer-BLAST software,[20] checking for primer specificity and avoiding known SNPs. Regions that form secondary structures, as predicted by Mfold,[21] were excluded as primer binding site. PCR was performed with Tempase Hot Start polymerase (VWR International, Leuven, Belgium) and amplicons were analysed via agarose gel electrophoresis. Sequencing reactions were performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with individual PCR primers as sequencing primers. The sequences were run at Eurofins Genomics (Ebersberg, Germany) and the results were analysed with BioEdit v7.2.5.[22] For missense variants, the evolutionary conservation of the affected amino acid was checked via ClustalW and quantified with the ConSurf web tool[23] and the functional effect of the substitution was predicted *in silico* with PROVEAN[24] and PolyPhen-2.[25] The Exome Variant Server (EVS),[26] Exome Aggregation Consortium (ExAC)[27] and Genome Aggregation Database (GnomAD)[27] databases were searched for orthologous variants in humans.

A *Bse*RI PCR-RFLP assay was developed to genotype exon 38 of *MYH7* (Acc. No.: NC_018728.3; exon 38 spans positions 76 166 287 to 76 166 382) for the c.5647G>A (p.(Glu1883Lys)) variant on gDNA and validated by sequencing. The assay was performed on DNA extracted from either whole blood using proteinase K digestion as described in Van Poucke *et al*.[28] or from heart tissue as outlined above in 200 additional cats. These were 125 domestic shorthairs and 25 cats each from the ragdoll, Maine coon and British shorthair breeds. The resulting fragments were evaluated via gel electrophoresis.

3.4 Results

Clinical features

The six year old domestic shorthair cat was presented to the university hospital with acute paralysis of first one and then also the other pelvic leg. Three weeks previously, he had developed a cough that did not respond to antibiotics. The owner had noted no other clinical signs. The only information about family members that the owner could provide was that the only litter mate, a female, had been found dead suddenly at the age of two years.

On inspection and physical examination the cat had tachypnoea, severe dyspnoea and cyanosis. Femoral pulses were absent and the hind legs were paralysed, cold and extremely painful when manipulated. Lung sounds were muffled ventrally and the dyspnoea made cardiac auscultation very difficult. The cat was hypothermic with a rectal temperature of 36 ºC. Focused thoracic sonography showed multiple B-lines and focused cardiac sonography showed subjective thickening of the left ventricular free wall. The cat was diagnosed with thromboembolism of the distal aorta most likely due to hypertrophic cardiomyopathy, with a suspicion of pulmonary oedema. No further examinations were performed because of the poor general condition of the cat. Because of the severity of the clinical signs and the poor prognosis, the cat was euthanized.

Pathological features

Pathological examination revealed subjectively hypertrophic skeletal muscles and an enlarged heart that weighed 27.2 gram (normal weight: < 20 gram).[29] The left ventricle felt firm on palpation and the left atrium was dilated. Transverse sectioning revealed concentric hypertrophy of the left ventricular wall with narrowing of the ventricular lumen. The thicknesses of the left ventricular free wall, interventricular septum and right ventricular free wall were 12, 9 and 3 mm, respectively. The lungs were congested and oedematous. Serohaemorrhagic fluid was present in the pleural cavity, trachea and nasal cavity. The kidneys showed multiple chronic infarctions.

Histopathological examination of the left ventricle myocardium revealed a diffuse hypertrophy

and karyomegaly of the cardiomyocytes with multifocal areas of increased branching. There were also numerous small fibrotic septa intersecting the myocardium (interstitial fibrosis) with adjacent myofibre disarray. The subendocardial region of the left ventricle showed a non-circumferential extensive fibrosis with few scattered small areas of chondroid metaplasia and areas with large aggregates of hemosiderin-laden macrophages (iron-positive on Prussian blue). This thick subendocardial layer stained red on Von Giesson and blue on both Masson's Trichrome and Alcian blue and showed a gradual increase in tissue density from luminal (loosely arranged collagen fibres) to myocardial (compact collagenous connective tissue). Immunohistochemistry for elastin revealed multifocal large areas of increased elastin deposition within the subendocardial layer. The gross and histopathological lesions are consistent with a hypertrophic cardiomyopathy with areas of substantial subendocardial replacement fibrosis. Histopathological images of the heart are given in Figure 1.

Genetic analysis

The qPCR assay was negative for both known *MYBPC3* c.91G>C and c.2455C>T variants. By sequencing the coding region of the cDNA, seven silent and four missense variants in *MYBPC3* and nine silent and one missense variant in *MYH7* were found. They were all archived in the EVA database (https://www.ebi.ac.uk/eva/?Study-Browser&browserType=sgv; project ID: PRJEB3.0318; analysis ID: ERZ795310) and are listed in Supplementary Table 5. Only one variant, *MYH7* c.5647G>A (p.(Glu1883Lys)), was found to change an amino acid that is highly conserved, both in its metazoan orthologues as in its feline and human paralogues, and predicted to be deleterious by PROVEAN (score: -2.613) and PolyPhen-2 (HumDiv score: 1.000, HumVar score: 0.978). This variant was absent in the 200 additionally screened cats and its human orthologue was counted once in the 31 396 alleles in the GnomAD database and not found in the other human databases.

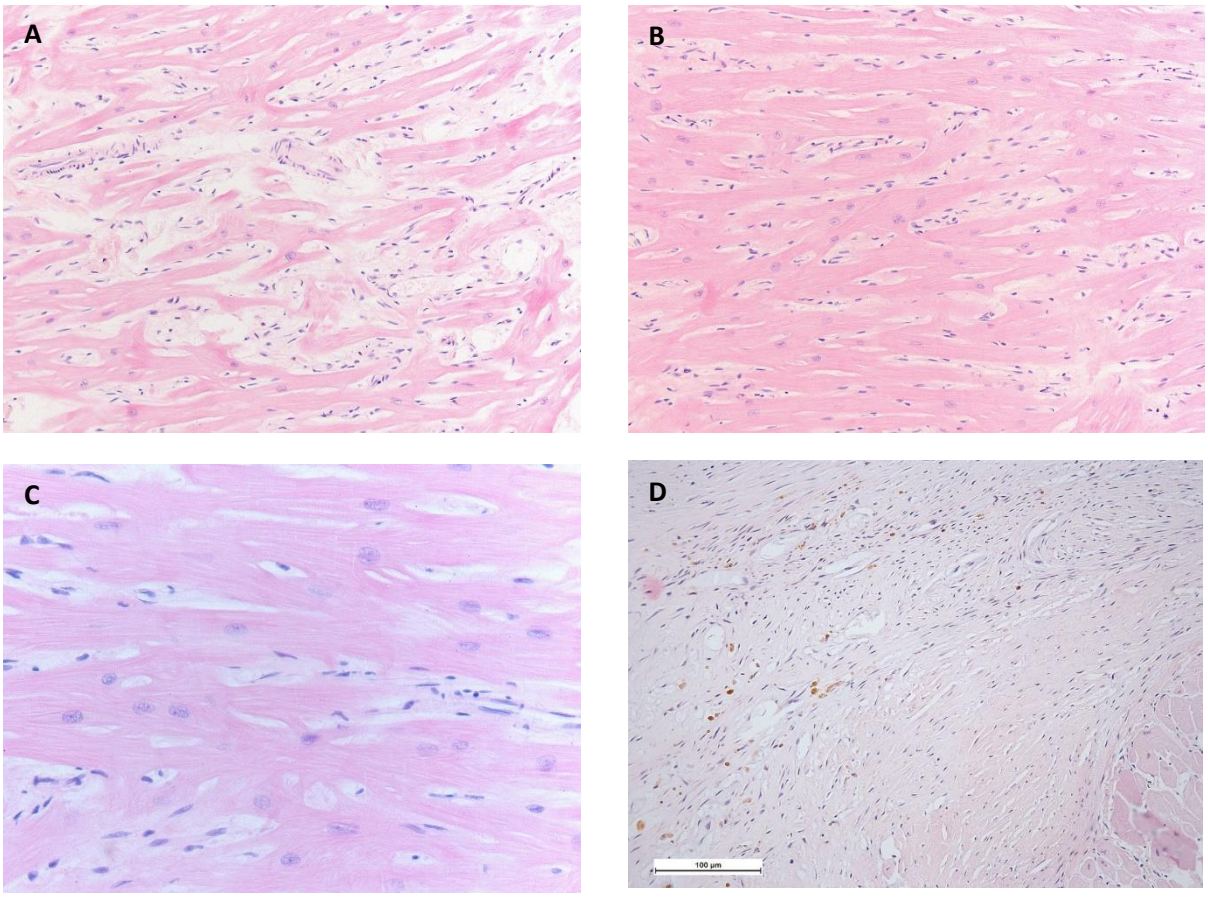

Figure 3.1 Histopathological images of the heart. **A** The cardiomyocytes show a disorganised pattern (myofibre disarray) around foci of interstitial collagen (interstitial fibrosis) and some blood vessels. Some nuclei are swollen (karyomegaly). H&E, original magnification x200. **B** Branching and myofibre disarray admixed with interstitial fibrosis and hypertrophic cardiomyocytes with karyomegaly. H&E, original magnification x200. **C** The cardiomyocytes display branching, karyomegaly and a disorganised pattern. H&E, original magnification x400. **D** The subendocardial region shows deposition of extracellular matrix (mainly collagen) admixed with neovascularisation, fibroblasts and large aggregates of hemosiderin-laden macrophages. There is a maturation to dense collagenous connective tissue in the proximity of the myocard. H&E, original magnification x200.

3.5 Discussion

The physical examination abnormalities in this cat were highly suggestive of thromboembolism of the distal aorta. Most cases of feline arterial thromboembolism are caused by underlying cardiac disease, most commonly HCM.[30] The respiratory signs were compatible with acute pain and with acute, severe congestive heart failure. The history of coughing might also be related to the heart disease, although cardiogenic coughing is rare in cats.[31] Ultrasound examination was limited by the critical condition of the patient, but was indicative for HCM.

Left ventricular hypertrophy can also be secondary to an underlying disease, such as hyperthyroidism and hypertension, and ante mortem diagnostic tests to reliably exclude these diseases were lacking. The lack of macroscopical lesions associated with these diseases the signalment of the cat and the severity of the heart disease are more compatible with primary HCM, but a secondary component was not fully excluded. Some rare causes of hypertrophy, such as myocarditis or infiltration lymphoma, were excluded by histological examination.

In cases of cardiomyopathy with severe fibrosis, there can be a thin line between HCM and restrictive cardiomyopathy, and there is evidence that the two conditions are different phenotypical expressions of the same genetic disease.[32] Restrictive cardiomyopathy can also occur secondary to radiation fibrosis, nutritional deficiencies or infiltrative diseases such as amyloidosis, hypereosinophilia, sarcoidosis or endomyocarditis.[33] In cats, it has also been suggested that it is the result of an end-stage HCM with myocardial failure or infarction.[34] Our case is compatible with a primary HCM because of the heart's increased weight, size, wall thickness and firmness. It also displayed all histological features predominant for HCM such as cardiomyocytes displaying hypertrophy, karyomegaly, branching and myofibre disarray, admixed with myocardial interstitial fibrosis.[35,36] The (sub)endocardial fibrosis was classified as a replacement fibrosis (secondary to ischemic infarction) as the histological features are consistent with chronic granulation/scar tissue and the presence of large aggregates of hemosiderin-laden macrophages is an indication of an old haemorrhage or infarct. The littermate's history of sudden death is also compatible with HCM, although no necropsy had been performed to identify the cause of death in that cat.[37]

Of the 21 variants that were found, only the five missense variants were further investigated, as silent variants are not associated with HCM.[2] The *MYBPC3* c.772G>A (p.(Val258Ile)), c.2765C>T (p.(Pro922Leu)) and c.3109G>A (p.(Ala1037Thr)) variants were not considered to be causal, as the effects of the amino acid substitutions were predicted to be benign and each variant was found in the reference sequence of the mouse, rat, horse and at least one species of the Felidae family. The *MYBPC3* c.220G>A (p.(Ala74Thr)) variant has been proposed earlier as a causative variant for HCM, but subsequent studies found that this variant is very common and not significantly associated with HCM.[38]

This left only the *MYH7* c.5647G>A variant as a possible causative variant, which has a human orthologue, NM_000257.3:c.5647G>A (p.(Glu1883Lys); rs121913652). This orthologue was described by Tajsharghi *et al.*[39] in a consanguineous human family in which three of four siblings were diagnosed with both HCM and MSM as adults. Two patients died of heart failure and the coding regions of *MYH7* in the third patient were sequenced. This patient was a homozygote for the variant, but its causality has been disputed because of limited evidence (see the discussion on ClinVar, variation ID: 14121).

The human and feline cases had comparable HCM, but apparently different degrees of skeletal muscle disease. The human patients displayed a short stature, thoracic scoliosis, calf hypertrophy in one case and progressive muscle weakness.[39] In contrast, the feline case had no musculoskeletal abnormalities other than the generalized muscle hypertrophy observed on necropsy. The owner had not observed muscle weakness. This comparative lack of muscle disease might be explained by a species difference in muscle fibre types. As type I muscle fibres, that express *MYH7*, make up a larger proportion of muscles in large animals such as humans than in smaller cats,[8] a defect in *MYH7* may have more severe consequences in humans. The muscular hypertrophy observed in this cat might have compensated the defect to an extent that allowed normal locomotor function. However, it is also possible that some degree of muscle weakness was present, but not recognized, as even severe myopathy in cats can go unnoticed by the owner and veterinarian.[40,41] Histopathology of skeletal muscles was not available to compare to the MSM phenotype in humans.

The human patient carrying the *MYH7* c.5647G>A variant was a homozygote and Tajsharghi *et al*. suggested an autosomal recessive pattern of inheritance for this variant.[39] However, both HCM and MSM generally show an autosomal dominant pattern of inheritance and this might also have been the case in this human family.[2,9] The two affected siblings that were not genotyped could be heterozygotes and it is not clear whether cardiomyopathy was ruled out in the parents, especially the father who died of stroke at age 58.[39] The cat described here was a heterozygote, consistent with an autosomal dominant pattern of inheritance. Because of the suspicion of HCM in the litter mate, it is considered unlikely that the causative variant was a *de novo* variant. The *MYH7* c.5647G>A variant is extremely rare in humans and the absence of this variant in the 200 additional cats suggests that it is also rare in cats.

The affected glutamic acid residue is part of the assembly competence domain (ACD), a C-terminal 29 amino acid region that has a distinct pattern of four negative charges surrounded by positive charges and is essential for sarcomeric thick filament assembly.[42] During the assembly process, the tails of myosin heavy chains form coiled coils, the coils dimerize tail-to-tail and then aggregate into filaments.[43] The tail-to-tail dimerization requires the correct functioning of the ACD, which is highly conserved in myosin-7 orthologues across species as well as in the striated muscle myosin heavy chain paralogues of cats and humans (Figure 2).[42,43]

The p.(Glu1883Lys) variant substitutes one of the four negative charges by a positive charge. *In vitro* assessment of this substitution shows a lower stability and higher solubility of the protein, indicating a reduced assembling capacity.[44] For a similar substitution (Glu1886Lys), it was shown that the extent of filament assembly is reduced to 60% compared to the wild type.[45] Expression of Glu1883Lys mutant protein in a *Drosophila* animal model causes ultrastructural thick filament misalignment and disrupted sarcomere structure in pupae. In adults, these abnormalities worsen and are complemented with hyaline bodies reminiscent of human MSM. These adults show a compromised ability to fly and jump, indicating severe functional effects of this variant, as predicted by computational and predictive data.[44]

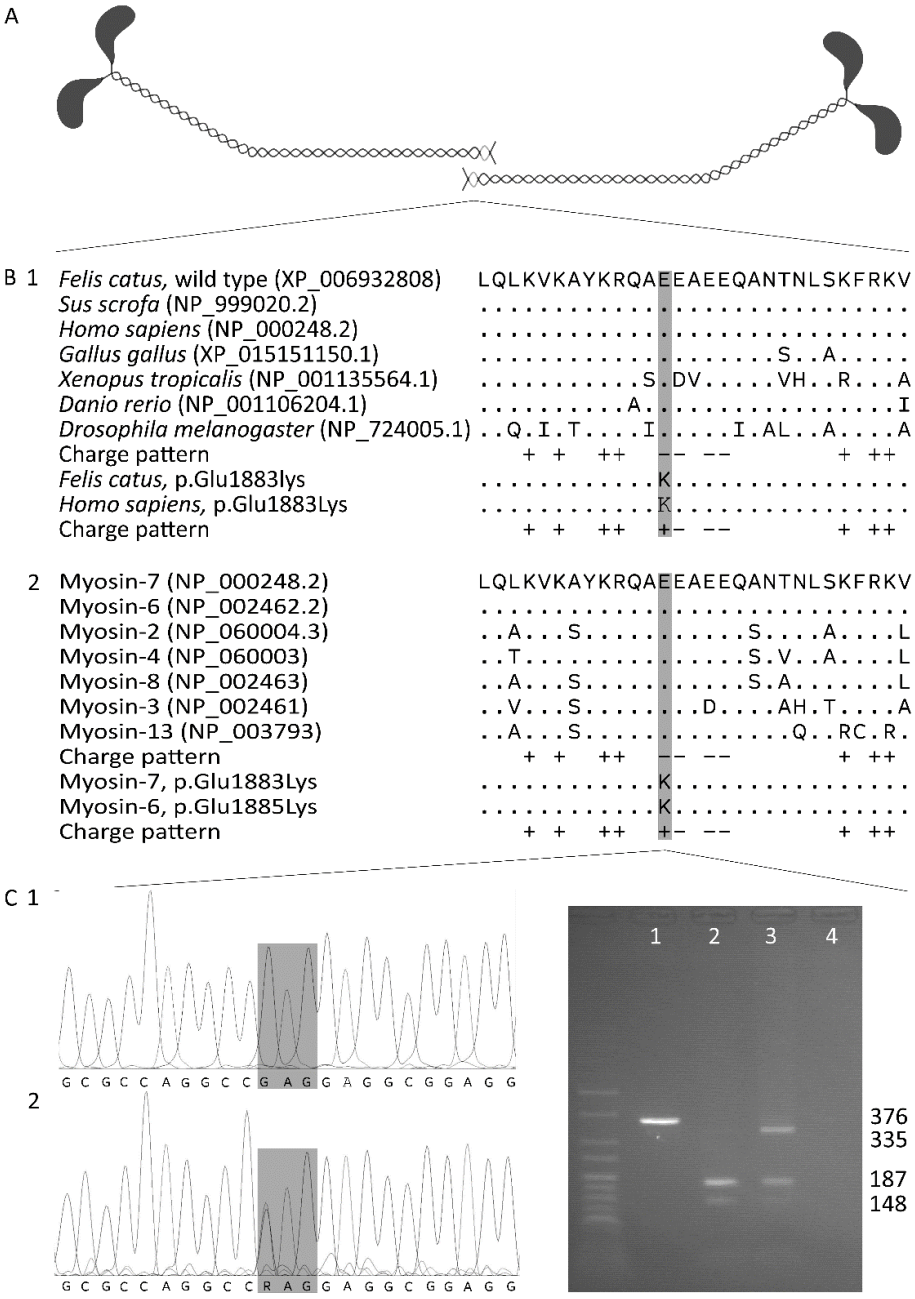

Figure 3.2 Position and evolutionary conservation of the ACD.**A**. Tail-to-tail dimerization of the coiled coils formed by myosin heavy chains. The ACD regions are shaded light-grey. **B**. The amino acid sequence and charge pattern of the ACD in myosin-7 (myosin heavy chain for *Drosophila*) orthologues across species (1) and skeletal and cardiac muscle myosin heavy chain paralogues in humans (2). Accession numbers are given in parentheses and identical amino acids are depicted as ".". The variants cause the substitution of a conserved, negatively charged glutamic acid residue by a positively charged lysine residue. **C**. Chromatograms of a homozygous wild type cat (1) and the heterozygous case (2). The variant changes codon 1883 from GAG to AAG. **D**. Agarose gel electrophoresis of an uncleaved amplicon (1), 376 bp long, the cleaved amplicons of a homozygous wild type cat (2) and the heterozygote case (3) and a negative control (4). The amplicon is always cleaved at an internal cleavage site in a large fragment of 335 bp and a small fragment of 41 bp. The wild type large fragment contains a second recognition site and is cleaved in fragments of 187 and 148 bp, while the variant large fragment does not contain a recognition site and remains intact. Two percent agarose gel with Hyperladder V.

In addition, a paralogous variant in *MYH6*, the major myosin heavy chain gene expressed in the cardiac atria,[7] causes an identical charge pattern disruption of the ACD with deleterious phenotypical effects in humans. This variant, NM_002471.3:c.5653G>A (p.(Glu1885Lys); rs760353963), is considered to be the causative variant in a family where Wolff-Parkinson-White syndrome segregates in an autosomal dominant pattern with incomplete penetrance.[46] It is also considered to have contributed to lethal congenital heart disease in a compound heterozygote who also carried another *MYH6* variant.[47]

By adding the data of the orthologous feline case to that of the already described human family, together with the updated population data, the recently described functional studies and the paralogous human *MYH6* variant, there is now enough evidence to classify the NM_000257.3:c.5647G>A (p.(Glu1883Lys)) variant as HCM-causing, according to the standards and guidelines for the interpretation of sequence variants.[48] This implies that other cats carrying this variant are also at risk of developing HCM. Given the variable penetrance of HCM, the exact outcome will depend on modifier variants and environmental factors. It is not known whether the described case represents a relatively mild or severe outcome.

This is the first report of HCM caused by a variant in *MYH7* in a cat. An earlier study sequencing sarcomeric candidate genes, including *MYH7*, in 14 HCM-affected cats did not identify any disease-causing variant.[49] A recent, more extensive study identified several candidate variants, but not in *MYH7*.[50] It might be interesting to investigate if this variant is also the causal variant in other domestic shorthair HCM cases.

Variants that cause HCM in humans are generally rare and the clinical significance of a variant found in human patients is not always clear.[1,2] The identification of an orthologous variant in a spontaneous animal model of HCM can support its causality in humans. The involvement of *MYH7*, in addition to *MYBPC3*, in feline HCM suggests that its similarity to human HCM extends to the genetic level. As most cases of feline HCM are idiopathic, further investigations on its aetiology are needed to confirm this genetic similarity. In addition to the phenotypic similarity, this similarity would make cats with HCM a suitable animal model for the development of preventive and therapeutic strategies for HCM.

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3.7 Supplementary file

Supplementary Table 1 Primers, probes, program and mixes of the qPCR assay.

Primers | probes	***MYBPC3* c.91G>C**	***MYBPC3* c.2455C>T**
Forward primer	5'-GAAGCCAAGGTCAGTG-3'	5'-GACCAGAGCTCCTGTC-3'
Reverse primer	5'-CTAGGCCATACTTGTCAC-3'	5'-CGTAGACTCGCATCTCA-3'
Wild-type probe	5'-FAM-TGTCTCGG**C**CTCGAA-BHQ1-3'	5'-FAM-TTCAGCC**G**CATCCACC-BHQ1-3'
Variant probe	5'-TEXASRED-TGTCTCGG**G**CTCGAA-BHQ2-3'	5'-HEX-AGTTCAGCC**A**CATCCACC-BHQ1-3'
qPCR program	**c.91G>C qPCR mix**	**c.2455C>T qPCR mix**
14'40" - 95 °C	4.9 µl H_2O	4.9 µl H_2O
00'20" - 95 °C	1.0 µl 10x Key Buffer	1.0 µl 10x Key Buffer
00'40" - 59 °C } 40x	1.0 µl Primers (5 µM each)	1.0 µl Primers (5 µM each)
Signal detection }	0.4 µl Wild-type probe (10 µM)	0.5 µl Wild-type probe (5 µM)
	0.4 µl Variant probe (10 µM)	0.3 µl Variant probe (5 µM)
	0.2 µl dNTPs (10 mM each)	0.2 µl dNTPs (10 mM each)
	0.1 µl Polymerase (5 U/µl)	0.1 µl Polymerase (5 U/µl)
	2.0 µl Template	2.0 µl Template
	10.0 µl Total volume	10.0 µl Total volume

Supplementary Table 2 PCR primer pairs with the location where each primer binds, the lengths of the amplicons and the respective PCR mixes and programs, as depicted in Supplementary Table 3. Locations are identified like in NC_018732.3 101324493..101342043 for *MYBPC3* and NC_018728.3 76165299..76187655 for *MYH7*.

***MYBPC3* cDNA primer pairs**	Location	Product length	PCR mix	PCR program
1f: 5'- TCCTTGGGTGGCCTGTGACT-3'	Exon 1	745	1	1
1R: 5'-TGCGAAAGTAGTCTGGGCATCTGT-3'*	Exon 6			
2f: 5'-TGACCCCATCGGCCTCTTTGTG-3'*	Exon 4	810	1	1
2r: 5'-GCACCCACGGACTCGAAGATGT-3'*	Exon 14			
3f: 5'-GGGCATGAAGCGAGACGAGAAGA-3'*	Exon 11	638	1	1
3r: 5'-ACTTGAACACCGCCTGGTCCTT-3'*	Exon 17			
4f: 5'-ACGGGCAGAGACACCACCTCAT-3'*	Exon 16	697	1	1
4r: 5'-GTGAAGATGCTGCGGTCCTTGGT-3'*	Exon 22			
5f: 5'-AGCTACGCCTGGATGTCCCTATCT-3'*	Exon 20	729	1	1
5r: 5'-ACTCCACGCTGTAGCCATCCAA-3'*	Exon 25			
6f: 5'-TGATTGAGGGCGTGGTGTATGAGA-3'	Exon 24	860	2	1
6r: 5'-AGTGCGACGGTAATGTTCCAAGACA-3'*	Exon 30			
7f: 5'-TGCTGCGGATCGAGAACATGGA-3'*	Exon 28	865	1	1
7r: 5'-AGGCCCGCTCACCTTAATTGC-3'	Exon 33			
***MYH7* cDNA primer pairs**	Location	Product length	PCR mix	PCR program
8f: 5'-TGCTCTGTCTTTCCTTGCTGCTCT-3'*	Exon 1	858	1	2
8r: 5'-TTCCGGTCGCCCCAAAATGGA-3'	Exon 9			
9f: 5'-GGGATCGCAGCAAGAAGGAGCA-3'*	Exon 7	747	1	2
9r: 5'-GCTTGGTCTCCAGGGTGGCATT-3'	Exon 14			
10f: 5'-CATGTACAAGCTGACGGGTGCCAT-3'*	Exon 12	958	1	
10r: 5'-GGGATGTGTAGAGCGCAAGTTGGT-3'*	Exon 18			
11f: 5'-GTCCTCCCTCAAGATGCTCAGTAACC-3'*	Exon 16	859	1	2
11r: 5'-GTTGTCTTGTTCCGCCTGCACTT-3'	Exons 22 & 23			

Supplementary Table 2 (continued)

***MYH7* cDNA primer pairs**	Location	Product length	PCR mix	PCR program
12f: 5'-GCTGCTGGGCTCCCTAGACATT-3'*	Exon 20	844	1	2
12r: 5'-GACTTTGGCCTTGGTCAGGGTGTT-3'	Exon 24			
13f: 5'-TGGAGAAGGAGAAGCATGCAACAGA-3'*	Exon 23	936	2	2
13r: 5'-GGCTGGTGAGGTCGTTGACAGAA-3'	Exon 28			
14f: 5'-AACCTGCAGCGTGTGAAGCAGAA-3'*	Exon 27	964	1	2
14r: 5'-GGACTTTCTCCAGCTCATGGATGGTT-3'	Exon 33			
15f: 5'-CCATCCAGAGGACAGAGGAGCTTGA-3'*	Exon 30	805	1	2
15r: 5'-TGGAGACCCTTGACTTGCTTCTGG-3'	Exon 34			
16f: 5'-AACCATCCATGAGCTGGAGAAAGTCC-3'*	Exon 33	615	1	2
16r: 5'-TGGTTGATGAGGCTGGTGTTCTGG-3'	Exons 35 & 36			
17f: 5'-CCGTGCCAACGACGACCTGA-3'*	Exon 35	918	2	2
17r: 5'-TGCTTCATCCAAAGGGGCTGCT-3'*	Exon 40			
***MYH7* gDNA primer pair for PCR-RFLP**	Location	Product length	PCR mix	PCR program
18f: 5'- GGTAACGACCACGGCGGGAGA-3'	Intron 37	376	1	3
18r: 5'- CGCTCCTCTGCCTCATCCAGCTC-3'	Exon 39			

*These primers were used as sequencing primers.

Supplementary Table 3 PCR and sequencing mixes and programs.

PCR mix 1	**PCR mix 2**	**Sequencing mix**
5.7 µl H_2O	4.7 µl H_2O	2.0 µl H_2O
1.0 µl 10x Key Buffer	1.0 µl 10x Key Buffer	2.0 µl 5x Sequencing buffer
1.0 µl Primers (5 µM each)	1.0 µl GC-rich	2.0 µl GC-rich
0.2 µl dNTPs (10 mM each)	1.0 µl Primers (5 µM each)	1.5 µl Sequencing primer (2 µM)
0.1 µl Polymerase (5 U/µl)	0.2 µl dNTPs (10 mM each)	0.5 µl RR-mix
2.0 µl Template	0.1 µl Polymerase (5 U/µl)	2.0 µl Template
10.0 µl Total volume	2.0 µl Template	10.0 µl Total volume
	10.0 µl Total volume	

PCR program 1	**PCR program 2**	**PCR program 3**	**Sequencing program**
14'00" - 95 °C	14'15" - 95 °C	14'30" - 95 °C	2'00" - 95 °C
01'00" - 95 °C	00'45" - 95 °C	00'30" - 95 °C	0'20" - 95 °C
01'00" - 65 °C (40x)	00'45" - 64 °C (40x)	00'30" - 66 °C (30x)	4'00" - 65 °C (30x)
02'00" - 72 °C	01'30" - 72 °C	01'00" - 72 °C	Hold - 4 °C
05'00" - 72 °C	05'00" - 72 °C	02'00" - 72 °C	
Hold - 15 °C	Hold - 15 °C	Hold - 15 °C	

Supplementary Table 4 Restriction digest mix and reaction conditions.

Restriction digest mix	**Reaction conditions**
1.0 µl NEBuffer 4	Temperature: 37 °C
2.0 µl *Bse*RI (5 U/µl)	Duration: > 6 hours
7.0 µl Template	
10.0 µl Total volume	

Supplementary Table 5 Variants found in *MYBPC3* and *MYH7* of the described case.

***MYBPC3* variants (XM_019812396.1)**			***MYH7* variants (XM_006932746.4)**		
c.220G>A	p.(Ala74Thr)	heterozygote	c.975T>C	p.(Asp325=)	heterozygote
c.414T>C	p.(Ser138=)	homozygote	c.1128C>T	p.(Asp376=)	heterozygote
c.772G>A	p.(Val258Ile)	heterozygote	c.1572T>C	p.(Ile524=)	heterozygote
c.1032T>C	p.(Arg344=)	heterozygote	c.1719G>A	p.(Pro573=)	heterozygote
c.1311C>T	p.(Gly437=)	heterozygote	c.3546G>A	p.(Thr1182=)	homozygote
c.1326C>T	p.(Ser442=)	heterozygote	c.4053A>G	p.(Thr1351=)	heterozygote
c.1956C>T	p.(Arg652=)	heterozygote	c.4308T>C	p.(Asn1436=)	heterozygote
c.2765C>T	p.(Pro922Leu)	heterozygote	c.4314C>T	p.(Ala1438=)	heterozygote
c.2847A>G	p.(Ala949=)	homozygote	c.4815G>A	p.(Thr1605=)	homozygote
c.3109G>A	p.(Ala1037Thr)	heterozygote	c.5647G>A	p.(Glu1883Lys)	heterozygote
c.3267A>G	p.(Gln1089=)	heterozygote			

Supplementary Table 6 Variants in *MYBPC3* and *MYH7* of the eight other HCM-affected cats where the coding regions of these genes were sequenced. These cats were 5 domestic shorthairs, 2 British shorthairs and one crossbreed domestic shorthair x Persian. The allele frequency in this group of 8 cats is given for each variant. None of these variants was considered to cause HCM. All variants were archived in the EVA database together with the variants in the described case (https://www.ebi.ac.uk/eva/?Study-Browser&browserType=sgv; project ID: PRJEB30318; analysis ID: ERZ795310).

***MYBPC3* variants (XM_019812396.1)**			***MYH7* variants (XM_006932746.4)**		
c.175G>A	p.(Ala59Thr)	0.0625	c.85C>A	p.(Arg29=)	0.0625
c.220G>A	p.(Ala74Thr)	0.125	c.975T>C	p.(Asp325=)	0.4375
c.311C>T	p.(Pro104Leu)	0.0625	c.1128C>T	p.(Asp376=)	0.25
c.414T>C	p.(Ser138=)	1.000	c.1572T>C	p.(Ile524=)	0.375
c.772G>A	p.(Val258Ile)	0.25	c.1719G>A	p.(Pro573=)	0.1875
c.1032T>C	p.(Arg344=)	1.000	c.1746C>T	p.(Tyr582=)	0.1875
c.1806C>T	p.(Asp602=)	0.25	c.1872T>C	p.(Tyr624=)	0.0625
c.1956C>T	p.(Arg652=)	1.000	c.2289G>A	p.(Val763=)	0.125
c.2095G>A	p.(Ala699Thr)	0.0625	c.2886C>T	p.(Ala962=)	0.0625
c.2607C>T	p.(Pro859=)	0.3125	c.2943A>G	p.(Glu981=)	0.125
c.2765C>T	p.(Pro922Leu)	0.9375	c.3132G>T	p.(Val1044=)	0.0625
c.2847A>G	p.(Ala949=)	0.875	c.3171C>T	p.(Gly1057=)	0.0625
c.2976C>A	p.(Leu992=)	0.0625	c.3459C>T	p.(Ala1153=)	0.1875
c.3109G>A	p.(Ala1037Thr)	0.9375	c.3546G>A	p.(Thr1182=)	0.1875
c.3126C>T	p.(Tyr1042=)	0.125	c.3813C>T	p.(Asn1271=)	0.125
c.3267A>G	p.(Gln1089=)	0.0625	c.4053A>G	p.(Thr1351=)	0.3125
c.3388A>G	p.(Ile1130Val)	0.0625	c.4200C>G	p.(Ala1400=)	0.125
c.3525C>T	p.(Asp1175=)	0.1875	c.4308T>C	p.(Asn1436=)	0.25
c.3799C>T	p.(Leu1267=)	0.0625	c.4314C>T	p.(Ala1438=)	0.125
			c.4509A>G	p.(Lys1503=)	0.8125
			c.4815G>A	p.(Thr1605=)	0.9375
			c.4959C>T	p.(Thr1653=)	0.3125
			c.5106G>A	p.(Ala1702=)	0.1875
			c.5550C>G	p.(Leu1850=)	0.25

Necropsy findings in the eight other HCM-affected cats

A male castrated domestic shorthair of unknown age had been euthanised for hind leg paralysis and dyspnoea. Necropsy revealed a hypertrophic heart (44 grams) with a narrowed left ventricular lumen, pulmonary oedema and mild general skeletal muscle hypertrophy.

A 3 year old, male castrated domestic shorthair had been euthanised for hind leg paralysis and dyspnoea. Necropsy revealed a hypertrophic heart (26 grams) with a narrowed ventricular lumen and subendocardial haemorrhages, a thrombus in the distal aorta, skeletal muscle hypertrophy, pulmonary oedema and multiple kidney infarctions.

A 7 year old, female domestic shorthair had been euthanised for hind leg paralysis. Necropsy revealed a hypertrophic heart (25 grams).

A 13 year old, female domestic shorthair cat had been euthanised for dyspnoea. Necropsy revealed a hypertrophic heart (24 grams) and pleural effusion.

A 7 year old, Persian x domestic shorthair crossbred cat had been found dead in the garden. Necropsy revealed a hypertrophic heart, pulmonary oedema, pleural effusion and multiple kidney infarctions.

A 2 year old, male castrated British shorthair was diagnosed ante mortem with HCM on echocardiography and later euthanised for congestive heart failure. No formal necropsy was performed, but the heart was excised for this study.

A 13 year old, male castrated British shorthair was diagnosed ante mortem with HCM on echocardiography and later euthanised for hind leg paralysis. No formal necropsy was performed, but the heart was excised for this study.

A 10 year old, male castrated domestic shorthair was diagnosed ante mortem with HCM on echocardiography and later euthanised for congestive heart failure. No formal necropsy was performed, but the heart was excised for this study.

4. The *TNNT2*:c.95-108G>A variant is common in Maine coons and shows no association with hypertrophic cardiomyopathy

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4.1 Abstract

Hypertrophic cardiomyopathy (HCM) is a common and potentially fatal heart disease in many cat breeds. An intronic variant in *TNNT2*, c.95-108G>A, was recently reported as the cause of HCM in the Maine coon. The aim of this study was to determine this variant's allele frequency in different populations and its possible association with HCM. Based on 160 Maine coon samples collected in Belgium, Italy, Sweden and the United States, the variant's allele frequency was estimated to be 0.32. Analysis of the 99 Lives feline whole genome sequencing database showed that the *TNNT2* variant also occurs in other breeds, as well as mixed-breed cats. Comparison of 31 affected and 58 healthy cats did not reveal significantly increased odds for HCM in homozygotes. Based on the combined evidence and in agreement with the standards and guidelines for the interpretation of sequence variants, this variant is currently classified as a variant of unknown significance and should not be used for breeding decisions regarding HCM.

4.2 Introduction

Hypertrophic cardiomyopathy (HCM; OMIA id: 000515-9685) is the most common heart disease in cats.[1,2] HCM is defined as a thickening of the left ventricular wall that is not secondary to abnormal loading conditions or systemic disease.[3,4] Many affected cats stay in the preclinical stage for a long time or their whole life, but can abruptly develop detrimental complications such as congestive heart failure, arterial thromboembolism or sudden death.[5] HCM is known as a genetic affliction in humans, and many feline cases are also suspected to have a genetic cause.[6-10] Although a genetic aetiology implies the possibility to select against the disease, there are several practical complicating factors. On the one hand, phenotypical screening for HCM requires echocardiography by an experienced echocardiographer and might still be challenging in some cases. Mildly affected cats may be hard to distinguish from healthy cats and healthy cats may still develop HCM at a later age.[11] On the other hand, genetic screening is also complicated because HCM is genetically heterogeneous.[12] To this date, several feline variants have been reported as disease-causing in the literature. Three of these

were found in the Maine Coon, a popular breed in which HCM occurs commonly and often leads to severe clinical signs at a young age.[13,14]

Two of these three variants are located in the myosin binding protein C3 (MYBCP3) gene (transcript reference sequence: ENSFCAT00000002530.5): c.91G>C and c.220G>A (also known as A31P and A74T).[15,16] Multiple studies have shown that the c.91G>C variant in a homozygous state strongly increases the risk of HCM,[9,17,18] but this association could not be validated for the c.220G>A variant.[18,19] A third variant, located intronic in the troponin T2, cardiac type (*TNNT2*) gene (transcript reference sequence: ENSFCAT00000061100.2), c.95-108G>A, was recently reported as the cause of cardiomyopathy in a Maine coon cat homozygous for this variant.[20] Based on one family, this variant was computationally predicted to be harmful, but its effect has not been further investigated with functional, clinical or population studies.

The aims of this study were to investigate the *TNNT2*:c.95-108G>A variant further by (i) estimating its allele frequency in Maine coon populations in multiple countries and (ii) examining its association with HCM, followed by an evaluation of its pathogenicity according to the American College of Medical Genetics (ACMG) standards and guidelines for the interpretation of sequence variants.[21]

4.3 Material and methods

Study design

The study was established as an international multicentre study consisting of two phases. In the first phase, Maine coon samples from genetic laboratories and veterinary clinics in Belgium, Italy, Sweden and the United States were genotyped to estimate the *TNNT2* variant's allele frequency. Additionally, the frequency of the allele was determined in Maine coon samples and samples from other breeds based on the 99 Lives Consortium database.[22] In the second phase, samples of Maine coon cats for which the HCM phenotype was known were used in a case-control study to determine the odds ratio for the presence of HCM in cats homozygous for the variant.

Power analysis

A power analysis was conducted to determine the minimal sample size necessary to detect a variant with an odds ratio of 5.0 (based on the ACMG guidelines[21]) and 19.4 (based on the *MYBPC3*:c.91G>C variant[18]). The analysis assumed a disease-prevalence of 15%[1] in the group without the disease-causing allele, a balanced number of affected and healthy cats and a desired power of 0.8. The required sample size per group was 32 (leading to a total of 64 cats) or 9 (leading to a total of 18 cats) for the odds ratios of 5.0 and 19.4, respectively.

Phenotyping

Phenotyping for HCM was done by echocardiography according to the American College of Veterinary Internal Medicine (ACVIM) consensus guidelines for the classification, diagnosis and management of cardiomyopathy in cats.[4]

Genotyping

DNA was isolated from blood or cardiac tissue samples from Maine coon cats. PCR primers specific for a 539 bp DNA fragment surrounding the *TNNT2*:c.95-108G>A variant, based on the Felis_catus_9.0 reference sequence, were designed with Primer3web version 4.1.0.[23] PCR products were confirmed by agarose gel electrophoresis and then Sanger sequenced. The resulting electropherograms were analysed in UGENE version 33 or Codon Code Aligner 8.0.2.

The 99 Lives feline whole genome sequencing database was filtered on genetic variation at position 42204052 of chromosome F1.

The cats with known phenotypes were also genotyped for the *MYBPC3*:c.91G>C variant by Sanger sequencing or by means of a TaqMan assay developed in-house. Cats that were homozygous for this variant were excluded from the association analysis. Details on the primers and reactions can be found in the supplementary file.

In silico analysis

Four online computational tools were used to predict the effect of the variant on the mRNA splicing of *TNNT2*: GENSCAN (http://hollywood.mit.edu/GENSCAN.html, organism: vertebrate),[24] SSPNN (https://www.fruitfly.org/seq_tools/splice.html, organism: Human or other),[25] ESEfinder (http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi, matrices: 5SS_U2_human, 3SS_U2_human, 5SS_U2_mouse and 3SS_U2_mouse)[26] and Netgene2 (https://services.healthtech.dtu.dk/service.php?NetGene2-2.42, organism: Human).[27] In addition, the analysis with the online tool ASSP (http://wangcomputing.com/assp/, default settings),[28] as performed in the original paper, was repeated. The genomic sequence of the *TNNT2* gene (ENSFCAG00000004613) was entered once unchanged and once with the variant (g.5476G>A) in each tool and the predicted splice sites of the reference and variant sequence were compared.

cDNA sequencing

Septal heart tissues were available as snap-frozen samples collected within minutes after cats had been euthanized at the University Animal Hospital in association with the Swedish University of Agricultural Sciences. Two carriers of the variant were identified; one healthy female aged 12.8 years euthanized due to age/arthrosis and behavioural issues/scratching, and one HCM-affected male in congestive heart failure aged 5.7 years. Tissue samples were collected from the same anatomical part of the heart for each cat, snap-frozen in liquid nitrogen, and stored at -80 °C until the date of processing.

Total RNA was isolated from these samples and reverse transcribed to cDNA. Primers spanning the cDNA sequence between exon 1 and exon 7 (based on ENSFCAT00000061100.2) were designed with Primer3web version 4.1.0. PCR products were confirmed by agarose gel electrophoresis and then Sanger sequenced. The resulting electropherograms were analysed in Codon Code Aligner 8.0.2. Details on the procedures, primers and reactions can be found in the supplementary file.

ACMG classification

The standards and guidelines for the interpretation of sequence variants by the ACMG were used to classify the variant as (likely) pathogenic, (likely) benign or of uncertain significance.[21] Every criterion from the guidelines that was relevant for the type of data in this study was evaluated.

Statistical analysis

All analyses were performed in R version 4.0.3. Allele frequencies were computed for each sample origin separately and the corresponding standard errors and 95% confidence intervals were calculated by normal approximation.[29,30] The significance of the differences in allele frequency between the sample origins was assessed with the chi square test and the association between the alleles and the year of birth of the cats with logistic regression.

The odds ratio for the presence of HCM was calculated for cats homozygous for the variant versus cats with the “other two genotypes” (heterozygous and homozygous for the wild type allele), based on the proposed autosomal recessive mode of inheritance in the original paper[20]. A logistic regression model was fitted with the genotype and the origin of the sample as covariates, with “other genotypes” as the reference level for genotype. The potential effect of time of sampling was also assessed. Significance of the covariate was determined with the likelihood ratio test and set at $\alpha \leq 0.05$.

4.4 Results

Allele frequencies

One hundred and sixty Maine coon samples, originating from Belgium, Italy, Sweden and the United States, were genotyped for the *TNNT2*:c.95-108G>A variant. Fifteen of these had been genotyped by whole genome sequencing, the other 139 were genotyped by Sanger sequencing. Both heterozygotes and homozygotes for the variant were found in all four countries. The country-specific allele frequencies ranged from 0.26 to 0.35 and the overall allele frequency in the Maine coon was estimated to be 0.32. The alleles were not significantly

associated with the country of origin (P = 0.5) or year of birth (P = 0.41). The samples and their genotypes are summarized in Table 1.

The 99-Lives database contains 296 cats from other breeds whose genotype for this variant was known.[22] The variant was found in the British shorthair, Devon rex, Persian, ragdoll, Siamese, Tennessee rex and Thai breeds. The variant was also found in random-bred cats at an allele frequency of 0.09.

Table 1 Allele frequency of the *TNNT2*:c.95-108G>A variant in Maine coon populations.

Country	N	Allele frequency	95% Confidence interval
Belgium	40	0.29	0.19 to 0.39
Italy	30	0.35	0.23 to 0.47
Sweden	50	0.36	0.27 to 0.45
United States	40	0.26	0.17 to 0.36
General	160	0.32	0.26 to 0.37

Association with HCM

The HCM phenotype was available for 93 cats, of which 32 were affected and 59 were healthy controls. One additional cat showed left ventricular wall thicknesses within range on echocardiography, but in combination with ventricular and atrial dilatation, focal thickening of the interventricular septum and systolic anterior motion of the mitral valve, suggesting end-stage HCM.[31] Another cat died of aortic thromboembolism before an echocardiography could be performed, but showed significant hypertrophy of the left ventricular walls on necropsy. Histopathological examination of the myocardium was performed for these two cats and revealed myofiber hypertrophy, myofiber disarray and interstitial myocardial fibrosis in both cases. Both cats were classified as HCM, bringing the total number of affected cats at 34.

Three affected cats were homozygous for the *MYBPC3*:c.91G>C variant and therefore excluded from the association analysis. Thirteen cats, six affected and seven healthy, were heterozygous for this variant and were retained for the analysis. The median age was 4.6 years (range: 0.7 to 15.2) and 7.1 years (range: 0.4 to 16.5) for the affected and control group, respectively. The phenotypes and genotypes are cross-tabulated in Table 2. The country of origin was a significant covariate (P = 0.007) and hence retained in the model. The odds ratio

for developing HCM in homozygous variant cats compared to other genotypes was estimated to be 1.47, with a 95% confidence interval from 0.39 to 5.51 and a P-value of 0.57.

Table 2 HCM phenotypes and genotypes for the *TNNT2*:c.95-108G>A variant.

	GG	**GA**	**AA**
HCM	12	14	5
Healthy	25	27	7
HCM Prevalence	0.33	0.34	0.42

A second analysis that used the 35 oldest healthy controls (median age: 9.17; minimum: 6.75), thereby minimizing a potential effect of the age in the control group, while still retaining 80% power, yielded a non-significant (*P* = 0.30) odds ratio of 2.43. As this *TNNT2* variant itself had no significant effect, a possible interaction with the *MYBPC3*:c.91G>C variant was investigated. No significant effect of (i) heterozygosity for the *MYBPC3*:c.91G>C variant (*P* = 0.51) or (ii) the interaction between this variant and the *TNNT2*:c.95-108G>A variant (*P* = 0.80) studied here on HCM status was found.

Predicted effects on splicing

The output of the splice site predictions is summarized in Table 3. GENSCAN does not give predicted splice sites, but only the predicted exons. ASSP, the tool that was used in the original report of the variant, [20] predicted the creation of a new splice acceptor site as a result of the *TNNT2*:c.95-108G>A variant. One of the other four tools, SSPNN, predicted the same effect. Utilisation of this splice site would add a sequence of 106 bases from intron 4 to the 5'-end of exon 5 (Figure 1A; exon numbered according to ENSFCAT00000052073.2), leading to a premature stop codon and loss of 80% of the protein's amino acids.[20] The other three tools predicted no change in splice sites as a result of the variant.

Table 3 Predicted splice sites for *TNNT2* by different programs.

Program	Donor sites	Acceptor sites	Effect variant
GENSCAN	15 exons		None
ASSP	163	212	*de novo* acceptor site
SSPNN	41	81	*de novo* acceptor site
ESEfinder	59	90	None
Netgene2	38	105	None

cDNA sequencing

mRNA was available for two Maine coon cats, one heterozygous for the variant and one homozygous wild type. Sanger sequencing of the corresponding cDNA showed a mixture of two different transcripts. These resulted from alternative splicing of exon 4, which was spliced out of the most common transcript, but retained in the less common transcript. Based on the electropherograms, the difference in RNA concentrations was most distinct in the heterozygous cat. No trace of the predicted extension of exon 5 caused by aberrant splicing was found.

ACMG classification

The relevant criteria for the classification of sequence variants according to the ACMG guidelines are summarized in Table 4.[21] None of the criteria that support

a pathogenic or a benign role for the variant are met, which leads to the classification of the variant as a “variant of uncertain significance”.

A

Exon 3 / Intron 3 / Exon 4

WT AAGAAGCCGCAGAAGGTAT...[2517 bases]...TCAGAAGAGGAGGACTGGAGAGAGGACGAAGACG

VT AAGAAGCCGCAGAAGGTAT...[2517 bases]...TCAGAAGAGGAGGACTGGAGAGAGGACGAAGACG

Intron 4

WT GT...[1830 bases]...TGGATCCTGGATGTTGCTGACCTCCATCCCCACCCCTCTCTGATCTGGGTT

VT GT...[1830 bases]...TAGATCCTGGATGTTGCTGACCTCCATCCCCACCCCTCTCTGATCTGGGTT

Intron 4 (continued) / Exon 5

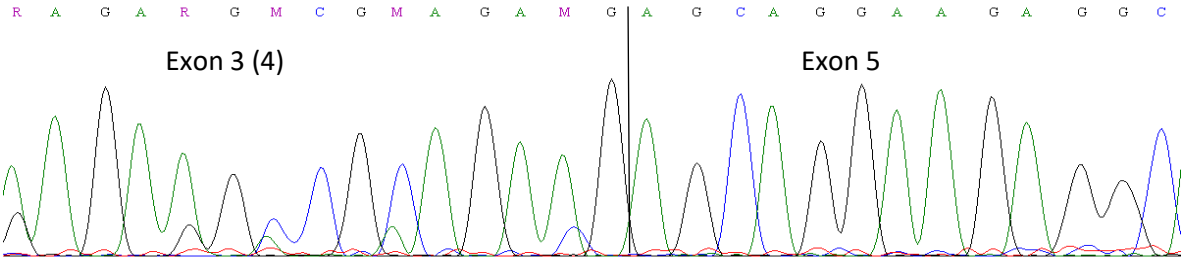
WT CTGCCTGCTAGCCTAAGTAAGGGCCTGGGTATTCACTGTTTCTGGCTCACTTCTCCAGAGCAGGAAGAGGC

VT CTGCCTGCTAGCCTAAGTAAGGGCCTGGGTATTCACTGTTTCTGGCTCACTTCTCCAGAGCAGGAAGAGGC

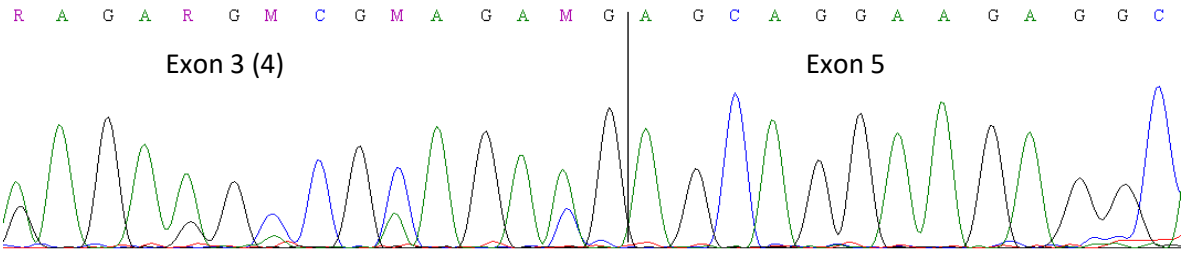
B

	Wild type cDNA		Variant cDNA	
	Preceding sequence	Exon 5	Preceding sequence	3'-part of intron 4
Exon 4 absent	AAGAAGCCGCAGAAG	AGCAGGAAGAGGC	AAGAAGCCGCAGAAG	ATCCTGGATGTTG
Exon 4 present	GAGAGGACGAAGACG	AGCAGGAAGAGGC	GAGAGGACGAAGACG	ATCCTGGATGTTG
Co-occurrence	**RAGARGMCGMAGAMG**	**AGCAGGAAGAGGC**	RAGARGMCGMAGAMG	ATCCTGGATGTTG

C



D



Legend

WT	Wild type sequence	A	The *TNNT2*:c.95-108G>A variant
VT	Variant sequence	AG	The splice site predicted by SSPNN
GGAAGAG	Exonic DNA	CCTAAG	Intronic DNA predicted to be incorporated
CATTCC	Intronic DNA	|	Boundary between exons

Figure 4.1 Domestic cat cDNA sequences of *TNNT2*. **A**: gDNA sequence of *TNNT2* from the start of exon 3 to the 5'-region of exon 5. The computational tools ASSP and SSPNN predict the c.95-108G>A variant creates a new splice site. **B**: cDNA sequences for wild type gDNA (without the predicted splice site, left column) and variant gDNA (with the utilization of the new splice site, right column). Because exon 4 is subject to alternative splicing, exon 5 can be preceded by either exon 3 (upper row) or exon 4 (middle row) in the cDNA sequence. Isolated cDNA can be a mix of two transcripts (bottom row) or one of the transcripts. The sequence that was actually observed, is depicted in bold.
C and **D**: Myocardial cDNA sequences of two cats carrying the *TNNT2*:c.95-108G>A variant, one heterozygote (**C**) and one homozygote (**D**). Transcripts containing only exon 3 and containing both exon 3 and 4 co-occurred (i.e. the bold sequence demonstrated in the bottom row of the left column of **B**). The incorporation of intronic DNA, as predicted on the basis of ASSP and SSPNN, was not found.

4.5 Discussion

This study aimed to further investigate the *TNNT2*:c.95-108G>A variant that was reported as a novel cause of cardiomyopathy in Maine coon cats as functional, clinical or population data were lacking.[20] A two-phase study was set up to calculate the allele frequency of the mutant allele and investigate its association with HCM in a large population.

The resulting allele frequency based on the first phase of the study was 0.32 overall. The high allele frequency in each of the investigated countries suggests that the variant is common in Maine coon populations around the world. Additionally, the variant was found in other breeds and mixed-breed cats, though generally at a lower allele frequency. Among these other breeds were the Siamese and Thai, two eastern breeds that are not closely related to the Maine coon,[32] suggesting that the variant emerged before the creation of these breeds.

In the second phase of the study, based on 90 phenotyped cats, the association between the variant and HCM was assessed. A non-significant odds ratio of 1.47 was calculated, while the minimal sample size based on the power analysis was exceeded. Furthermore, the HCM-prevalence was similar for the three genotypes separately. As such, the original association was not reproduced. The significant association of HCM with the country of origin can most likely be explained by differences in the numbers of cases and controls that were sequenced by the participating laboratories. As the data of the original paper conflicted with what was found here, a check was performed based on the ACMG consensus guidelines for the interpretation of sequence variants to weigh the evidence supporting classification of this variant as pathogenic or benign.

Table 4 Summary of relevant variant classification criteria.

Criterion	Result	Remark	Conclusion
Null variant in a gene where LOF is a known mechanism (PVS1)	LOF possible in *TNNT2*, LOF not confirmed on mRNA or protein level		Not fulfilled
Significant OR > 5.0 (PS4)	OR = 1.41, P = 0.61		Not fulfilled
Multiple lines of computational evidence (PP3/BP4)	2/5 predicted harmful effects, 3/5 did not	Should not be used with contradicting results	Not fulfilled
Observed in healthy adult with full penetrance expected at early age (BS2)	11-year-old healthy homozygote	Full penetrance of feline HCM cannot be assumed	Not fulfilled
Allele frequency > 5% (BA1) or higher than expected (BS1)	Allele frequency = 32%	No suitable cut-off for the allele frequency available	Validity of criterion questionable

The original criterion name as mentioned in Richards *et al*.[21] was added in parentheses. LOF = loss of function, OR = odds ratio, HCM = hypertrophic cardiomyopathy.

A first criterion, providing very strong evidence for pathogenicity according to these guidelines, is loss of function caused by a splicing variant. This is on the condition that loss of function is a known disease mechanism for the gene and that the splice effect is confirmed on the mRNA or protein level.[21] The mechanism by which the variant was proposed to cause cardiomyopathy is that it creates a novel splice site, causing the loss of over 80% of the protein's amino acids [20]. The involvement of *TNNT2* in human HCM is well-established and both nontruncating and truncating variants in this gene have been shown to cause disease.[33] In this light, loss of function is also a possible disease mechanism for this gene in cats. However, no functional RNA or protein evidence was provided in the original article. As such, the conditions for this criterion to be valid were not met. The sequencing of cDNA from the heart of a heterozygote in our study showed no evidence of aberrant splicing either. In addition, the *in silico* support for the splice site effect of the variant was limited as the prediction programs gave different results. When the results of prediction programs vary, the ACMG criteria state that they should not be used to classify a variant as benign or pathogenic.[21]

Statistical association with the disease is considered strong evidence for pathogenicity, but only if the odds ratio is higher than 5.0 and statistically significant.[21] These criteria were also not met for the *TNNT2*:c.95-108G>A variant; the point estimate was far lower, both compared to the 5.0 threshold and relative to the odds ratio of the established disease-causing *MYBPC3*:c.91G>C variant (which was 19.4).[18]

An allele frequency higher than 5% is a stand-alone criterion to classify a variant as benign for rare Mendelian disorders in humans.[21] It has been demonstrated that even in humans, imposing a strict cut-off is not ideal for every situation.[34] Considering the high prevalence of HCM in cats and the different population structure of purebred cats compared to humans, this cut-off is likely too strict. For example, the *MYBPC3*:c.91G>C variant, a cause of HCM, has an allele frequency of 0.18 in Maine coons.[35]

A cut-off higher than expected for the disorder is considered strong evidence for a benign classification.[21] A disease-specific cut-off for a random sample can be calculated if the disease prevalence and penetrance are known and genetic heterogeneity is taken into account.[34] This

method is difficult to apply here, as the sample was not randomly selected and the disease prevalence in the sample seems higher than in the general population. The genetic heterogeneity of HCM, which is immense in humans,[8] is mostly unknown in cats and therefore hard to take into account here. To avoid overinterpretation of a possibly unreliable figure, we chose not to calculate an alternative to the cut-off of 5%.[21,36]

The detection of a variant in a homozygous state in a healthy adult is also a strong criterion for classification as benign, if full penetrance is expected at an early age.[21] Incomplete and age-dependent penetrance is a well-known characteristic of HCM in humans.[8, 37] and also in cats, this is well known for the *MYBPC3*:c.91G>C variant.[9,17,18] Although this criterion is thus not ideal for HCM, the oldest cat homozygous for the mutant allele in our population, was still healthy on echocardiography at the age of 11 years, which already corresponds to the median longevity of Maine coons.[38,39] HCM is thought to generally develop before this age, but as the age of diagnosis ranges from 6 months to 21 years, it cannot be fully ruled out that this cat may still develop HCM.[5,40] As such, this criterion that would support classification of the variant as benign is also insufficiently supported. Combined, neither the criteria supporting pathogenicity, nor those supporting benign classification are met. Hence, this variant should be classified as a variant of uncertain significance according to the ACMG guidelines.

There are some potential limitations linked to study design. The obtained allele frequency estimates could be biased as the samples used in this study were collected in the context of genetic screening and/or scientific investigation. They therefore do not form a completely random sample of the client-owned Maine coon population. The samples were also collected over a long period of time (the oldest sampled cat was born in 1991) and the allele frequency may fluctuate over time, even though no significant association was found with year of birth (P = 0.55). Given that the allele frequency was very high, the true allele frequency will probably still be substantial even if this is an overestimation.

A complicating factor in multicentre studies is that diagnostic tests are made by different persons using different equipment and possibly slightly different methods. This is also relevant in the current study, as echocardiographic measurements of ventricular wall dimensions are

subject to interobserver variation.[41] The problem was partially mitigated by including the country of origin as a variable in the logistic regression analysis. As the diagnosis in this study was a binary variable (HCM or healthy) and equivocal cats were excluded from the analysis, small differences in measurements would probably have resulted in the same classification and not impacted the analysis for most cats. It is nevertheless possible that some borderline cases that were classified as equivocal in one centre would have been classified as healthy or affected in another, or vice versa.

Furthermore, the association analysis was a comparison between homozygotes and other genotypes, as HCM caused by this variant was reported to have an autosomal recessive mode of inheritance.[20] The control group was generally older than the affected group, but it contained some young animals that may develop HCM at a later age. However, all but one control (a homozygous wild type cat) were older than the proband described by McNamara *et al*.[20] at the age of diagnosis. If this proband showed a typical manifestation of the cardiomyopathy caused by homozygosity of the *TNNT2*:c.95-108G>A variant, expectations are that the older homozygotes in our study would be affected.

Finally, cardiac cDNA was available for only one cat carrying the variant, a heterozygote. Sequencing of cardiac cDNA from more cats carrying the variant, including homozygotes for the variant, would allow a more confident assessment of the variant's effect on splicing. The alternative splicing is similar to that in humans, where the orthologue of feline exon 4 is present in fetal isoforms but not in the main adult isoform.[24] There is no indication that this splicing is related to the *TNNT2*:c.95-108G>A variant.

Altogether, the current evidence is insufficient to classify this variant as pathogenic or benign, which implies this variant has to be classified as a variant of unknown significance. As such, this variant should not be used in clinical decision making, according to the ACMG guidelines.[21] Extrapolating these guidelines to companion animals, we advise against the use of this variant in breeding decisions, especially as the variant's high allele frequency means that such use can impact many breeding decisions.

4.6 References

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4.7 Supplementary file

Genotyping *TNNT2*:c95-108G>A

Primer sequences (Belgium, Italy, Sweden, USA)

Forward primer:	5'-CCTCACCTTCAGCCTCTTCT-3'
Reverse primer:	5'-CGCACCCTAACACACTCCTA-3'

M13'-tag for primers in BigDye sequencing kit (Sweden)
M13 forward sequence: 5′-TGTAAAACGACGGCCAGT-3′
M13 reverse sequence: 5′-CAGGAAACAGCTATGACC-3′

PCR mix (Belgium)

5.7	µl Water
1.0	µl 10x Key Buffer
1.0	µl Primers (5 µM each)
0.2	µl dNTPs (10 mM each)
0.1	µl TEMPase Hot Start DNA Polymerase (5 U/µl)
2.0	µl Template DNA
10.0	µl Total volume

PCR program (Belgium)

95.0 °C	14:30	
95.0 °C	0:30	35 x
59.0 °C	0:30	
72.0 °C	1:00	
72.0 °C	5:00	

PCR mix (Italy)

13.6	µl Water
2.0	µl 10x TaqGold Buffer
1.2	µl $MgCl_2$ (2.5 mM)
0.6	µl Primers (10 µM each)
0.8	µl dNTPs (10 mM each)
0.2	µl AmpliTaq Gold Polymerase (5 U/ul)
1.0	µl Template DNA
20.0	µl Total volume

PCR program (Italy)

95.0 °C	2:00	
95.0 °C	0:30	30 x
61.5 °C	0:30	
72.0 °C	0:50	
72.0 °C	5:00	

PCR mix (Sweden): BigDye Direct Cycle Sequencing Kit

1.5	µl Primer mix (0.8 µM each)
5.0	µl BigDye PCR Primer Mix
2.5	µl Water
1.0	µl Template DNA (4 ng/µl)
10.0	µl Total volume

PCR program (Sweden)

95.0°C	10:00	
96.0°C	0:03	35 x
59.0°C	0:15	
68.0°C	0:30	
72.0°C	2:00	

PCR mix (USA)

39.3	µl Water
5.0	µl 10x PCR Rxn buffer
1.5	µl Mg (50 mM)
2.0	µl Primers (10 µM each)
1.0	µl dNTPs (10 mM each)
0.2	µl Platinum Taq DNA Polymerase (5 U/µl)
1.0	µl Template DNA
50	µl Total volume

PCR program (USA)

94.0 °C	2:00	
94.0 °C	0:30	30 x
55.0 °C	0:30	
72.0 °C	1:00	
72.0 °C	5:00	

PCR products from Italy were purified and sequenced at Eurofins Genomics (Ebersberg, Germany), those from the USA were sequenced at the Cornell Genomics Facility (Ithaca, NY, USA). PCR products from Belgium were sequenced by the reaction below and the results were read out at Eurofins Genomics.

Sequencing mix (Belgium)

3.0	µl Water
2.0	µl 5x Sequencing Buffer
1.5	µl Forward primer (2 µM)
1.0	µl GC-rich
0.5	µl BigDye Terminator RR-mix
2.0	µl PCR product
10.0	µl Total volume

Sequencing program (Belgium)

95 °C	2:00	
95 °C	0:20	30 x
60 °C	0:10	
65 °C	4:00	

Sequencing mix (Sweden)

2.0	µl BigDye Sequencing Mix
1.0	µl BigDye M13 forward or reverse primer
10.0	µl PCR product
13.0	µl Total volume

Sequencing program (Sweden)

37°C	15:00	
80°C	2:00	
96°C	1:00	
96°C	0:10	25 x
50°C	0:05	
60°C	1:15	

Terminator clean up (Sweden)

45.0	µl SAM Solution
10.0	µl XTerminator Solution
13.0	µl sequencing product
68.0	µl Total volume

The samples were mixed by vortexing at 2 500 rpm for 20 min and centrifuged at 1 000 G for 2 min prior to capillary electrophoresis on a 3 500 DNA Analyzer (Applied Biosystems™, Waltham, MA, USA).

Genotyping *MYBPC3*:c.91G>C

The samples used in this study were collected by many different institutes over a long period of time. As they were often genotyped for the *MYBPC3*:c.91G>C variant (first reported in 2005) at the time of collection, many different protocols have been used and the exact protocol was not known for all samples. We therefore cannot provide details that cover all the samples used in this study. Cats that were homozygous for this variant were excluded from the analysis on the association between the *TNNT2*:c.95-108G>A variant and HCM.

cDNA sequencing

Briefly, septal tissue, less than 25 mg, was cut into smaller pieces on ice. Homogenization was performed in CK14-tubes (Bertin Technologies, Montigny-le-Bretonneux, France) for 2×20 s with a 10 s delay at 6 000 rpm with the Precellys Evolution tissue homogenizer (Bertin Technologies). The tissue was homogenized in lysis buffer followed by extraction according to manufacturer's recommendations for the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany). Samples were stored at 80 °C until further processed.
iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) was used according to manufacturer's recommendations for reverse transcription of 0.5 µg input of total RNA/reaction.

Primer sequences

Forward primer:	5'-TGTCTGACGTGGAAGAGGTG-3'
Reverse primer:	5'-GCCATCTTCAGTTTCTTTAGCTTC-3'
M13-tag forward:	5′-TGTAAAACGACGGCCAGT-3′
M13-tag reverse:	5′-CAGGAAACAGCTATGACC-3′

The procedures for the sequencing of cDNA were identical to those for the genotyping of the *TNNT2*:c95-108G>A variant in Sweden, except that the annealing temperature was 59 °C.

5. Genetic aspects of corneal sequestra in a population of Persian, Himalayan and exotic cats

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5.1 Abstract

Corneal sequestra are ophthalmic lesions that are remarkably common in Persian, Himalayan and exotic cats. In this study, the genetic aspects of this disease were investigated in a population of cats originating from a single cattery. Odds ratios were calculated for parents with affected offspring. The heritability of (owner-reported) corneal sequestra was estimated with a Markov chain Monte Carlo procedure. Well-phenotyped cases and controls were used for a genome-wide association study. Data from 692 cats originating from the cattery, of which 61 were affected, were used. Cats from two specific mothers had significantly higher odds for developing corneal sequestra, but no significant effect of the fathers was found (after correction for the mothers). The heritability of corneal sequestra was estimated to be 0.96. A genome-wide association study with 14 cases and 10 controls did not reveal an associated chromosomal region. The large effect that genetic factors had on the development of corneal sequestra in this study suggests that selective breeding could be an effective way to reduce the prevalence of this condition in these cat breeds.

5.2 Introduction

Corneal sequestrum is an ophthalmic condition that is virtually unique to cats, as only a few cases have been described in horses[1] and dogs.[2] They are characterized by a local degeneration and brown-black discoloration of the cornea of variable size and severity. In mild cases, the sequestrum can spontaneously slough and heal, while more severe cases can persist for years or even progress to corneal perforation.[3] The level of discomfort varies from no signs of pain to extreme discomfort with epiphora, blepharospasm and photophobia.[4] In mild cases, the treatment can be limited to analgesia, but many sequestra are so deep and painful that they require surgical removal.[3] Surgical removal of a sequestrum consists of a keratectomy with or without the use of a grafting material depending on the depth of the sequestrum.

The age of onset varies from a few months to 17 years, but most cases occur between two and seven years.[4] Both sexes are affected equally. A breed predisposition in Persian cats and

the related Himalayan and Exotic breed has been widely reported.[3,5-7] These breeds also have a higher risk of being bilaterally affected.[3,5] Other breeds reported to be predisposed are the Siamese, Birman and Burmese.[8,9] A prevalence of 2.4% (28 out of 1161) was found in a population of cats presented to a veterinary teaching hospital, making it the most common presumed hereditary or breed-related ocular disease in this population.[10]

The pathogenesis of corneal sequestra remains mostly unclear. Trauma and infections, most notably Feline Herpesvirus I (FHV-1), have been proposed as aetiologies affecting all breeds.[3,4,11] The high prevalence of entropion and trichiasis in brachycephalic cats, which can cause chronic irritation of the cornea, may partially explain the breed predisposition.[3,7,12] Another suggested predisposing factor is that the facial morphology of these cats, with prominent globes and shallow orbits, can lead to lagophthalmos, exposure of the central cornea and tear film evaporation.[7,8] Other possible explanations are breed-specific lower corneal sensitivity[13] and defects in the tear film,[14] metabolism[4] or the corneal epithelium.[15] An autosomal recessive pattern of inheritance has been suggested,[16] but no pedigree data have been published to support this. The aim of this study was to investigate the influence of the parents, heritability and association with genomic regions of corneal sequestra on the basis of a large pedigree.

5.3 Materials and methods

Data collection

All data and samples originated from a single cattery of Persian, Himalayan and exotic shorthair cats. The date of birth, sex, sequestrum status (affected or not) and parentage of all kittens, born between 2002 and 2021, were provided by the breeder, as well as the pedigrees of the breeding animals. The offspring was classified as affected if the breeder was informed by the owners that the cat was diagnosed with a sequestrum or had undergone surgical treatment for a corneal sequestrum. The offspring was classified as healthy if the breeder was informed by the owners that the cat was healthy or if no information on corneal sequestra was available.

Parent risk analysis

For each dam with affected offspring, the proportion of affected offspring was compared to that proportion among the total offspring of all other dams. Offspring born after 2019 were excluded from this analysis, as the youngest affected animals were born in 2019. An odds ratio and its confidence interval were calculated for each dam and Fisher's exact test was used to determine statistical significance. The same procedure was followed for the three sires.

As the dams with the highest proportion of affected animals among their offspring had all been mated to the same sire, the effect of the sire could be confounded by the effect of the dams. To allow correction for this confounding, the dams were transformed to a binary variable based on the analysis above: high risk (odds ratio > 1 and $P < 0.05$) and low risk (odds ratio < 1 and/or $P > 0.05$). A logistic regression model was fit for the full dataset with sire, dam (as a binary variable) and year of birth as predictive variables for corneal sequestra. Significance of the covariates was determined with the likelihood ratio test.

Heritability estimate

The heritability of corneal sequestra was estimated on the basis of the phenotypic data from the kittens born in the cattery and their ancestry data available from the pedigree of the dams and sires. The phenotype was modelled as the outcome of a logistic regression model containing the year of birth, sex and estimated breeding value of the animal and a random residual contribution. The estimated breeding value was computed as the additive genetic standard deviation, multiplied by the Mendelian sampling term and a random gametic effect, plus the average estimated breeding value of the parents. The Mendelian sampling term was calculated as the square root of the diagonal of the additive relationship matrix constructed with the optiSel package. The heritability was estimated as the additive genetic variance divided by the additive genetic variance plus the residual variance, which is assumed to be 1 for a binary trait.[17]

The values of the parameters were estimated via a Bayesian approach using Markov chain Monte Carlo in the Stan programming language, as described by Cai *et al*.[18] The priors for the

intercept, year and sex coefficient were drawn from a normal distribution with mean zero and standard deviation 4 (normal (0, 4)) and the prior for the residual error from normal (0, 1). To avoid bias from the choice of the prior value of the additive genetic standard variation, four different distributions were used to draw priors from: a Cauchy distribution with mean 0 and standard deviation 2.5 (Cauchy (0, 2.5)), as suggested by Cai *et al*.,[18] Cauchy (0, 10), normal (0, 10) and uniform (0, 20). Only positive values from these distributions were used. For each prior distribution, four parallel chains were run for 60 000 iterations. The first 30 000 of these were discarded as burn-in and every 30th value was sampled from the next 30 000 iterations. An example of the Stan scripts is included as a supplementary file.

Genome-wide association study (GWAS)

A subset of the population was used for a genome-wide association study to identify chromosomal locations associated with corneal sequestra. Cases were defined as cats that had undergone surgery for a corneal sequestrum or were diagnosed with a corneal sequestrum on eye exam during at the time of sampling. Controls were defined as cats that were at least two years old and declared free of corneal sequestra on eye exam at the time of sampling. Eye exams were performed by a board-certified veterinary ophthalmologist, certificate in veterinary ophthalmology or veterinarian with experienced ophthalmology knowledge. Affected animals suspected of FHV-1 infection based on the clinical history were excluded as cases for the GWAS.

DNA was extracted from whole blood on EDTA by the phenol-chloroform method.[19] The DNA was quantified with the Qubit 4.0 fluorometer and at least 500 ng from each sample was used for genotyping on the Illumina Infinium iSelect 63k Cat DNA SNP genotyping array.[20]

Data from the GWAS were analysed in PLINK v1.90b6.24.[21] Variants and animals with a genotyping rate below 95% were excluded from the analysis, as were variants with more than one Mendelian error or a minor allele frequency below 5%. Association between the allele and the phenotype was assessed with Fisher's exact test and P-values were corrected by Holm's

method. Population stratification was assessed by a multidimensional scaling plot of the first two principal components and a QQ plot of the uncorrected P-values.

5.4 Results

From 2002 to 2021, 757 kittens were born in the cattery from 43 dams and three sires. Sixty-one of these cats (8.1%), 35 males and 26 females (57% and 43% of the affected cats, respectively), were known to have developed a corneal sequestrum. The oldest of the affected cats was born in 2006, the youngest in 2019.

Effect of the parents

Twenty-one dams and all three sires had at least one affected animal among their offspring. Two dams had an odds ratio significantly higher than 1 when their offspring was compared to that of all other dams: 13.0 ($P < 0.001$) for dam 1 and 2.94 ($P = 0.011$) for dam 2. Dams 2 and 3 were known to be affected, all other dams were (as far as known) healthy. The data of the 21 dams with affected offspring are shown in Table 1.

All three sires had affected offspring. Sire 1 had a significantly increased odds ratio of 2.07 ($P = 0.014$), sire 2 had an odds ratio significantly lower than 1 (0.45, $P = 0.032$) and sire 3 did not significantly deviate from the general population, as shown in Table 2.

Table 1 Offspring born after 2019 of dams that had at least one affected offspring.

Dam	Number of cases	Number of controls	Odds Ratio	95% CI	P-value
1	17	17	13.0	5.8 to 29.2	< 0.001
2	9	33	2.94	1.17 to 6.7	0.011
3	4	12	3.39	0.77 to 11.7	0.053
4	1	34	0.275	0.007 to 1.70	0.24
5	1	2	4.9	0.082 to 96	0.26
6	2	11	1.80	0.189 to 8.5	0.35
7	2	11	1.80	0.189 to 8.5	0.35
8	1	4	2.45	0.049 to 25.3	0.39
9	4	27	1.47	0.362 to 4.4	0.52
10	4	28	1.41	0.349 to 4.3	0.53
11	2	37	0.51	0.058 to 2.07	0.57
12	2	15	1.31	0.142 to 5.8	0.67
13	2	17	1.15	0.126 to 5.0	0.69
14	1	17	0.57	0.013 to 3.74	1
15	1	14	0.69	0.016 to 4.7	1
16	1	18	0.53	0.013 to 3.50	1
17	1	15	0.64	0.015 to 4.3	1
18	1	13	0.75	0.017 to 5.1	1
19	1	12	0.81	0.019 to 5.6	1
20	3	36	0.80	0.153 to 2.66	1
21	1	9	1.08	0.024 to 8.0	1

CI = confidence interval. Odds ratios rounded according to Cole.[22]

Table 2 Offspring of the three sires born after 2019.

Sire	Number of cases	Number of controls	Odds Ratio	95% CI	P-value
1	45	342	2.07	1.12 to 4.0	0.014
2	9	166	0.45	0.189 to 0.94	0.032
3	7	86	0.77	0.285 to 1.77	0.7

CI = confidence interval. Odds ratios rounded according to Cole.[22]

There is a risk of confounding as dams were usually mated to only one specific sire. For example, dams 1 and 2 were always mated to sire 1 and their high odds may partially explain the increased odds for sire 1. To model the dams and sires together, the dam variable was transformed into a binary variable with dams 1 and 2 as “high risk” and all other dams as “low risk”. A logistic regression model with corneal sequestrum state in function of sire and this binary dam variable was fit. The year of birth was not included, as this was not statistically significant (P = 0.32), while the binary dam variable was retained because of its strong significance ($P = 1.1 \times 10^{-12}$). Compared to sire 3, there was no significantly higher risk or cats sired by sire 1 (P = 0.50) or sire 2 (P = 0.20).

Heritability

Every prior distribution resulted in a Gelman-Rubin statistic of 1 for all parameters in the model, indicating a good convergence of the four chains. The mean posterior heritability estimates were very similar for the different priors, ranging from 0.96 to 0.98 (Table 3). The Cauchy (0, 2.5) had the widest 95% posterior interval for heritability, ranging from 0.76 to 1. The parameter coefficients for year of birth and sex were negative for all prior distributions, suggesting a higher risk for older cats and male cats. However, the 95% posterior interval for the sex parameter always firmly included zero, indicating much uncertainty around this estimate.

Table 3 Heritability estimates using four different prior distributions.

Prior distribution	Heritability estimate	95% posterior interval	Gelman-Rubin statistic
Cauchy (0, 2.5)	0.96	0.76 to 1	1
Cauchy (0, 10)	0.98	0.88 to 1	1
Normal (0, 10)	0.98	0.86 to 1	1
Uniform (0, 10)	0.98	0.89 to 1	1

Genome-wide association study

A GWAS was performed with fourteen cases and ten controls. Among the cases were dams 2 and 3 and among the controls sire 1; all other samples were from cats born from the cattery. The cases had a median age of 6 years at the time of sampling (range: 0 to 11 years) and the controls had a median age of 6 years (range: 2 to 10 years). All animals had call rates over 99%.

After filtering out variants with a low call rate (<0.95), more than one Mendelian error or a minor allele frequency < 0.05, 37 595 variants remained. The lowest obtained raw P-value was 1.8×10^{-4} (Figure 5.1) and after correction for multiple testing, all P-values were adjusted to 1. Excluding the four youngest control cats (2 to 4 years at the time of sampling) decreased the corrected P-value of the top variant (ChrA1.146883627) to 0.24, but did not change other corrected P-values.

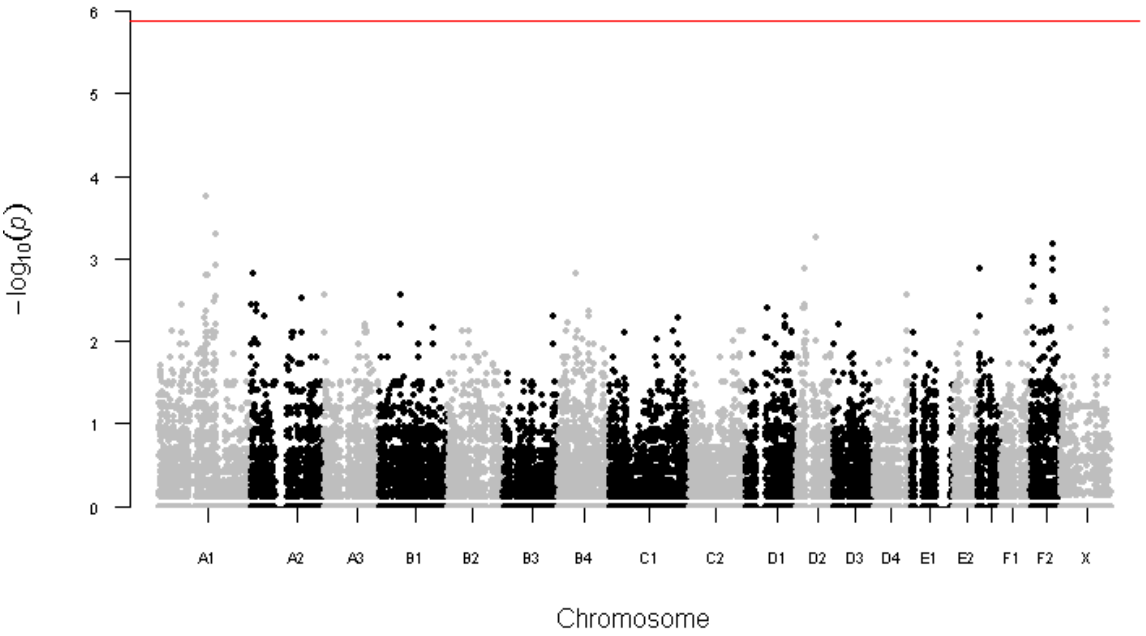

Figure 5.1 Manhattan plot of the P-values obtained with Fisher's exact tests. The genome-wide significance (red line) is set at 1.3×10^{-6} (= 0.05/37 595 tests).

Breeding decisions: parental and litter phenotypes

A summary of the prevalence of corneal sequestra is provided in Table 4. As all sires were healthy, there were no litters where both parents were affected. The prevalence among progeny when one of the parents was affected (dams 2 and 3) was 22%. When the parents themselves were unaffected, the prevalence was lower, i.e. 6.9%. Exclusion of affected parents from breeding would have decreased the number of potential sires and dams to 44, i.e. 96% of the total of 46 breeding animals.

Table 4 Prevalence of corneal sequestra in function of the parental phenotypes.

Parental phenotype	2 parents affected	1 parent affected	2 parents healthy
n affected/ total progeny (%)	/	13/58 (22%)	48/699 (6.9%)

5.5 Discussion

Two out of 43 dams (4.7%) had given birth to 26 of a total of 61 affected cats (40%) and the odds for having a corneal sequestrum were significantly higher for the offspring of these two dams. The importance of the dam implies that genetic factors have a substantial influence on the development of the disease. There was no sex predisposition and the proportion of affected

animals in most litters was lower than is expected for an autosomal recessive (or dominant) pattern of inheritance, unless the penetrance is very low. An autosomal dominant pattern of inheritance furthermore seems unlikely as only two out of 20 dams with affected offspring and none of the sires were known to be affected. Based on the distribution of affected cats, a complex pattern of inheritance is more likely.

The high heritability estimates further suggest that the development of corneal sequestra in cats from this cattery is mostly influenced by genetic factors and only very little by environmental factors. In theory, heritability estimates are population- and environment-specific, but in practice, heritability estimates for the same trait in different populations are often similar.[23] It is therefore likely that corneal sequestra also have a high heritability in other populations of Persian cats that suffer from this condition. A heritability close to 1 is compatible with the notion of an autosomal recessive pattern of inheritance as suggested by Vawer,[16] but also with other Mendelian patterns of inheritance or a polygenic segregation.[23]

A complication for the interpretation of heritability estimates is that the estimate can be inflated by a shared environment of closely related animals. In this study all animals were raised in the same cattery followed by scattering of the cats over a large number of owners at the age of 13 weeks. This makes a systematic environmental effect between litters unlikely, except for the first six weeks after birth, when the kittens stay with their mother. There is also no indication of a change of the environment over time as year of birth was not significant. If the environment in which the cats were raised underwent important changes over time, the incorporation of the year of birth into the model would have provided a correction for this.

A GWAS was conducted to search for loci that are associated with the development of corneal sequestra. No statistically significant association was found and no single locus stood out from the others. This finding is also compatible with a complex pattern of inheritance involving multiple genetic loci that are difficult to detect in this limited sample. On the other hand, it cannot be ruled out that a major gene that influences the disease was missed because of the small sample size for the GWAS.

Segregation analysis could be used to determine which model of inheritance can best explain the observed distribution of the disease. However, software programs that can perform a segregation analysis, such as the SEGREG program in the S.A.G.E. package, cannot analyze pedigrees that contain loops. Due to multiple instances of inbreeding among the ancestors and matings of related dams with the same sire, pedigree loops were highly prevalent in this dataset. These loops could only be broken by drastically altering and reducing the pedigree. As the value and reliability of analysing such a highly altered pedigree is questionable, no segregation analysis was performed.[24]

The high heritability of corneal sequestra and the disproportional number of affected kittens from some of the parent animals imply that selection of breeding animals can have a marked effect on the prevalence of the disease in future generations. As no disease-causing variant was identified, breeders without a statistical background or access to someone that calculates estimated breeding values, will often base themselves on the phenotype of the parents. The prevalence of corneal sequestra among the offspring of unaffected dams was 6.9%, somewhat lower than the prevalence of 8.1% in all offspring, but far lower than the prevalence of 22% when one of the parents itself was affected (Table 4). Therefore, a first reduction seems to be possible by excluding affected parents. The exclusion of breeding animals with only one affected animal among many descendants or with affected distant relatives is more controversial, as excessive exclusion of cats may reduce genetic variation of the breed. For example, implementing this strategy on the pedigree at hand would result in the exclusion of all sires and 21 dams used for breeding. The late onset of the disease can be an obstacle to the successful implementation of a breeding program, as high risk parents may only be identified after breeding. Regular ophthalmologic examination of breeding animals and breeding at a later age might somewhat mitigate this problem.

This study was limited by the reliance on reported data and lack of clinical information of some of the cats. Despite the fact that the breeder has contact on a regular basis with the owners of cats born in the cattery. Some owners may never report the presence of a corneal sequestrum. In case of a discrete corneal sequestrum, few to no clinical signs can be present and may thus

be unnoticed or deemed clinically unimportant by the owner. However, it seems reasonable to assume that such underreporting is independent of parentage of the cat and therefore does not substantially bias the heritability estimate or odds ratio calculations. The absence of corneal sequestra in the control cats of the GWAS was confirmed on ophthalmological examination by a board-certified veterinary ophthalmologist, certificate in veterinary ophthalmology or veterinarian with experienced ophthalmology knowledge. This makes false negative phenotypes unlikely, although incomplete and age-related penetrance of disease-causing allele(s) cannot be ruled out. To control for age-related penetrance, we repeated the GWAS with the youngest control cats excluded. This altered the results somewhat, but did not lead to statistical significance for the top variant and did not have a notable effect on neighbouring variants.

On the other hand, false positive cases due to phenocopies caused by infection (FHV-1) or chronic irritation, for instance due to entropion, were also possible. Once again, it seems reasonable to assume that secondary environmental causes are independent from parentage and therefore do not substantially bias the heritability estimate or odds ratio calculations. Cases suspected to be caused by FHV-1 infection were excluded from the GWAS on the basis of their clinical history. PCR tests for herpesvirus DNA were not performed, as these are likely to be false negative for sequestrum-causing infections that have been resolved at the time of testing or might be false positive for infections that have developed or reactivated after the formation of a sequestrum. Cats with nasal entropion were not excluded as cases, as entropion is commonly seen in Persian cats. Lateral entropion of the lower eyelid was not identified in any case.

The small sample size gave the GWAS limited power. Simulations assuming a autosomal recessive pattern of inheritance and a perfect association between a marker and the causative variant yield a statistically significant result as long as all affected cats are homozygous for one variant and up to seven controls are heterozygotes (the others homozygous for the other variant). In the presence of complicating factors, such as phenotypical misclassification, incomplete penetrance, recombination or simply more heterozygotes in the control group, this

significance is lost. This limited robustness makes it difficult to distinguish a true lack of association from a negative result due to limited power.

Corneal sequestration was treated as a binary variable in this study, as animals were classified as either affected or healthy. Using an ordinal classification with multiple levels of severity might allow a more nuanced investigation of the disease, but to the author's knowledge, an objective multigrade classification system for corneal sequestra has not yet been developed. For the purposes of genetic research, variables such as whether the cat was bilaterally affected, the age of onset or the extensiveness of the lesion (if recorded) might be used to divide the affected cats into groups of different severity.

Data from the cattery studied here showed that parent animals influence the odds of being affected by a corneal sequestrum in their offspring and that this disease has a high heritability. No association between the disease and a genomic locus could be established, suggesting a complex mode of inheritance.

Future studies may investigate the genetic aspects of corneal sequestra in broader and more diverse populations to see if the findings of this study can be confirmed. A GWAS with a larger sample may identify one or more loci that are implicated in the development of corneal sequestra. Using old control cats might further increase the power of a GWAS by overcoming age-related penetrance. Further phenotypical studies might help to determine the optimal age for screening and breeding. However, based on the data available, breeding advice can be provided helping to reduce the prevalence of corneal sequestra.

5.6 References

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5.7 Supplementary file

```
// Stan model based on the script by Cai et al. (2019).

// Being affected by corneal sequestrum is modelled in function of:

// additive relationships, year of birth and sex (0 = male, 1 =
female).

// The uniform distribution is used for drawing priors for the prior
of the

// additive genetic standard variation.

data {

int<lower=0> nrec; // number of observations

int<lower=0> nanim; // number of animals in the pedigree

int<lower=0> maxan; // maximum number of animals = nanim + 2 unknown
parents

int<lower=0> phsire; // id for unknown sire

int<lower=0> phdam; // id for unknown dam

int<lower=0,upper=1> y[nrec]; // phenotype: 1 = affected, 0 =
healthy

real sqd[nanim]; // Mendelian sampling coefficient

int<lower=0,upper=1> sexF[nrec]; // sex: 0 = male, 1 = female

int<lower=0> year[nrec]; // year that the animal was born

int<lower=0> id[nanim]; // ids in pedigree

int<lower=0> sire[nanim]; // sire ids

int<lower=0> dam[nanim]; // dam ids

int<lower=0> pid[nrec]; // id vector linking animals to a phenotype

}

parameters{

vector[nrec] rese; // residual error

real b0; // intercept

real by; // coefficient for year

real bF; // coefficient for sex

vector[nanim] gam; // standard normal gametic effects

real sg; // additive genetic standard deviation

}

transformed parameters{

vector[maxan] ebv; // vector of all estimated breeding values (ebv)

real<lower=0,upper=1> h2;
```

```
h2 = sg*sg/(sg*sg+1); // heritability

ebv[phsire] = 0.0; // ebv of unknown sires (set to 0)

ebv[phdam] = 0.0; // ebv of unknown dams (set to 0)

for(k in 1:nanim){

ebv[k] = gam[k]*sqd[k]*sg + (ebv[sire[k]] + ebv[dam[k]])/2;

}

}

model {

for(i in 1:nrec){

y[i] ~ bernoulli(inv_logit(b0 + by*year[i] + bF*sexF[i] +
ebv[pid[i]] + rese[i]));

}

// Priors for model parameters

gam ~ normal(0,1);

b0 ~ normal(0,4);

by ~ normal(0,4);

bF ~ normal(0,4);

rese ~ normal(0,1);

sg ~ uniform(0,20); // this version of the script uses the uniform
distribution

}
```

6. Discussion

6.1 The genetics of feline HCM

The *MYH7*:c.5647G>A variant in a domestic shorthair cat, described in chapter three, was the first detected spontaneous HCM-causing variant in *MYH7* in a non-human animal. The variant was never found in another animal in our laboratory and was also not found in a study sequencing 103 HCM-affected cats.[1] This indicates that the allele frequency of the variant is low and accounts for only a small subset of the feline cases of HCM. This is reminiscent of the genetic makeup of human HCM, where most causative variants (including the orthologue of this feline variant) are extremely rare.[2] With the current limited knowledge of the aetiology of HCM in mixed breed cats, no firm conclusions can be drawn about the genetic heterogeneity of HCM-causing variants. However, these random bred cats form an enormous population whose reproduction is far less regulated than that of their purebred conspecifics or, in the case of (semi-)feral cats, not regulated by humans at all. This makes it less likely that one or a few harmful alleles can obtain a high frequency, suggesting that a considerable part of the high prevalence of HCM in these cats might be due to many individually rare variants.

In contrast, HCM-causing variants with a high allele frequency may occur in purebred cats as a result of the smaller population and the breeding practices in these breeds. This is most likely in breeds where HCM has a remarkably high prevalence or where severe cases that may be attributed to homozygosity are common, such as the Maine coon, ragdoll and sphynx. The *MYBPC3*:c.91G>C and c.2455C>T variants have been identified as common HCM-causing variants in the Maine coon[3] and ragdoll,[4] respectively. Several studies have shown a significant odds ratio or relative risk >5 for HCM in Maine coons homozygous for the c.91G>C variant.[5-7] Fewer studies have been carried out in ragdolls, but homozygosity for the c.2455C>T variant significantly increases the risk of cardiac death.[8] An orthologous variant, as well as different missense variants affecting the same codon, have been reported as causes of HCM in humans.[9,10] A formal classification according to the ACMG guidelines is lacking and further analyses might be required to meet the criteria for classification as pathogenic. In both breeds,

HCM patients have been identified that do not carry one of these variants,[6,11] leaving open the possibility that other HCM-causing variants occur in these breeds.

A third variant in *MYBPC3*, c.220G>A, was also suggested to be a cause of HCM in Maine coons, but this was only published in a conference abstract and its clinical relevance has been questioned.[12] Subsequent research did not demonstrate an increased risk for either heterozygotes or homozygotes for this variant.[6] Nevertheless, two recent papers suggest that the latter variant does cause HCM, but they provide only indirect evidence that is no more than suggestive[13] or statistically flawed.[14] Another suggestion for the Maine coon, a variant in *TNNT2*[15] was extensively investigated in chapter four and classified as a variant of unknown significance.

In a recent paper, a variant in the *ALMS1* gene was reported as a cause of HCM in sphynx cats.[16] This was based on a statistical association and similarities with human diseases. The putative disease-causing variant in this gene was present in most affected cats and almost absent in control cats. However, because all affected cats but none of the control cats were sphynxes, it is impossible to determine whether the variant is associated with HCM or simply with the sphynx breed. Very little is known about the function of *ALMS1*, but disease-causing variants in this gene cause Alström syndrome in humans. This multisystemic disorder can involve dilated or restrictive, but not hypertrophic cardiomyopathy. In addition, it often involves retinal degeneration, hearing loss, obesity, type 2 diabetes, hepatic and renal dysfunction, short stature, respiratory problems and additional endocrine disorders,[17,18] which were not noted in the HCM-affected sphynxes. Any genome contains variants for which a disease-related narrative can be constructed,[19] so this (limited) overlap between the human and feline phenotypes is insufficient evidence for the involvement of *ALMS1* in feline HCM. Therefore, the causality between the variant in *ALMS1* and HCM in sphynxes has not been proven.[20]

6.2 Is HCM a monogenic disease?

Much of the work on the genetics of feline HCM, including this dissertation, relies on the assumptions that human HCM is a monogenic disease and that this can be extrapolated to cats. In some human families, the association between HCM and a sarcomeric variant has indeed been established beyond doubt.[21] However, the incomplete penetrance[22] and variable expression of some HCM-causing variants indicates that the disease can be strongly influenced by other genetic and/or environmental factors.[23] Mouse models in which human HCM-associated variants have been knocked-in generally fail to develop the HCM phenotype, but it is not known whether this is because these variants on their own are insufficient to cause HCM[23] or because of physiological differences between humans and mice.[24] Furthermore, a direct link between the effects of variants on the sarcomeric proteins and the histological and clinical phenotypes of HCM is lacking. Some common features in HCM patients, such as elongation of the mitral valve leaflets, have no clear connection to sarcomeric dysfunction at all.[23]

In addition, a large portion of the human patients has no family history of HCM. In the majority of these patients (70%), no causative variant is identified by genetic screening, and this is also the case for 22% of the patients with a family history.[25] A whole genome sequencing study in HCM patients with no disease-causing variant in the exons of sarcomeric genes could identify such variants in the introns of these genes or in genes associated with syndromes that involve cardiac hypertrophy, but only in a minority of these patients.[26] Attempts to find new HCM-causing variants in non-sarcomeric genes have been mostly unsuccessful and these variants make up less than 1% of the variants known to cause HCM.[27,28]

The inability to identify a causative variant in a large portion of the human HCM patients challenges the paradigm of HCM as a monogenic disease of the sarcomere. Several disease models have been proposed as alternatives for the classic monogenic assumption. HCM may be a spectrum of cardiac disorders with different aetiologies, ranging from monogenetic diseases via oligogenic disorders to complex diseases influenced by many pathogenic variants of small effects.[29] Environmental influences may also cause ventricular hypertrophy that is

clinically indistinguishable from HCM.[23] Instead of the prevailing notion of autosomal dominant inheritance, some cases of human HCM may be explained by autosomal recessive inheritance.[30]

In cats, the cause of most HCM cases is simply not known.[20] Sequencing the exons of sarcomeric genes often does not yield a causative variant, as shown for *MYBPC3* and *MYH7* for eight of the affected cats in the study of chapter three and for the eight sarcomeric genes in 14 affected cats in a study by Meurs et al.[31] This shows that HCM in cats can also occur without a causative variant in the coding regions of sarcomeric genes, perhaps even more than in humans. In human patients, the probability of a positive genetic test is higher when patients are younger and have more severe HCM.[32] There is some similarity in cats, where the ragdoll and Maine coon, the breeds with known variants, are notorious for their early and severe cases.[33,34] However, this may also be due to high rates of homozygosity for their common disease-causing variants, and the genetics of mild HCM cases at old age remain largely unexplored.

6.3 Variant classification in veterinary genetics

The above discussion of feline HCM variants shows that this field of study is full of controversy. Testing breeding animals for variants with unclear clinical relevance may unnecessarily reduce the genetic variation of a breed. In the long run, controversies around variants and retractions of genetic tests may erode the confidence of breeders, owners and veterinarians in genetic tests and reduce their motivation to use them for the benefit of animals.

Veterinary geneticists would benefit from clear criteria to determine whether variants cause disease or not. Standards and guidelines for canine clinical genetic testing laboratories have been published,[35] but contain no concrete criteria to evaluate potentially disease-causing variants. As explained in chapter one and applied in chapters three and four, such guidelines have been provided for human clinical genetics by the American College of Medical Genetics and Genomics.[36] Many elements from these guidelines are directly applicable in the investigation of monogenic diseases in companion animals.

A problematic criterion is the variant frequency in a control population. According to this criterion, variants found at a frequency > 5% in the general population are always considered benign.[36] This is not suitable for feline medicine, especially when a single breed is under investigation. Due to their reproductive isolation, breeds differ in allele frequency not only for variants associated with breed-specific traits,[37] but also for pathogenic variants[38] and variants of uncertain significance.[39] It is therefore good practice to use breed-specific control populations. Due to the breeding practices outlined above, pathogenic variants can have frequencies much higher than the 5% cut-off suggested for humans.

For example, variants causing polycystic kidney disease in Persians and exotics,[40] retinal degeneration in Abyssinian and Somali[41] and HCM in Maine coons,[38] occur at allele frequencies of 17.3%, 17.4% and 18.0%, respectively.

Another, more flexible population criterion is an allele frequency that is higher than expected on the basis of the disease prevalence, which is considered strong evidence for a benign interpretation.[36] This criterion is applicable in feline medicine in cases where unbiased estimations of the disease prevalence and the variant's allele frequency are available. However, the expected allele frequency also depends on the penetrance, which increases the expected frequency when incomplete, and genetic heterogeneity of the disease, which decreases the expected frequency.[42]

6.4 Implications for breeding

The *MYH7*:c.5647G>A variant was established as an HCM-causing variant in chapter three. As the affected cat was a heterozygote, an autosomal dominant pattern of inheritance is assumed. Accordingly, any cat carrying this variant is expected to develop HCM, as is approximately 50% of its offspring. Such cats are better excluded from breeding, but may not yet have developed detectable HCM at the time of breeding. With this in mind, a DNA test based on this variant seems useful for breeders. However, the variant was only found in a single cat in this study and not found in a later study[1] and thus seems to have a very low allele frequency. Furthermore, if there is a population where this variant does occur at a relevant

frequency, it is likely to be a population of domestic shorthair cats, where pre-breeding DNA screening is generally not done. Development and commercialization of such a DNA test therefore seems of little practical value. In the case of a screening panel, where thousands of variants are tested on a microarray and the extra cost of testing one variant is low, it may be interesting to include this variant for research purposes.[43]

The *TNNT2*:c.95-108G>A variant was discussed in chapter four. This variant was classified as a variant of uncertain significance, because there was insufficient evidence to classify it as either (likely) pathogenic or (likely) benign. Variants of uncertain significance are not considered “actionable” in human medicine, meaning that they should not influence decision making in treatment or reproduction.[36,44] In line with this rule, the *TNNT2*:c.95-108G>A variant should not be used for breeding decisions in cats and offering a screening test based on this variant is inappropriate.

So far, the only tests that are suitable to reduce the risk of HCM are tests for the *MYBPC3*:c.91G>C and c.2455C>T variants, in Maine coons and ragdolls, respectively. These genetic tests should be restricted to their respective breeds, as they are virtually absent in other breeds.[6,45] As the absence of these variants does not exclude the possibility of developing HCM, it is recommended to complement this with echocardiography in cats that are selected for breeding on the basis of their DNA tests. In breeds in which no common HCM-causing variants are known, echocardiography is the only available method for HCM screening.

Under the assumption that HCM follows an autosomal dominant pattern of inheritance,[46] as it does in humans, all animals that test positive for an HCM-causing variant have an increased risk of HCM in their offspring and should be excluded from breeding. However, an increased risk could only be proven for homozygous Maine coons[5-7] and ragdolls[8] and the high allele frequencies mean that these heterozygotes form a substantial portion of each.[38,45] Therefore, both heterozygous and homozygous wild type cats can be used for breeding, as long as they are negative for HCM on echocardiography and heterozygous cats are only mated to wild type cats (Figure 6.1).[47]

Cats that show left ventricular hypertrophy should be investigated for possible underlying causes. Cats with a diagnosis of HCM or equivocal echocardiographic results should be excluded from breeding.[48] Breeders have the duty to provide their cats with life-long care or a responsible new owner if they are excluded from breeding.[49] As the proportion of cats that have HCM at breeding age was estimated to be 3-5%, exclusion of HCM-affected cats should not have a strong impact on genetic diversity.[48]

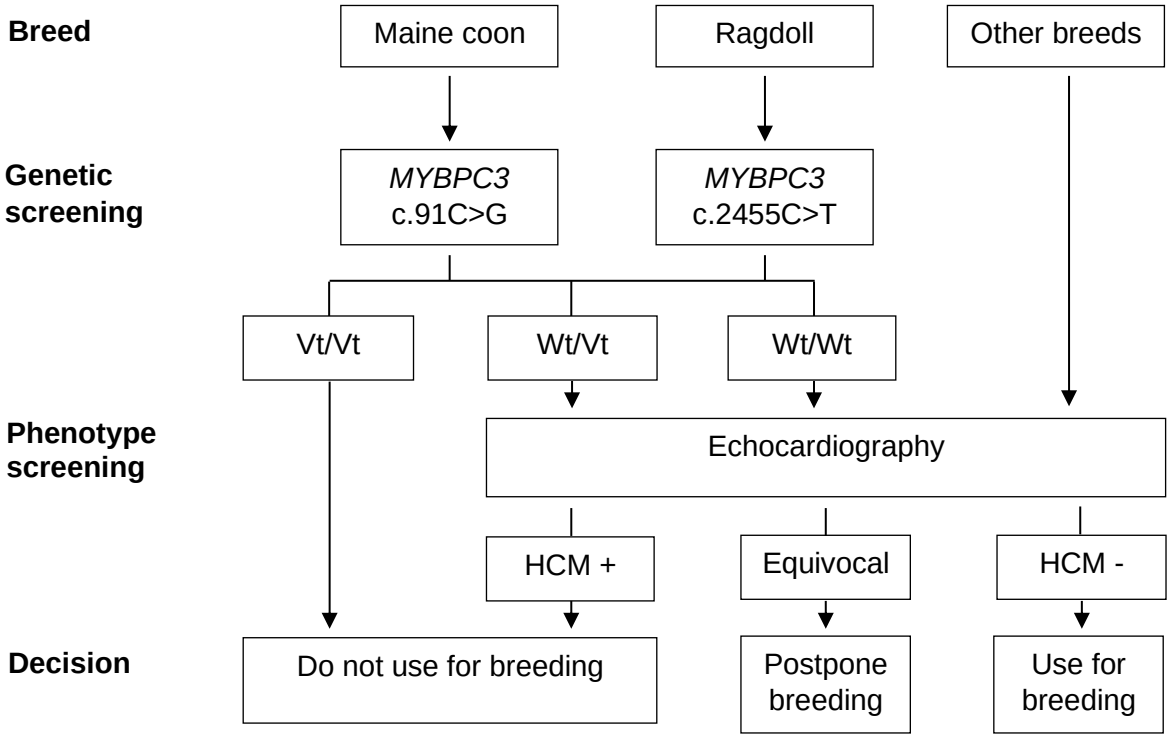

Figure 6.1 Recommendations for screening and breeding to reduce the prevalence of HCM in cats. Vt/Vt = homozygous variant, Wt/Vt = heterozygous, Wt/Wt = homozygous wild type.[50]

Genetic tests cannot be developed for corneal sequestra as long as no causative variant is identified. Considering that the proportion of affected cats was much lower than one out of four for most dams, it seems unlikely that one variant with appreciable penetrance is responsible for this disease. A complex pattern of inheritance seems more probable, but would mean that selection on the basis of a single variant is unlikely to markedly decrease the prevalence of corneal sequestra. Today the simplest alternative to a DNA test remains the traditional selection based on phenotype: animals that have developed corneal sequestra should be excluded from breeding. Examination by a board certified veterinary ophthalmologist or a veterinarian trained as an eye scheme examiner is the preferred method of phenotypical

assessment before breeding.[51] As most affected cats develop a sequestrum between two and seven years of age and 41% between the ages of two and four,[52] it would be ideal to postpone breeding to the age of four.

A more precise estimate of the genetic risk that a breeding animal conveys to its offspring can be based on the phenotypes of relatives. This allows to exclude high risk breeding animals who are themselves unaffected, but the exclusion should be balanced with the need to maintain genetic diversity. For this reason, the breeding advice formulated in chapter five did not involve excluding cats with only one affected kitten among their offspring. The selection process can be enhanced by the computation of estimated breeding values, but this will require expert assistance for most breeders and associations and seems unrealistic at the moment. When arrays are used to screen breeding cats[43], this opens up the possibility of genomic selection against corneal sequestra.

6.5 Future perspectives

Despite the efforts of veterinary geneticists, the vast majority of the feline HCM cases remain unexplained. Only two variants, *MYBPC3*:c.91G>C and c.2455C>T, are suitable for genetic testing because they commonly occur in their respective breeds and their pathogenicity is supported by convincing evidence. These tests are practical tools for breeders. As such, research leading to the discovery of new disease-associated variants is what can be most easily implemented.

It is however not easy. Studies on widespread genetic diseases in animals often assume that the same variant is responsible for most cases in a breed, but this is not necessarily true for HCM. The genetic basis may be heterogeneous and include multiple rare variants and not all cases may be monogenic. In human medicine, a family history of HCM and/or sudden cardiac death is the strongest predictor of successful genetic testing.[32] If this is also true for cats, pedigree-based studies are more likely to yield success than association studies with randomly included cases and controls from a breed.

In light of the limited success of genetic testing in some groups of human patients and the recent suggestions for alternative explanations for these cases, it may be unrealistic to expect that sarcomeric variants can explain all cases of feline HCM. Future studies on unexplained HCM in humans[23] may yield new insights into multigenic or environmental causes of left ventricular hypertrophy that are also applicable to feline patients. Meanwhile, clinical and epidemiological studies in cats may identify new diseases that mimic HCM. As with the relatively recent definition of transient myocardial thickening as a separate disease entity[53], it is possible that other phenocopies are identified in the future.

The genetics of feline HCM has been plagued with controversies and recent claims on the basis of weak evidence may further harm the credibility of this field of study. Veterinary guidelines for variant classification can help to avoid such controversies by developing widely accepted standards of evidence. The ACMG guidelines and their expansions and modifications are a suitable starting point,[36,54] but modifications are necessary, as illustrated by the issues with allele frequencies discussed above. Formulation and publication of such guidelines by a broad international collaboration of veterinary geneticists can help to make them as universally applicable and acceptable as possible. Adoption of these guidelines by databases like OMIA would help to stimulate their implementation.[55]

The study in chapter five did not result in the identification of a chromosomal region that can be linked to corneal sequestration. This may be caused by insufficient power of this study due to its small sample size. Similar association studies with larger sample sizes may succeed in identifying one or several regions that are associated with the disease. A recent GWAS with a custom microarray was successful in identifying one region associated with eosinophilic keratoconjunctivitis with a moderate sample size of 15 cases and 40 controls.[56] Whether this is also feasible for corneal sequestration, will depend on the genetic architecture of the disease. If a variant with a large effect size can be identified by GWAS, a DNA test for this variant can be developed to reduce the risk of corneal sequestra in future generations of cats. If one or more variants with a small effect size can be identified, genetic testing for these

variants will probably have limited utility. Nevertheless, the genes in which these variants are located may give interesting clues about the pathogenesis of the disease.

The late age of onset of most corneal sequestra hampers selection on the basis of phenotype, especially when the phenotype of offspring is to be taken into account. Future phenotypical studies might identify early signs of or relevant risk factors for the disease. For example, the reduced lipid content of tear fluid in both the affected and contralateral unaffected eye of affected cats may indicate a predisposing factor for the development of a sequestrum.[57] Further investigations of this phenomenon might identify biomarkers for cats that are at risk of developing sequestra. Selection on the basis of such biomarkers might be more efficient than on the basis of sequestra that develop later in life.

6.6 Conclusion

This thesis contains several new insights into the genetics of feline heart and eye diseases. A novel HCM-causing variant was identified in *MYH7*, providing the first evidence of the involvement of this gene in cats. An intronic *TNNT2* variant as a cause of HCM was examined more extensively than in the original report. This variant turned out to have a high allele frequency and showed no association with HCM and no aberrant splicing effects. Finally, it was shown that the risk of having a corneal sequestrum was influenced by the parents and that this disease has a high heritability.

Besides these successes, some investigations did not achieve the intended result. The screening of *MYBPC3* and *MYH7* in eight other HCM-affected cats did not result in a causative variant. This is in accordance with the knowledge of genetics in human HCM, where patients may have HCM-causing variants in other sarcomeric genes or have no identifiable cause of HCM. The classification of the variant in *TNNT2* as a variant of uncertain significance seems unsatisfactory as a final classification. Further studies involving functional splicing assays or family segregation may provide enough evidence for a definitive classification. However, the classification as variant of uncertain significance has important practical consequences, as testing for this variant is discouraged. The GWAS with corneal sequestration patients did not

identify associated variants, which is in line with the hypothesis of a complex disease, but may also be attributed to a lack of power.

The findings in this thesis support the hypothesis that feline HCM can be caused by variants in sarcomeric genes. This strengthens the notion that screening of these genes in HCM patients may result in the identification of similar variants that, when common, may be used in genetic testing. Simultaneously, they are a warning that the results from such screening should be interpreted carefully. The evidence for the involvement of genetic factors in corneal sequestration can be taken as a starting point for further genetic investigations that might elucidate more about the aetiology of this disease. The results from this thesis can be used by breeders to make better decisions regarding genetic testing for HCM and to develop a selection strategy against corneal sequestration.

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Summary

Genetic diseases are a common problem in cats. The most common heart disease in cats, hypertrophic cardiomyopathy (HCM), is possibly a monogenic disease, as it is in humans. Knowledge of genetic variants that cause HCM can be used to breed animals with a lower risk of HCM. Currently, only two HCM-causing variants in cats have been identified and the cause of most cases of feline HCM remains unknown. Corneal sequestra are eye lesions that are frequently seen in brachycephalic cats. The cause of these lesions is unknown and there are virtually no comparable cases in other species. Given the strong predisposition of certain breeds, genetic factors may play an important role in this disorder.

In chapter three, nine cats that were diagnosed post mortem with HCM were screened for a possible cause of HCM. RNA from heart tissue was transformed to cDNA and this was used to determine the coding sequences of two genes that contain most HCM-causing variants in humans, namely *MYBPC3* and *MYH7.* In one of these cats, a variant in *MYH7*, c.5647G>A, was found as the cause of HCM. This variant causes an amino acid substitution of a highly conserved, negatively charged glutamic acid by a positively charged lysine in an important functional domain. This variant has been described before as a cause of HCM in a human patient, has a damaging effect according to computational predictions and animal experiments and seems to be extremely rare in both humans and cats. On the basis of these findings, this variant was classified as pathogenic.

A variant in an intron of *TNNT2* was investigated in chapter four. This variant was presented as a cause of cardiomyopathy in Maine coons in an earlier publication, but only on the basis of a single patient and its parents and computational predictions. The allele frequency of this variant in Maine coons was estimated to be 32% on the basis of samples from Belgium, Italy Sweden and the USA, and also occurs in other breeds and in random-bred cats. No statistical significant association between this variant and HCM was found. Furthermore, predictions of different software programs gave contradictory results and predicted splicing abnormalities were not confirmed in mRNA of cats carrying the variant. As there was insufficient evidence to

classify this variant as either pathogenic or benign, it was classified as a variant of uncertain significance, that should not be used in reproductive or treatment decision making.

The genetic basis of corneal sequestrum was investigated in chapter five. Kittens of certain parents were found to have a higher risk of developing corneal sequestra and this disorder was estimated to have a heritability of at least 0.96. A genome-wide association study did not yield any variants associated with corneal sequestrum. The disease thus seems to be strongly influenced by genetic factors and to follow a complex pattern of inheritance, although a monogenic pattern of inheritance cannot be fully excluded.

The two investigated variants are unsuitable for the development of genetic tests, given the rareness of the variant in *MYH7* and the lack of evidence for the pathogenicity of the variant in *TNNT2.* The studies do show the importance of criteria to determine whether variants are pathogenic. The guidelines by the American College of Medical Genetics could be used to develop such guidelines in veterinary medicine. The high heritability of corneal sequestrum implies that the incidence of the disease can be reduced by selection. This can be done on the basis of the phenotype of breeding animals and their offspring, but more advanced possibilities are selection based on estimated breeding values or genomic selection.

Samenvatting

Genetische ziekten zijn een veelvoorkomend probleem bij katten. De meest voorkomende hartaandoening bij katten, hypertrofe cardiomyopathie (HCM), is mogelijk een monogene aandoening, zoals dat ook bij mensen wordt gezien. Kennis van genetische varianten die HCM veroorzaken, kan gebruikt worden om dieren met een lager risico op HCM te fokken. Tot op heden zijn slechts twee HCM-veroorzakende varianten bij katten gekend en blijft de oorzaak in de meeste gevallen van HCM bij katten onbekend. Corneasekwesters zijn oogletsels die veel gezien worden bij brachycefale katten. De oorzaak hiervan is onbekend en er zijn vrijwel geen gelijkaardige gevallen bij andere diersoorten. Gezien de sterke predispositie van bepaalde rassen is het mogelijk dat genetische factoren een grote rol spelen in deze aandoening.

In hoofdstuk drie werd bij negen katten, die post mortem gediagnosticeerd waren met HCM, gezocht naar een mogelijke genetische oorzaak van HCM. RNA uit hartweefsel werd omgezet naar cDNA en op basis hiervan werden de coderende sequenties van de twee genen bepaald waarin bij mensen de meeste HCM-veroorzakende varianten voorkomen, namelijk *MYBPC3* en *MYH7*. Bij een van de katten werd een variant in *MYH7*, c.5647G>A, gevonden als oorzaak van HCM. Deze leidt tot een aminozuursubstitutie van een sterk geconserveerd, negatief geladen glutaminezuur door positief geladen lysine in een belangrijk functioneel domein. De variant is al eerder als oorzaak van HCM bij een menselijke patiënt vastgesteld, heeft volgens computersimulaties en dierexperimenten een schadelijk effect en lijkt zeer zeldzaam bij zowel mens als kat. Op basis hiervan werd deze variant geclassificeerd als pathogeen.

In hoofdstuk vier werd een variant in een intron van het gen *TNNT2* onderzocht. Deze variant was in een eerder publicatie voorgesteld als oorzaak van cardiomyopathie in Maine coons, maar enkel op basis van één patiënt en zijn ouders en computervoorspellingen. De variant bleek een allelenfrequentie van 32% te hebben in Maine coons in België, Italië, de Verenigde Staten en Zweden, en ook voor te komen in andere rassen en rasloze katten. Er kon geen statistisch significante associatie tussen de variant en HCM worden vastgesteld. Ook gaven voorspellingen van verschillende computerprogramma's tegenstrijdige resultaten en werden

voorspelde afwijkingen van de splicing niet bevestigd in mRNA van katten met deze variant. Omdat er onvoldoende bewijs was om deze variant als pathogeen of goedaardig te classificeren, is het een variant van onzekere significantie, die niet gebruikt moet worden bij beslissingen rond voortplanting of medische behandeling.

In hoofdstuk vijf werd de genetische basis van corneasekwesters onderzocht. Kittens van bepaalde ouders bleken een groter risico te hebben om corneasekwesters te ontwikkelen en deze aandoening bleek een erfelijkheidsgraad van minstens 0.96 te hebben. Bij een genoombrede associatiestudie werden geen varianten gevonden die met corneasekwesters geassocieerd zijn. De aandoening lijkt dus sterk beïnvloed te worden door erfelijke factoren en lijkt een complex overervingspatroon te volgen, hoewel monogene overerving niet volledig kon worden uitgesloten.

De twee onderzochte varianten zijn ongeschikt voor de ontwikkeling van genetische testen, wegens de zeldzaamheid van de variant in *MYH7* en het gebrek aan bewijs voor pathogeniciteit van de variant in *TNNT2*. Het onderzoek toont wel het belang aan van criteria om te bepalen of varianten pathogeen zijn. De richtlijnen van het American College of Medical Genetics zouden een goede basis kunnen zijn om zulke richtlijnen te ontwikkelen voor de diergeneeskunde. De hoge erfelijkheidsgraad van corneasekwesters impliceert dat de incidentie van deze ziekte sterk verminderd kan worden door selectie. Dit kan in eerste instantie op basis van het fenotype van de ouderdieren en hun nakomelingen. Meer geavanceerde mogelijkheden zijn selectie op basis van geschatte fokwaarden en genomische selectie.

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Curriculum vitae

Tom Schipper was born in Winschoten, The Netherlands, at the 20th of June 1992. He obtained his bachelor's degree at the University of Antwerp in 2015 and graduated as a veterinarian at Ghent University in 2018. In the context of his master thesis, he investigated the genetics of feline hypertrophic cardiomyopathy at the university's Laboratory of Animal Genetics. With a grant provided by the Special Research Fund, this research was extended into a PhD project. The project involved the search for new disease-causing variants, the evaluation of a published variant and an exploration of the genetics of feline corneal sequestrum. Tom is author and co-author of multiple scientific publications in international peer-reviewed journals, presented his findings at the International Conference on Canine and Feline Genetics and Genomics and supervised students of the Faculty of Veterinary Medicine during their master thesis. He currently works as a teaching assistant at the Laboratory of Gene Therapy and pursues a master in education.