Genetic characterization of a Ragdoll family affected by hypertrophic cardiomyopathy

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Abstract

Hypertrophic cardiomyopathy (HCM) is a common cardiac disease among felines, resulting in myocardial hypertrophy and subsequent decreased cardiac function. Consequently, cats may develop congestive heart failure and/or arterial thromboembolism. The disease is inherited and various mutations in sarcomeric genes have been associated with feline HCM. Known gene variants have been reported in the genes myosin binding protein C3 (MYBPC3), myosin heavy chain 7 (MYH7), troponin T2 (TNNT2) and Alstrom syndrome protein 1 (ALMS1).

The aim of this master thesis was to examine the occurrence of genomic variants, known to be associated with feline HCM, that possibly could explain the high HCM penetrance among members of an affected Ragdoll cat family.

Samples from eight cats from a Ragdoll family affected by HCM and an additional sample from an unrelated Ragdoll affected by HCM were included in this study. Previously collected blood samples from seven cats, swabs from the oral cavity from one cat and cardiac tissue from one cat were included and used for DNA analysing. Sequence specific primers were designed or used from previous research, quality checked and used for sequencing and subsequent analysing to detect any genetic variants in the analysed regions of the cats' DNA.

All family members affected by HCM were homozygous for the Ragdoll breed-specific gene variant in *MYBPC3* (p.R820W). None of the healthy family members were homozygous for the gene variant, however, two healthy cats were heterozygous. Four of eight family members were heterozygous for the *TNNT2* gene variant, however, all the *TNNT2* heterozygous family members were healthy. All HCM affected family members were negative for the gene variant in *TNNT2*. All family members were negative for the other gene variants. The unrelated Ragdoll cat was negative for all tested gene variants.

Results from this study indicate that the Ragdoll breed-specific variant in *MYBPC3* is a probable cause of the high prevalence of HCM among the family members of the present Ragdoll family. In this study, there were a strong association between family members homozygous for this gene variant and family members affected by HCM. However, the possibility that an unknown or novel gene variant is causing the high prevalence of HCM among this Ragdoll family, cannot be excluded. In this study population, there were no associations between the gene variant in *TNNT2* and HCM among the Ragdoll cats.

Keywords: Hypertrophic cardiomyopathy, Ragdoll, Genetics, Myosin binding protein C3, Myosin heavy chain 7, Cardiac troponin T, Alstrom syndrome protein 1

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Abbreviations

ALMS1	Alstrom syndrome protein 1
ATE	Arterial thromboembolism
CHF	Congestive heart failure
HCM	Hypertrophic cardiomyopathy
MCO	Maine Coon
MYBPC3	Myosin binding protein C3
MYH7	Myosin heavy chain 7
RAG	Ragdoll
TNNT2	Cardiac troponin T

1. Introduction

Hypertrophic cardiomyopathy (HCM) is a disease primarily affecting the cardiac muscle (Luis Fuentes *et al*. 2020). It is characterized by hypertrophy of the myocardium, causing thickening of the left ventricular walls (Paige *et al*. 2009). The hypertrophy can further lead to a reduced diastolic function and dilatation of the left atrium (Linney *et al*. 2014). Cats may consequently develop congestive heart failure (CHF) (Kittleson & Côté, 2021) and/or arterial thromboembolism (ATE) (Payne *et a*. 2013).

Hypertrophic cardiomyopathy is a comparably common feline disease with a prevalence of approximately 14.7% among shelter cats in the UK (Payne *et al*. 2015). The disease occurs in cat families, including Ragdoll cats, which is a breed where the familiar HCM has been studied. Genetic factors have been identified to contribute to the development of HCM and various mutations in sarcomeric genes have been associated with the presence of disease (Freeman *et al*. 2017). Known gene variants associated with feline HCM have been reported in the genes coding for myosin binding protein C3 (*MYBPC3*), myosin heavy chain beta (*MYH7*), cardiac troponin T (*TNNT2*) and ALMS1 protein (*ALMS1*).

The liability for developing HCM is often more pronounced in homozygous cats compared to heterozygous cats. Ragdoll cats homozygous for the breed-specific variant in the *MYBPC3* gene develop the disease at an earlier age compared to heterozygous cats (Meurs *et al*. 2007). Genetic testing (Luis Fuentes *et al*. 2020) and echocardiographic screening (Häggström *et al*. 2016) used for breeding purposes can thereby reduce the prevalence of the disease among Ragdolls.

In this master thesis, samples from eight cats from a Ragdoll family affected by HCM were examined. An additional sample from an unrelated Ragdoll affected by HCM was added for comparative reasons of gene variants.

The aim of this study was to examine the occurrence of genomic variants, known to be associated with feline HCM, that possibly could explain the high HCM penetrance among members of this affected Ragdoll cat family.

2. Literature Review

2.1 Feline hypertrophic cardiomyopathy

Feline cardiomyopathy is a primary myocardial disease affecting both function and structure of the cardiac muscle. The cardiomyopathy is, depending on phenotype, categorized into following groups: hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM), dilatated cardiomyopathy (DCM), arrhythmogenic cardiomyopathy (ARVC) and a nonspecific phenotype (Luis Fuentes *et al*. 2020). The hypertrophic phenotype is the most common form (Ferasin *et al*. 2003) and it is also the most common type of heart disease in domestic cats (Freeman *et al*. 2017).

Hypertrophic cardiomyopathy is characterized by thickened left ventricular walls due to myocardial hypertrophy. The distribution of hypertrophy in the left ventricle differs between cats. Some cats show a diffuse hypertrophy while others have an asymmetrical and regional thickening of the left ventricle wall (Paige *et al*. 2009). The histologic features of HCM include myofiber disarray, interstitial fibrosis and abnormal intramural coronary arteries (Fox 2003). The left ventricular hypertrophy results in abnormal relaxation during diastole along with an increased ventricular stiffness (Varma & Neema, 2014). The diastolic function decreases, leading to a reduced cardiac output caused by a reduced ventricular filling (Linney *et al*. 2014). Cats may respond to this situation by tachycardia (Kittleson & Côté, 2021) or bradycardia (Luis Fuentes *et al*. 2020). The intracardial filling pressure increases and can further lead to left atrial dilatation (Linney *et al*. 2014). An increased pressure in the pulmonary veins consequently cause pulmonary edema and/or pleural effusion, *i.e.* CHF (Kittleson & Côté, 2021).

Genetic factors have been identified to contribute to the development of feline familial HCM (Luis Fuentes *et al*. 2020). In people, >1500 mutations have been associated with the HCM phenotype, including those present in genes coding for sarcomeric, ion channel and desmosomal proteins (Lopes *et al*. 2013). All the genes that hitherto have been implicated in feline HCM codes for mainly sarcomeric proteins. The sarcomere, which is the smallest contractile unit of the myocyte, is

the fundament of cardiac contraction. The sarcomere consists of thin actin filaments and thick myosin filaments (Henderson *et al*. 2018). The sarcomere consists of different zones: the Z-disc that forms the borders of the sarcomere, the I-band that consists of actin filaments, the M-band that contain cross-linked myosin filaments and the A-band that comprises both actin and myosin filaments (Wang *et al*. 2021). The interaction between myosin and actin filaments causes the Z-discs and the M-band being drawn towards each other, leading to contraction of the sarcomere (Henderson *et al*. 2018).

When diagnosing HCM, it is important to rule out other disease that may affect the heart, because, left ventricular hypertrophy can also occur secondary to certain systemic diseases. This can be seen in systemic hypertension, caused by hyperthyroidism or chronic renal insufficiency (Lesser *et al*. 1992). Left ventricular hypertrophy is also associated with hypersomatotropism (Borgeat *et al*. 2018). Felines diagnosed with HCM and felines diagnosed with left ventricular hypertrophy caused by chronic systemic hypertension show similar ultrasound finding as cats with HCM (Sampedrano *et al*. 2006). This underscores the importance of excluding diseases causing secondary left ventricular hypertrophy before establishing the diagnosis. Cardiomyopathy may also be idiopathic, meaning that the cause of the disease in unidentified (Ferasin *et al*. 2003). Genetic mutations are suspected as causative in these cases.

2.1.1 Prevalence of hypertrophic cardiomyopathy

In a study done by Paige *et al*. (2009), the prevalence of HCM among 103 apparently healthy domestic shelter cats in the UK was 14,6% (Paige *et al*. 2009). Another study by Payne *et al*. (2015) showed similar results, with a prevalence of 14,7% among 780 apparently healthy domestic shelter cats. The study showed that the prevalence of HCM increases with age (Payne *et al*. 2015). In pure-bred cats, breeds affected by HCM include Ragdoll, Maine Coon, Sphynx and Persian (Freeman *et al*. 2017). The prevalence of HCM in pure-bred cats has been described to be considerably lower compared to the UK domestic shelter cats (Häggström *et al*. 2016).

2.1.2 Clinical presentation

Hypertrophic cardiomyopathy commonly occurs subclinically without any present or previous signs of illness (Fox *et al*. 2018). In a retrospective study where 61 cats were diagnosed with HCM, the most common clinical signs were systolic heart murmur, dyspnoea, tachycardia, lethargy and gallop rhythm (Ferasin *et al*. 2003). In this study, a heart murmur was auscultated in 72.1% of the cats. Other more uncommon signs included hypotension, arrhythmias and hindlimb paresis.

As the disease progresses, two potential complications are the development of CHF and ATE. In a retrospective study of cats diagnosed with HCM, CHF was present in 33% of the cats and evidence of ATE was present in 6% of the cats (Payne *et a*. 2013). Sudden cardiac death is also an outcome that can occur (Borgeat *et al*. 2014).

2.1.3 Stages of hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy is a progressive disease (Luis Fuentes *et al*. 2020). Staging of felines with HCM is made depending on presence of clinical signs and echocardiographic findings. Cats predisposed (*e.g*. belonging to an affected family), but not yet showing clinical or echocardiographical signs of cardiomyopathy, are categorized into stage A. Stage B includes cats with echocardiographic evidence of cardiomyopathy, most commonly left ventricular hypertrophy, but not yet showing overt signs of disease. Stage B is subdivided into stages B1 and B2. Stage B1 includes cats with normal or near normal left atrial size and these cats are at a low risk of developing ATE or CHF. Felines with a higher risk of developing these complications are staged as B2 and these cats present with left atrial enlargement. Presence of left atrial dilatation is accordingly important to evaluate for differentiating between B1 and B2. Stage C includes cats that show clinical signs of CHF or ATE. At this stage, the condition can be treatable in cats with CHF with heart failure medication. The benefit of treatment of cats with ATE is variable depending on site and severity of occlusion (see below). If response to the treatment of CHF is insufficient despite standard medication, the cats are classified into stage D.

2.1.4 Diagnosis

Auscultation of the heart is of great importance at the physical examination (Luis Fuentes *et al*. 2020). Even though the diagnosis of HCM cannot be established by auscultating the heart, abnormalities may indicate a heart abnormality and indicate that further diagnostic workup is warranted. In case of abnormal findings *e.g.* heart murmur, gallop sound or arrhythmia, echocardiography is indicated.

Ultrasound examination is commonly used for diagnosing HCM. The diagnosis of HCM should be made based on subjective findings that are supported by measurements. The myocardial thickness and the atrial size can be subjectively assessed. Many cases of HCM also presents with abnormal motion of the anterior mitral valve leaflet, called systolic anterior motion (SAM), causing dynamic left ventricular outflow tract obstruction (DLVOTO) and mitral valve regurgitation (Paige *et al*. 2009). Other findings may include spontaneous echocardiographical contrast (“smoke”), intracardiac thrombus and, in the case of CHF, pleural/ pericardial effusion and pulmonary B-lines (“rockets”) (Ferasin *et al*. 2003).

During echocardiographic examination, the thickness of the left ventricular wall and the interventricular septum are measured during diastole. A previously commonly used universal cut off value used for diagnosing left ventricular hypertrophy is when the left ventricular free wall (LVFW) or the interventricular septum (IVS) in diastole is ≥6mm. (Sampedrano *et al*. 2006; Payne *et* al. 2010; Payne *et al*. 2015; Ferasin *et al*. 2003; Borgeat *et al*. 2015; Paige *et al*. 2009). However, later research shows that body size has a clinically relevant impact on the echocardiographical cardiac measurements. Compared to normally sized cats, larger cats have larger hearts and smaller cats have smaller hearts. Body size is clinically usually estimated by using body weight. Accordingly, it is therefore of great importance to take body weight into consideration when establishing normal reference ranges for echocardiographical measurements. Significant non-linear associations between body weight and measurements of *e.g*. left ventricular internal diameter (diastole), left ventricular free wall (diastole) and left ventricular free wall (systole) have been found. An increase in the body weight is associated with an increase of these measurements and the association is best described by allometric scaling. Body weight based normal reference ranges have thereby been established based on allometry (Häggström *et al*. 2016).

For an example of an echocardiographic image of a negatively screened cat, see figure 1. For an example of an echocardiographic image of a cat affected by HCM, see figure 2.

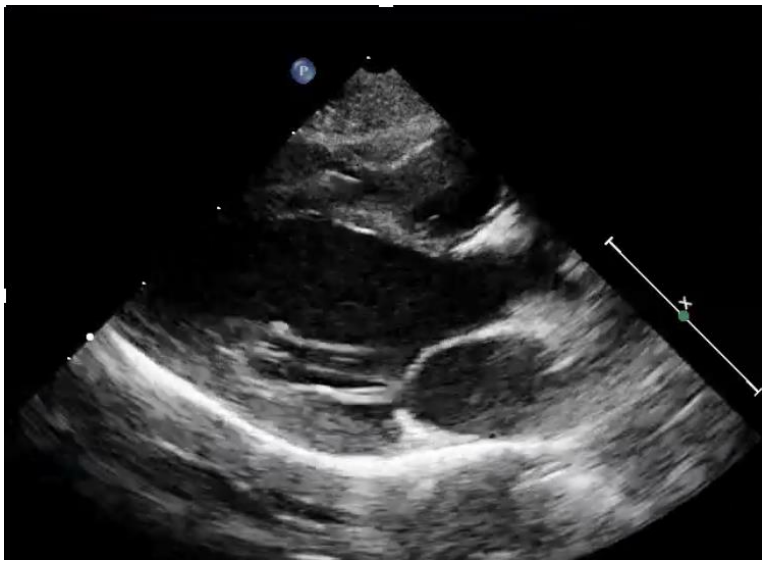

Figure 1. Echocardiographic image of a cat negatively screened for HCM. Photo: Jens Häggström

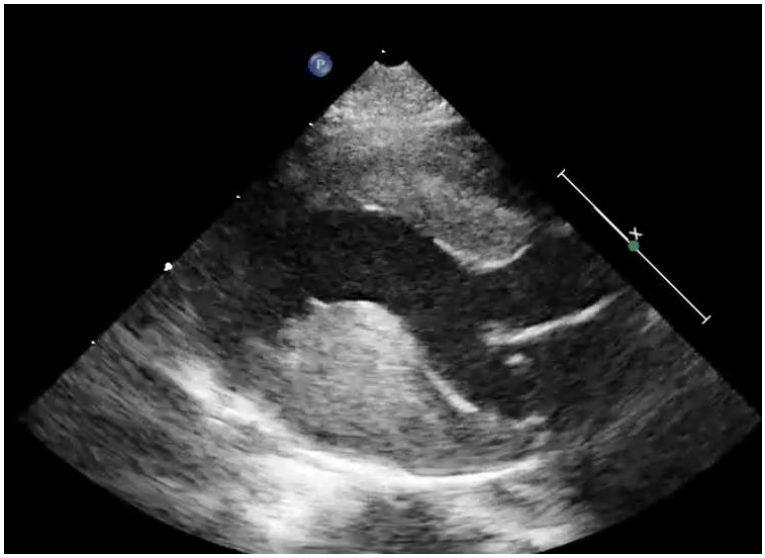

Figure 2. Echocardiographic image of a cat affected by HCM. Photo: Jens Häggström

Thoracic radiography is comparably insensitive in detecting HCM in cats. However, it is useful to examine the consequences of the disease, such as pulmonary edema and/or pleural effusion. Hypertrophic cardiomyopathy can be suspected in case of radiographic findings indicating cardiomegaly, pulmonary edema and pleural effusion. However, cardiomegaly has been reported to be present in only 59% of affected cats in a study including radiographs from 61 cats diagnosed with HCM (Ferasin *et al*. 2003). Results showed that 23% of the cats presented with signs of pulmonary edema and 6.6% showed indications of pleural effusion.

In addition, there are other diagnostic methods working as a complement to echocardiography. Analysing cardiac biomarkers such as NT-proBNP (Fox *et al*. 2009) and Troponin-I (Herndon *et al*. 2008) can provide information that can be useful for differentiating between respiratory distress secondary to a cardiac respectively noncardiac origin, which can be valuable in instances where echocardiography is not available. (Fox *et al*. 2009; Herndon *et al*. 2008)

For definitive diagnosis of HCM, histopathological examination is required (Kitz *et al*. 2019). At histopathological examination of cardiac tissue from cats diagnosed with HCM, findings include e.g., disarrangement of myocytes, fibrosis, interstitial enlargement, leukocyte infiltration and myocyte degeneration.

2.1.5 Treatment

Treatment recommendations of cats affected by HCM is dependent on stage. There are no recommendations for medical treatment of cats in stage B1. In stage B2, where a higher risk of developing ATE occur, treatment with thromboprophylaxis

such as clopidogrel is recommended (Luis Fuentes *et al*. 2020). In case of arrhythmia, beta blocker medication such as atenolol decreases the heart rate and murmur grade in cats with subclinical HCM (Jackson *et al*. 2015).

Cats in stage C, with CHF, require a more aggressive treatment (Luis Fuentes *et al*. 2020). In the case of acute heart failure, intravenous diuretic medication such as furosemide, along with oxygen administration, thoracocentesis and sedation are different options available depending on the patient's need. Pimobendan can be considered orally in cats without evidence of significant left ventricular outflow tract obstruction.

In cases suffering from ATE, treatment with analgesia (preferably an opioid agonist) and anticoagulant therapy (heparin) is recommended (Luis Fuentes *et al*. 2020). Occlusion of a smaller vessel or a frontal leg artery may be treatable, but treatment of a larger visceral, cerebral or the femoral arteries is usually unethical owing to the suffering and poor outcome.

Regarding stage C and treatment of chronic CHF, furosemide is given orally for treatment of pulmonary edema and pleural effusion (Luis Fuentes *et al*. 2020). Angiotensin-converting enzyme (ACE) inhibitors may be added in some cases. However, there were no improvement in clinical or echocardiographic parameters in cats affected by HCM that were treated with enalapril (Rush *et al*. 1998) nor an improved survival (King *et al*. 2019). Antithrombotic treatment is given prophylactic in the case of moderate to severe dilatation of the left atrium. Pimobendan may be used, but again, in the absence of significant left ventricular outflow tract obstruction (Luis Fuentes *et al*. 2020).

Similar treatment as in stage C is recommended for stage D (Luis Fuentes *et al*. 2020). Torasemide may replace furosemide if treatment with furosemide is insufficient. Additionally, food containing a high salt content should be avoided.

2.1.6 Prognosis

The prognosis for felines affected by HCM has been evaluated in different studies. The median survival time from diagnosis to death was 1276 (0 to 3617) days in a study where 127 cats with HCM were examined (Payne *et al*. 2010). Factors contributing to a higher risk of cardiac death include for instance a higher age, lack of heart murmur, gallop sound, arrythmia, congestive heart failure, arterial thromboembolism, left atrium dilatation (>16 mm) and hypertrophy of the left ventricle (≥9 mm) (Payne *et* al. 2013).

In a study including cats diagnosed with a nonobstructive or an obstructive form of HCM, the risk for cardiovascular death was 6.7% one year after study entry, 22.8%

5 years after study entry and 28.3% ten years after study entry. This study showed that once felines affected by HCM developed cardiovascular morbidity, the time from cats showing clinical signs to cardiovascular death was 1,3 years (Fox *et al*. 2018). The long-term prognosis of HCM may be viewed as poor, due to the progression of the disease. However, because not all affected cats are showing overt clinical signs of disease, the prognosis should be viewed as more complex. In a retrospective study of cats diagnosed with HCM between September 2001 and February 2011, 78% of the 217 cats with available data for follow-up were subclinical (Stage B). At the time of follow-up, 29% of the cats were 10-15 years old and 13.4% of the cats were > 15 years (Trehiou-Sechi *et* al. 2012). This underscores that cats may live for several years with HCM and still be subclinical. The study also shows that cats diagnosed with HCM may have a normal life span.

2.2 Genetics

A definition of a mutation is when the genetic variant occurs in ≤1% of the population (Marian 2021). A mutation is a modification of the nucleotide sequence and can be an alternation in a single or multiple nucleotides. Genetic variants can be pathogenic, benign or of unknown significance. Pathogenic genetic variants can affect the proteins' function and structure and by that lead to disease.

Hypertrophic cardiomyopathy in felines is a hereditary disease regarded to have an autosomal dominant inheritance pattern, however it is believed to have an incomplete penetrance (Gil-Ortuño *et al*. 2020). An autosomal dominant inheritance means that development of disease only requires one copy of the pathogenic gene variant to occur.

There are genetic variants in different genes, described as a cause of HCM. Causal associations between sarcomeric gene variants and presence of cardiomyopathy have been found (Freeman *et al*. 2017). Additional genetic variants in genes such as ion channel genes and desmosomal genes, have also been associated with the HCM phenotype in people (Lopes *et al*. 2013).

Ragdolls homozygous positive for the *MYBPC3*-variant p.R820W have been found to develop the disease at an earlier age compared to cats that are heterozygous for the gene variant (Meurs *et al*. 2007). There are additional examples supporting that development occur earlier in cats homozygous for a certain gene variant compared to heterozygous cats. One example, is a study done on Maine Coon cats regarding the breed-specific variant p.A31P of the *MYBPC3* gene, where severe disease was developed earlier on in the homozygous cats (Meurs *et al*. 2005).

2.2.1 Myosin binding protein C3

Myosin binding protein C3 is important for the interaction between myosin and actin (Finley & Cuperman, 2014). The protein is an essential part of the sarcomere and contraction of the cardiac muscle (Marian 2021). Numerous gene variants associated with familial HCM in humans have been identified in *MYBPC3* (Carrier *et al*. 1997).

In a study done by Meurs *et al*. (2007), 21 Ragdoll cats diagnosed with HCM were examined (Meurs *et al*. 2007). The study identified a gene variant in *MYBPC3*. This variant is referred to as c.2455C>T, meaning that in position 2455 of the coding sequence of the gene, C had mutated into a T. This resulted in a change from the amino acid arginine to tryptophan in position 820 of the protein (p.R820W). The variant of the protein, named p.R820W, was detected in all affected cats and was not found in any of the healthy cats in the control group.

Another genetic variant associated with inherited HCM in domestic cats has previously been found in the same gene as the p.R820W variant (Meurs *et al*. 2005). This variant (p.A31P) was detected in Maine Coon cats affected by HCM. By performing DNA sequencing a change in a base pair in codon 31 was discovered. G was replaced with C, leading to a change in the amino acid from alanine to proline (p.A31P).

The p.R820W *MYBPC3*-variant identified by Meurs *et al*. (2007) is believed to be a breed-specific gene variant in the Ragdoll cat. The prevalence of the gene variant in a population of 236 Ragdoll cats was 33.9% (Borgeat *et al*. 2014). The *MYBPC3*-variant in the Maine Coon cat (p.A31P) is also considered to be breed-specific (Meurs *et al*. 2005). The prevalence of this gene variant in a population of 2744 Maine Coon cats was 41.5% (Mary *et al*. 2010). In another study, the prevalence of the same gene variant was 16.33% among 49 Maine Coon cats (Sukumolanan & Petchdee, 2022). The same gene variant, c.2460C>T, has later been identified in *MYBPC3* in humans as well. This causes a change in the amino acid from arginine to tryptophan (p.R820W) and has also been associated with HCM in humans (Ripoll Vera *et al*. 2010).

A significantly larger proportion of Ragdoll cats carrying the *MYBPC3* p.R820W variant have a left ventricular hypertrophy compared to cats that are genotype negative for the mutation (Borgeat *et al*. 2015). Homozygous Ragdoll cats are more likely to die due to cardiac death and are more prone to die at a younger age compared to heterozygous Ragdoll cats or wild-type cats (Borgeat *et al*. 2014). This supports the hypothesis that p.R820W is a cause of inherited HCM. By performing genetic testing for known variants, recommendations for breeding can be provided.

Genetic testing can help estimating the risk of developing the disease (Luis Fuentes *et al*. 2020).

2.2.2 Myosin heavy chain 7

The abbreviation *MYH7* stands for a gene coding for the protein myosin heavy chain beta (Schiaffino & Reggiani 2011). This protein is an isoform of myosin heavy chain and is present in cardiac muscle. Myosin molecules are fundamental parts of the sarcomere, essential for generating contraction of the cardiac muscle.

Schipper *et al*. (2019) performed genetic analyses on a six-year-old male domestic shorthair cat (Schipper *et al*. 2019). The cat presented with dyspnea, cyanosis, tachypnea and paralysed hindlimbs. The cat was diagnosed with arterial thrombo-embolism, and because the ultrasound showed a thickened wall in the left ventricle, HCM was considered a probable caused. Both pathological and histopathological examination of the left ventricle indicated hypertrophy.

Genetic analyses were performed and the genes *MYBPC3* and *MYH7* were sequenced (Schipper *et al*. 2019). One genetic variant that altered an amino acid and that was assumed to be damaging was found in *MYH7*. The variant, c.5647G>A, led to a change in the amino acid from glutamic acid to lysine (p.E1883K). The cat was heterozygous, indicating the inheritance to be autosomal dominant.

This gene variant (c.5647G>A) has a human orthologue reported in a family where three out of four siblings had developed HCM. Only one of the siblings were sequenced, since the other two had died due to heart failure. The sibling analysed was homozygous for the variant (Tajsharghi *et al*. 2007 see Schipper *et al*. 2019). Multiple gene variants in *MYH7* have been associated with HCM in humans and it is one of the most common genes causing HCM in humans (Tesson *et al*. 1998).

2.2.3 Troponin T2

A case reported by McNamara *et al*. (2020) supports the possibility of an additional gene variant causing inherited HCM in domestic cats. In the study a Maine Coon cat affected by HCM was tested for mutations by performing whole genome sequencing. The cat was negative for the breed-specific p.A31P variant in *MYBPC3*. Instead, a new genetic variant was identified, located in an intron of the gene *troponin T2* (*TNNT2*) (McNamara *et al*. 2020). Cardiac troponin T (TNNT2) is an important part of the thin filaments located in the myocytes in the cardiac muscle. The protein connects the troponin complex with the protein tropomyosin, essential for the function of the myocytes (Marian 2021). Cardiac troponin T is one of the genes most frequently causing HCM in humans. Numerous gene variants

lead to an insufficient protein and have been associated with development of HCM. Most gene variants are missense or deletion mutations (Marian & Roberts 2001). The variant found in the Maine Coon cat was believed to be a possible cause of HCM in this cat. The hypothesis was that this variant, c.95-108G>A, may change *TNNT2* splicing, leading to a non-functional protein (McNamara *et al*. 2020).

In contrast, a study done by Schipper *et al.* (2022) indicates that the intronic variant in *TNNT2* appears not to have any strong association with HCM in Maine Coon cats (Schipper *et al*. 2022). In the study, the cats positive for the gene variant did not have the altered splice site predicted by McNamara *et al*. (2020), however there was an alternation in splicing. In this study, various criteria for determining if the variant was benign or pathogenic were examined. None of the criteria investigated to determine statistical association with HCM were met. This indicates that even though the genetic variant seems not to have any association with HCM in felines, some data indicates that this cannot be excluded.

2.2.4 Alstrom syndrome protein 1 gene

The function of the *Alstrom syndrome protein 1 gene* (*ALMS1*) gene is not entirely identified (Álvarez-Satta *et al*. 2015). Various functions that have been acknowledged include for instance impact on the ciliary function, intracellular membrane traffic, energy metabolism, signalling pathways of cilia and cell cycle control.

Genetic variants in the *ALMS1* gene in humans are associated with Alstrom syndrome (Brofferio *et al*. 2017). The disease affects multiple organs and has a clinical manifestation including cardiomyopathy, obesity, type 2 diabetes mellitus and retinal dystrophy.

Meurs *et al*. (2021) reported a novel gene variant in the *ALMS1* gene in Sphynx cats associated with HCM (Meurs *et al*. 2021). The genetic variant was found in chromosome A3: 92439157 in exon 12 (according to ensembl c.10126G>C), leading to a change from the amino acid glycine to arginine (p.G3376R). The gene variant was predicted to change the structure of the protein, contributing to a deleterious variation of the protein. Unfortunately, this study did not include a sufficient quantity of Sphynx cats nor Sphynx cats without HCM, which means that allele frequency in this population is unknown and the association between the variant and HCM unproven.

2.3 Hypertrophic cardiomyopathy in humans

In humans, HCM is an inherited disease with >1500 gene variants discovered in eleven genes encoding sarcomeric proteins (Freeman *et al*. 2017). Commonly the

mutations consist of a change in a single amino acid, resulting in an altered protein function and structure. The disease may be caused by a previously known mutation or due to *de novo* mutations (Maron *et al*. 2012). The prevalence of HCM in the general young adult population has been estimated to be 1:500 (Maron *et al*. 1995). The prevalence of individuals carrying HCM causing genes, however, has been estimated to be 1:200 (Semsarian *et al*. 2015).

The clinical presentation among HCM patients is diverse (Freeman *et al*. 2017). Most HCM patients have non or mild symptoms initially when diagnosis is made. This indicates that humans, similar to cats, may have HCM asymptomatically. The most common symptoms include dyspnea, lethargy and chest pain (Maron *et al*. 1999). Due to an insufficient relaxation of the myocardium, some patients may experience exercise intolerance and dyspnea during physical activity. Some patients develop severe complications such as arrythmias, heart failure and sudden cardiac death (Marian 2021).

2.4 Sequencing methods

Various techniques can be utilized for sequencing DNA (determining the order of the nucleotide bases A, T, C and G in a targeted DNA region).

2.4.1 Direct sequencing of DNA

Sanger sequencing is a method used for direct sequencing and often used in studies with a candidate gene approach (Hu *et al*. 2021). Chain-terminating and fluorescently labeled dideoxynucleotides is added for sequencing of the DNA complementary to the selected template. Subsequently, gel electrophoresis is used for separating the fragments according to size. For this procedure, capillary electrophoresis might be used.

2.4.2 Whole genome sequencing

Whole genome sequencing (WGS) is a method used for sequencing the entire genome of an organism (Hu *et al*. 2021). Illumina's technology is referred to as a next-generation sequencing (NGS) technique used for WGS. Before sequencing, DNA is prepared, fragmentated and selected for size length, followed by addition of sequencing adapters and sample specific barcodes through PCR. This generates sample specific sequencing libraries that can be normalized and pooled with other samples prior to WGS.

The process of sequencing includes clonal amplification and sequencing (Hu *et al*. 2021). During clonal amplification, DNA fragments bind to flow cell surfaces and

PCR is used for amplification of the DNA fragments. For sequencing, a method is used where DNA-polymerase allows fluorescent nucleotides to incorporate on the DNA chain. During sequencing, multiple cycles are run, and during all cycles one fluorescent nucleotide is added to the DNA chain. The fluorescent nucleotides result in a signal that is imaged, and data analyses are subsequently performed, allowing for the sequence of the DNA to be determined.

3. Material and Methods

3.1 Laboratory work

Nine previously collected samples from Ragdolls were included in this study. Eight of the samples were from cats from a Ragdoll family highly affected by HCM. An additional sample from an unrelated Ragdoll affected by HCM was included. Blood samples from seven cats, swabs from the oral cavity from one cat and cardiac tissue from one cat were available from previous sampling.

3.1.1 Primer designing

Sequence specific primers were designed for amplification of regions of previously known gene variants associated with HCM in the genes *MYBPC3*, *MYH7* and *ALMS1*. Primers were designed by usage of the program Primer 3 (https://primer3.ut.ee/ 221017). Primers for the p.A31P gene variant identified in *MYBPC3* in the Maine Coon cat breed (*MYBPC3*$_{MCO}$) were available from previous projects at the department. Primer sequences for the known gene variant in *TNNT2* was used from Schipper *et al*. (2022).

3.1.2 Optimizing DNA-protocol

PCR and subsequent gel electrophoresis were performed with the forward and reverse primers designed for *MYBPC3*$_{RAG}$, *MYH7* and *ALMS1*. This was performed for optimizing the protocol (see table 2) regarding temperature, which was used for later sequencing. Standard PCR was performed using the AmpliTaq Gold™ DNA Polymerase kit (Applied Biosystems, Waltham, MA, USA) according to manufacturer's instructions. The total volume used for PCR was 12.5 µl, with final concentrations of the primers and polymerase enzymes at 0.2 µM and 1.25 U, respectively. In addition, 200 µM of each dNTP and an adjusted Mg^{2+} concentration of 2.5 mM were used during the reaction.

Table 1. Primer sequences designed by usage of Primer 3 or provided from the department.

Gene	Forward primer	Reverse primer	Annealing T_m
MYBPC3$_{RAG}$ a	AGCAATGTGGGTGAGGACTC	ATGGCATTGACCGCGTAGA	58°
MYBPC3$_{RAG}$ b	CCCAAGATCAGCAATGTGGG	GGACCCGGATGTAAATGCCT	58°
MYBPC3$_{MCO}$	TCTCATAGAGCCACTGAAGCATTA	CTCAGAACTTTCCCTACTTCCACA	59°
MYH7 a	CCCTCCTCACTCCTAACCCT	CAGCTTGTTGACCTGGGACT	58°
MYH7 b	CCCTCCTCACTCCTAACCCT	TGACATGCGGTGACTAGTGG	58°
ALMS1 a	TGGACCTGTTATTGTGCCCC	TCCAGCCAATCACCACAGAA	58°
ALMS1 b	CCCCTTCTGATCACACTGCA	ACTGAGAAGGACGGCTTAGC	58°
ALMS1 c	TCTGTCATTTGCTTCTCCACCT	ACTGAGAAGGACGGCTTAGC	58°
ALMS1 d	CCCCTTCTGATCACACTGCA	AGAAGCCTGTGAAGCATTTTGA	58°

Table 2. PCR protocol for AmpliTaq Gold kit.

Number of cycles	Action	Time	Temperature (°C)
1x	Enzyme activation and denaturation	10 min	95
35x	DNA denaturation	15 s	95
	Primer annealing	30 s	58
	Extension	45 s	72
1x	Final extension	5 min	72
1x	Hold	Infinity	4

A 1% agarose gel was mixed for a standard DNA-gel electrophoresis. 0.5 g agarose and 50 ml tris-borate-EDTA (TBE) buffer were mixed and boiled until the agar had melted. The mix was cooled down to 50° and 5 µl of GelRed was subsequently added and mixed. The gel was poured into the tray and combs for generating sample wells were placed in the tray. The samples were mixed (5 µl sample with 1 µl loading buffer) and 5 µl of the mix were added into the wells. 5 µl of a 1 kb DNA-ladder were separately added into two wells as fragment references. The gel was run at 70V for 45 minutes and was photographed using ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA, USA).

3.1.3 Sequencing

Before sequencing, the DNA samples were quantified by using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNA samples were diluted to a concentration of 4 ng/µl. Sanger sequencing was performed with the designed primers (see Table 1) with orientation specific M13-tags added to each primer (see Table 3). The primers selected from Table 1 were *MYBPC3$_{RAG}$ b*, *MYBPC3$_{MCO}$*, *MYH7 b*, *ALMS1 a* and *ALMS1 b*.

Table 3. Following M13-sequence tags were added to respectively primer sequence.

M13 primer added to forward strand	M13 primer added to reverse strand
5’ TGTAAAACGACGGCCAGT 3’	5’ CAGGAAACAGCTATGACC 3’

The protocol for BigDye™ Direct Cycle Sequencing Kit (Applied Biosystems) was used according to manufacturer’s recommendations. In brief, a two-step PCR was performed, where PCR 1 was performed for region specific amplification and PCR 2 was performed for cycle sequencing. Purification of the sequencing products followed by capillary electrophoresis with (Applied Biosystems) was performed.

3.1.4 Sequencing analyses

Sequencing analyses were performed using the program CodonCode Aligner (downloaded 221031, https://www.codoncode.com/aligner/download.htm). The files from the sequencing were uploaded to the program and the forward and reverse strands were aligned. The sequences were inspected for presence of the known gene variants earlier mentioned.

3.2 Echocardiographic examination

The diagnosis of HCM among some of the family members in this study had previously been established by a standard echocardiographic examination (Häggström *et al.* 2016). At the echocardiographic examination, the heart was evaluated in both 2D- and 2D-guided M-mode. 2D echocardiography allows a real-time evaluation of the heart permitting a subjective assessment of cardiac anatomy and function. The M-mode technique was used for left ventricular measurements of e.g. wall thickness, (Carerj *et al.* 2003) whereas the left atrial to aortic root ratio was used to measure left atrial size in 2D mode.

During echocardiographic examination, various imaging planes were used for evaluation of the heart (Thomas *et al*. 1993). The right parasternal long axis view of the left ventricular out-flow tract is one example of imaging planes used for echocardiography in cats. Additional examples of imaging planes are the right parasternal short axis view at the level of the papillary muscles and the right parasternal short axis view at the level of the aortic valve and left atrium.

For the diagnosis of HCM, the left ventricular wall was subjectively evaluated and measured for hypertrophy (Luis Fuentes *et al*. 2020). This was done by studying both 2D-images and 2D-guided M-mode images. The thickness of the left ventricular wall was measured at end diastole in right parasternal short axis view (Hansson *et al*. 2002) and a leading-edge-to-leading-edge approach was used (Häggström *et al*. 2015). The following measurements were made: end-diastolic interventricular septal thickness (IVSd and IVSS), end-diastolic and end-systolic left ventricular free wall thickness (LVFWd and LVFWs), left ventricular internal dimension at end-diastole and end-systole (LVIDd and LVIDs).

Additional measurements made in M-mode included left ventricular fractional shortening (LV FS%). The LV FS% is an index of left ventricular systolic function. Measurements additionally made in 2D imaging include left atrial to aortic ratio (LA/Ao) and left atrial diameter. Additional important findings that were evaluated, included presence of *e.g.* abnormal geometry, thrombus and abnormal wall motion (Luis Fuentes *et al*. 2020). Furthermore, the presence of SAM and dynamic left ventricular outflow tract (LVOT) obstruction (Lamont *et al*. 2002) was evaluated in 2D and color mode loops (Häggström *et al*. 2015).

4. Results

This study included nine Ragdoll cats. Eight of these cats were family members and among these, four cats had previously been diagnosed with HCM. Seven of the eight family members were female. Three females and the male had been diagnosed with HCM. The four healthy family members were between 3-7 years old. Three of the affected family members died at the age of 1-3 years. For one of the affected family members, the status in unknown for whether the cat is alive. The unrelated Ragdoll cat in this study was a female, previously diagnosed with HCM. This cat died at the age of 1 year.

4.1 Gel electrophoresis

PCR and gel electrophoresis were performed for the primers designed for the genes *MYBPC3*$_{RAG}$, *MYH7* and *ALMS1* (see table 1). PCR was also performed for the previously available Maine Coon specific p.A31P gene variant in *MYBPC3* (*MYBPC3*$_{MCO}$). The results of the gel electrophoresis showed distinct single bands visible for all designed primers (see figure 3). This indicates a specific primer annealing and a successful amplification. Since all primers were sufficient for amplifying the correct DNA regions, one primer for each gene variant was chosen for the sequencing.

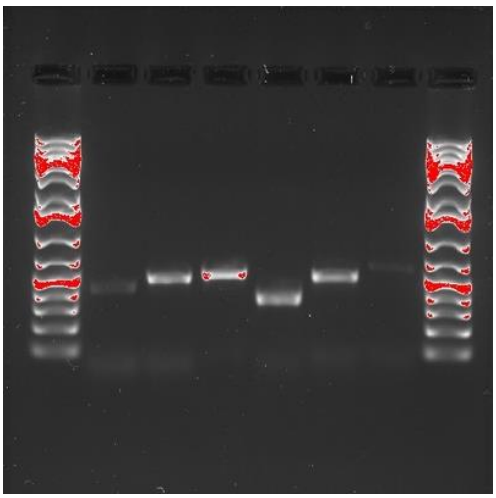

Figure 3. Image of the gel run with samples from the PCR of the designed primers (see table 1). Ladder was loaded into the left and right well. The primers were added in following order: MYH7 a, MYH7 b, ALMS1 a, MYBPC3$_{RAG}$ *a, MYBPC3*$_{RAG}$ *b, MYBPC3*$_{MCO}$.

Results from the sequencing of ALMS1 a were insufficient as the position of the gene variant was not included in the amplified sequence. New sets of primers for the requested position were therefore designed (see ALMS1 b, ALMS1 c, ALMS1 d in Table 1). The results of the second gel electrophoresis showed distinct single bands visible for the primers ALMS1 b, ALMS1 c and ALMS1 d (see Figure 4). Since all primers were sufficient for amplifying the DNA, one primer-pair was chosen for the subsequent sequencing.

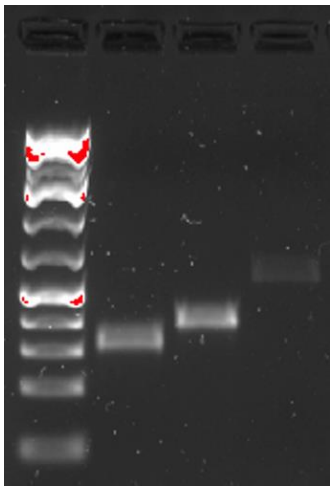

Figure 4. Image of the gel run with samples from the PCR of the designed primers (see table 1). Ladder was loaded into the left well. The primers were added in following order: ALMS1 b, ALMS1 c, ALMS1 d.

4.2 Sequencing

Samples from eight family members from a Ragdoll family and an additional sample from a Ragdoll affected by HCM were sequenced for previously known gene variants associated with feline HCM.

Four out of the eight family members in this study had previously been diagnosed with HCM. Results from the sequencing showed that all of the family members affected by HCM were homozygous for the Ragdoll breed-specific gene variant in *MYBPC3* (*MYBPC3*$_{RAG}$). Two of the healthy family members were negative for the gene variant and two healthy family members were heterozygous. The HCM affected Ragdoll cat, not related to any of the family members, was negative for the gene variant in *MYBPC3*. For the number of Ragdoll cats negative, heterozygous and homozygous for the Ragdoll breed-specific mutation in *MYBPC3*, see table 4.

Table 4. Number of Ragdoll cats negative, heterozygous and homozygous for the Ragdoll breed-specific gene variant in MYBPC3.

	Female			Male		
	Negative	Heterozygous	Homozygous	Negative	Heterozygous	Homozygous
Normal	2	2	-	-	-	-
HCM	1	-	3	-	-	1

Results from the sequencing of the gene *TNNT2* showed that four of the eight family members were heterozygous for the gene variant. All family members heterozygous for the *TNNT2* variant were healthy. The four family members affected by HCM were negative for the variant. None of the family members in this study were homozygous. The unrelated HCM affected Ragdoll cat was also negative for the gene variant in *TNNT2*. For the number of Ragdoll cats negative, heterozygous and homozygous for the *TNNT2* gene variant, see table 5.

Table 5. Number of Ragdoll cats negative, heterozygous and homozygous for the TNNT2 gene variant.

	Female			Male		
	Negative	Heterozygous	Homozygous	Negative	Heterozygous	Homozygous
Normal	-	4	-	-	-	-
HCM	4	-	-	1	-	-

Results from the sequencing of the known gene variants in *MYH7* and *ALMS1*, along with the Maine Coon breed-specific gene variant in *MYBPC3* (*MYBPC3$_{MCO}$*) showed that all cats in the study were negative for these gene variants. The sequence for the Maine Coon breed-specific variant was unreadable in one of the family members.

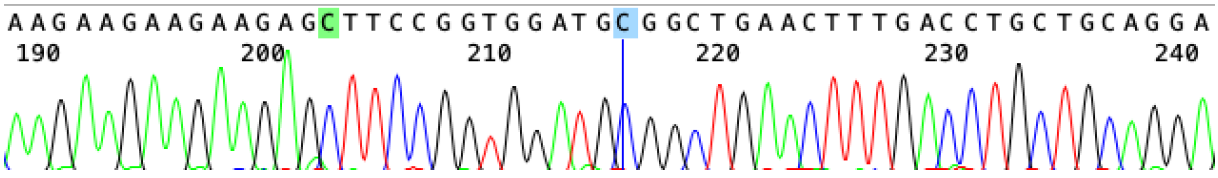

Figure 5. Image of a sequence from a cat negative for the Ragdoll breed-specific gene variant in MYBPC3. In position 2455 of the coding sequence of the gene, this cat does not have any mutation from the nucleotide C to T, meaning that this cat has a normal coding sequence. The highlighted position in the gene sequence shows a blue peak indicating the nucleotide C (normal). The image of the sequence is taken from the program CodonCode Aligner.

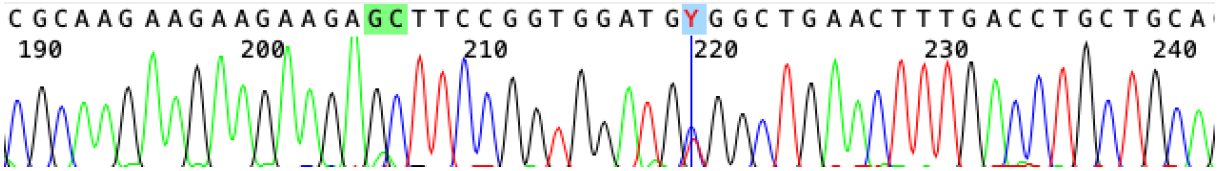

Figure 6. Image of a sequence from a cat heterozygous for the Ragdoll breed-specific gene variant in MYBPC3. In position 2455 of the coding sequence of the gene, this cat has a mutation from the nucleotide C to T in one of the cat's alleles. This means that this cat is heterozygous for the gene variant. The highlighted position in the gene sequence shows a blue peak indicating the nucleotide C (normal) and a red peak indicating the nucleotide T (mutated nucleotide). The ''Y'' indicates that the nucleotides in this position are a C and a T. The image of the sequence is taken from the program CodonCode Aligner.

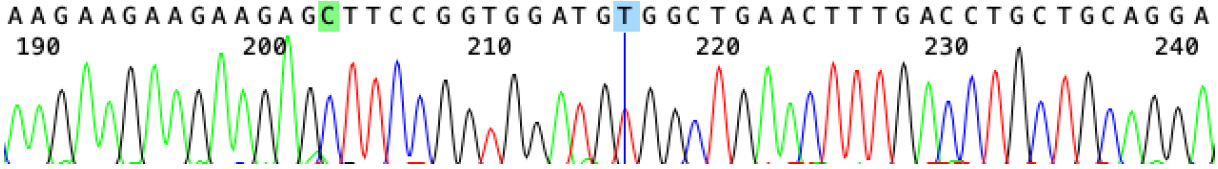

Figure 7. Image of a sequence from a cat homozygous for the Ragdoll breed-specific gene variant in MYBPC3. In position 2455 of the coding sequence of the gene, this cat has a mutation from the nucleotide C to T in both alleles. This means that this cat is homozygous for the gene variant. The highlighted position in the gene sequence shows a red peak indicating the nucleotide T (mutated nucleotide). The image of the sequence is taken from the program CodonCode Aligner.

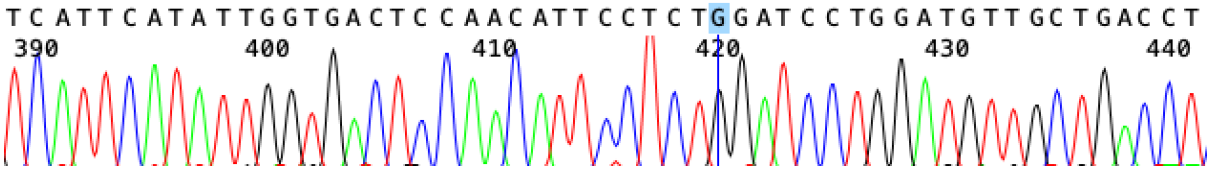

Figure 8. Image of a sequence from a cat negative for the gene variant in TNNT2. In position 95-108 of the coding sequence of the gene, this cat does not have any mutation from the nucleotide G to A, meaning that this cat has a normal coding sequence. The highlighted position in the gene sequence shows a black peak indicating the nucleotide G (normal). The image of the sequence is taken from the program CodonCode Aligner.

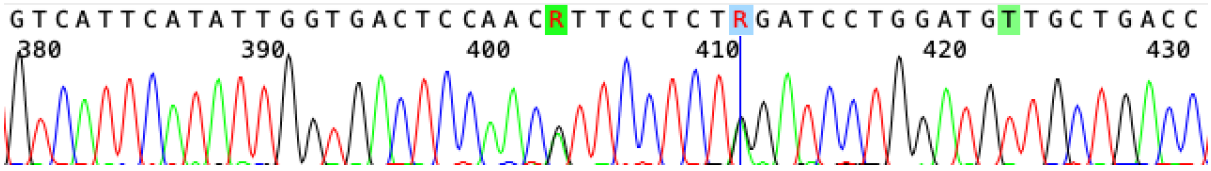

Figure 9. Image of a sequence from a cat heterozygous for the gene variant in TNNT2. In position 95-108 of the coding sequence of the gene, this cat has a mutation from the nucleotide G to A in one of the cat's alleles. This means that this cat is heterozygous for the gene variant. The highlighted position in the gene sequence shows a black peak indicating the nucleotide G (normal) and a green peak indicating the nucleotide A (mutated nucleotide). The ''R'' indicates that the nucleotides in this position are a G and an A. The image of the sequence is taken from the program CodonCode Aligner.

5. Discussion

Hypertrophic cardiomyopathy is the most common cardiac disease among domestic cats, and it is associated with known gene variants in *MYBPC3*, *MYH7*, *TNNT2* and *ALMS1*. Results from this study showed occurrence of the Ragdoll breed-specific gene variant in *MYBPC3* (p.R820W) among some of the family members. All family members affected by HCM were homozygous for the gene variant. Two of the healthy family members were heterozygous and two of the healthy family members were negative for the gene variant. Four of eight family members were heterozygous for the gene variant *TNNT2*. All these cats were healthy. The HCM affected cats in this study were all negative for the variant. All family members in this study were negative for the further tested genetic variants in *MYH7*, *MYBPC3*$_{MCO}$ and *ALMS1*. The sequence for the Maine Coon breed-specific gene variant (p.A31P) was unreadable in one of the family members. Because the remaining family members were negative for the gene variant, it is likely that this cat is negative for the variant as well. However, the conclusion about this speculation cannot be made without repeating the sequencing for this cat. The HCM affected Ragdoll cat not related to the studied Ragdoll family was negative for all tested gene variants.

Results from this study therefore indicate that the Ragdoll breed-specific mutation in *MYBPC3* is a probable cause of the high prevalence of HCM among the family members. The prevalence of the gene variant was 33.9% in a population of Ragdoll cats in a study (Borgeat *et al*. 2014). Because the gene variant is reported comparably common, it cannot be excluded that the presence of the gene variant among the cats is a coincidence, instead of a cause of HCM among the family members. However, because of the evident connection between homozygous family members and family members affected by HCM, it seems likely that the gene variant is a cause, rather than a coincidence. Previous publications have shown the association between the Ragdoll breed-specific gene variant in *MYBPC3* and feline HCM (Meurs *et al*. 2007). The results from this master thesis support this association.

Similar to previous research, it was clear that family members homozygous for the Ragdoll specific mutation in *MYBPC3* were more severely affected by HCM compared to family members heterozygous or negative for the gene variant. In this

study, none of cats heterozygous or negative for the gene variant had developed echocardiographic signs of HCM. However, it cannot be excluded that the heterozygous or negative cats will develop HCM in the future. These results are comparable to those reported by Meurs *et al*. (2007), stating that Ragdolls homozygous for the *MYBPC3*-variant p.R820W have been found to develop HCM at an earlier age compared to cats that are heterozygous. In this study, the average age of diagnosis of the homozygous cats were 21 months. The heterozygous cats had an average age of 30 months by the time for which diagnosis was made (Meurs *et al*. 2007). In this master thesis, the healthy cats were between 3-7 years old. Meanwhile all HCM affected cats besides one (with an unknown status), died at the age of 1-3 years.

A similar association between genotype and phenotype has been discovered in the Maine Coon cat breed, where an association between *MYBPC3*$_{MCO}$ carriers and Maine Coon cats affected by HCM has been identified (Meurs *et al*. 2005). However, no cat in this master thesis was positive for this gene variant. According to previous research, this variant is breed-specific for the Maine Coon cat (Meurs *et al*. 2005) and the results in this master thesis were therefore expected.

Another gene variant included in this master thesis was the variant in *TNNT2*. All HCM affected cats in this study were negative for the gene variant. The four healthy family members were all heterozygous for the variant. In this study, there were thus no associations between the gene variant in *TNNT2* and HCM among the Ragdoll cats. These results therefore indicate that the gene variant in *TNNT2* is not a probable cause of the high prevalence of HCM among the family members.

The possibility that another gene variant is contributing to the high prevalence of HCM among this Ragdoll family, cannot be excluded. An unknown or novel gene variant could be contributing to the disease among family members. Because the unrelated Ragdoll cat was negative for the HCM associated gene variants, conclusions can be made that other HCM causing variants most likely exist in the breed as well. However, conclusions about which potential gene variant and if the affected Ragdoll family has it as well, is unknown.

The results in this study clearly indicate the severe consequences of mating two cats that are carriers of the Ragdoll breed-specific gene variant in *MYBPC3*. Therefore, it underscores the great importance of following breeding recommendations. Because familial feline HCM has a genetic cause, breeding cats that have been negatively screened for the disease can limit the occurrence of HCM in the breed. Echocardiographic screening is used for breeding purposes and can thereby reduce the prevalence of the disease among Ragdoll cats (Häggström *et al*. 2016). A

commercial genetic test for the Ragdoll specific mutation in *MYBPC3* is also available (Luis Fuentes *et al*. 2020).

Sanger sequencing was used in this study for direct sequencing with a candidate gene approach (Hu *et al*. 2021). The unrelated Ragdoll cat in this study was negative for all tested genetic variants. Because this cat did not have any of the previously known gene variants associated with feline HCM, further sequencing for additional gene variants would be of great interest. The whole genome sequencing method would allow for this cat's entire genome to be studied (Hu *et al*. 2021), allowing the possibility of discovering a potential novel genetic alternation causing HCM in this cat.

Analysing an individual single nucleotide polymorphism (SNP) that is associated with a specific disease is a method for genetic testing (Wu *et al*. 2010). At SNP genotyping, a genetic locus is selected for analysing for the SNP, working as a genetic marker (Shen *et al*. 2009). This could have been an alternative method instead of the sanger sequencing. SNP genotyping, however, is in general a limited test as it particularly tests for a single gene variant in a specific locus. Because SNP genotyping was not possible for the selected gene variants for this study and because sanger sequencing was available, sanger sequencing was the best alternative and the method of choice.

In order to draw a more reliable conclusion about affected family members being homozygous for the Ragdoll specific *MYBPC3* gene variant, including additional family members would have been required. Including additional unrelated Ragdolls affected by HCM could also have increased the reliability of the result, indicating that the identified gene variant in this family is the probable cause of the high prevalence of HCM.

Other potential methods could have been used to draw a more reliable conclusion about the genetic cause of HCM among the family members. Whole genome sequencing would have allowed for other potential novel gene variants to have been discovered. As mentioned previously, it would also have been preferable to include a larger number of family members in this study. Because only eight family members were included, there is a possibility that the study result is not representative for the whole Ragdoll family. The family members included in this study were selected because previous samples were available from the University Animal Hospital at Swedish University of Agriculture Sciences. Since only a few family members were selected and since they were not selected at random, there is a possibility that the selection might impact the results, leading to selection bias. Another possible source of error in the study might be inaccuracy in the laboratory work because of lack of experience with the selected methods and equipment. This

might for example be the usage of an incorrect primer or an incorrect DNA sample, as well as an incorrect quantity of a substance.

The sequence for the Maine Coon breed-specific gene variant was unreadable in one of the family members. From this cat, DNA was collected from cardiac tissue. A poor quality of the DNA from the preparation of the cardiac tissue is a probable explanation of the unreadable sequence.

Numerous gene variants associated with familial HCM in humans have been identified in *MYBPC3* (Carrier *et al*. 1997), *MYH7* (Tesson *et al*. 1998), *TNNT2* (Marian & Roberts 2001) and *ALMS1* (Brofferio *et al*. 2017). This has led to the interest of sequencing these genes in the search for gene variants associated with feline HCM, often by direct sequencing with a candidate gene approach. In the future, it could therefore be interesting to sequence additional human HCM associated genes for gene variants in felines as well. In humans, >1500 genetic variants have been associated with the HCM phenotype, including those present in genes coding for sarcomeric, ion channel and desmosomal proteins (Lopes *et al*. 2013). All the genes that hitherto have been associated with feline HCM have been identified in sarcomeric genes. Studying ion channel and desmosomal genes in felines could therefore be of interest for future research.

5.1 Conclusion

The results of this study showed that all HCM affected family members were homozygous for the p.R820W *MYBPC3*-variant. None of the healthy family members were homozygous for the variant. Results from this master thesis therefore indicate that the Ragdoll breed-specific gene variant in *MYBPC3* could be a probable cause of HCM among family members, with results indicating that cats homozygous for the gene variant have an increased risk of developing the disease. The results of this master thesis did not show any association between HCM among the studied Ragdoll cats and the gene variants in *TNNT2*, *ALMS1*, *MYBPC3*$_{MCO}$ and *MYH7*.

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Popular Science Summary

Hypertrophic cardiomyopathy (HCM) is a common disease in domestic cats (affecting about 14.7%) that involve the heart muscle. The disease leads to a muscle thickening of the heart walls and may thereby lead to a reduction in the heart function. As a consequence, cats may develop heart failure and/or suffer from sudden death. The diagnosis of the disease is usually made by ultrasound examination.

Research has shown that the disease is inherited, and some cat breeds have a higher risk of developing HCM, including Ragdoll cats. There are known alternations in the DNA, so-called gene variants, that have been associated with HCM among cats. Known gene variants associated with HCM in cats have been found in the genes *myosin binding protein C3* (*MYBPC3*), *myosin heavy chain 7* (*MYH7*), *troponin T2* (*TNNT2*) and *Alstrom syndrome protein 1* (*ALMS1*).

Hypertrophic cardiomyopathy is also a disease affecting humans. As well in humans, it is an inherited disease. In humans, the disease affects one in five hundred individuals.

In this study, samples from eight cats from a Ragdoll family affected by HCM were examined. Four out of the eight family members had the disease. An additional sample from an unrelated Ragdoll affected by HCM was added for the possibility of comparing results. The aim of this study was to examine if any of the family members have any of the known gene variants that possibly could explain why four out of the eight family members had HCM.

Primers that bind to specific regions of the DNA were designed for the known gene variants in the genes *ALMS1*, *MYBPC3* and *MYH7*. Previously designed primers for *TNNT2* and a gene variant in *MYBPC3* were used as well. In *MYBPC3*, a Ragdoll breed-specific and a Maine Coon breed-specific gene variant were included in the study. Laboratory work was done and DNA from earlier collected samples was used to see if the studied cats had any of the gene variants. During the lab, the primers will bind to the DNA regions for which they are designed, permitting for the regions to be studied. For each studied gene in each cat, a file of the sequence

(the order of the nucleotide bases A, T, C and G) will be obtained and inspected for the presence of the known gene variants.

The results from this study showed that some of the family members had the Ragdoll breed-specific gene variant in *MYBPC3* and the known gene variant in *TNNT2*. Regarding the gene variant in *MYBPC3*, the results showed that all sick family members were homozygous (had two out of two copies showing the gene variant). The cats that were heterozygous (had one out of two copies showing the gene variant) or negative for the gene variant were all healthy. Regarding the results of the *TNNT2* variant, the four healthy family members were heterozygous for the variant and the four sick family members were negative. There were therefore no associations between the gene variant in *TNNT2* and HCM among the family members. The gene variant in *TNNT2* is thus not a probable cause of the high prevalence of HCM in this family. The unrelated Ragdoll cat that was included was negative for all tested gene variants. This indicates that other HCM causing variants most likely exist in the breed as well. There could therefore be an unknown or novel gene variant causing HCM in this cat.

The result from this study shows a connection between cats being homozygous for the Ragdoll breed-specific gene variant and cats that develop HCM. This is consistent with earlier research, stating that Ragdoll cats homozygous for their breed-specific gene variant develop the disease at an earlier age compared to cats that are heterozygous. In this study, the healthy cats were between 3-7 years old. Meanwhile all HCM affected cats besides one (with an unknown status), died at the age of 1-3 years. These results clearly indicate the severe consequences of mating two cats that are carriers of the gene variant. It therefore highlights the importance of following breeding recommendations and testing cats for the disease before breeding.

The results of this master thesis indicate that the Ragdoll breed-specific gene variant in *MYBPC3* is a probable cause of HCM among four of the eight family members. However, it cannot be excluded that an unknown gene variant is contributing to the penetrance of disease among the studied Ragdoll cats.

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