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Hypertrophic cardiomyopathy in Maine Coon cats in The Netherlands

*The significance of the MYBPC3-*A31P *mutation and other known causative mutations, and the utility of N-terminal pro-brain natriuretic peptide in the diagnosis of this condition.*

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Abstract

Background: Hypertrophic cardiomyopathy (HCM) is the most common cardiomyopathy in cats and it is postulated to inherit as an autosomal dominant trait. Thus far, two mutations in the *MYBPC3* gene are known. However, the relationship between genotype and phenotype is unclear. The gold standard test to diagnose HCM is echocardiography. However, this method has limited availability and is operator dependent. Measurement of N-terminal pro-brain natriuretic peptide (NT-proBNP) has been reported to be valuable in detecting HCM in cats.

Objectives: The aims of this study were to fully phenotype Maine Coon cats from the Dutch population, to establish the phenotype-genotype correlation within these cats, and to measure NT-proBNP and compare these results with echocardiography as a screening test for the diagnosis of HCM

Methods: Maine Coon cats (n=30) were classified using echocardiography as phenotypically healthy (n=19), or cats with an equivocal phenotype (n=3), or cats with HCM (n=8). Plasma NT-proBNP concentrations were measured in 33 Maine Coon cats. A total of 44 Maine Coon cats have been genotyped.

Results: Based on echocardiography, 22 cats classified as healthy and eight as HCM. The HCM mutation A31P was found in four (66.7%) of the healthy cats, one of the equivocal cats (16.7%), and one (16.7%) of the HCM cats. The A74T mutation was found in twelve (80.0%) of the healthy cats and three (20.0%) of the HCM cats. HCM allele frequencies did not differ significantly between 'healthy' and 'HCM' groups (p=0.64 for A31P; p=0.54 for A74T). NT-proBNP concentrations ranged between <24 pmol/l and 278 pmol/l (median 31 pmol/l) in healthy cats and ranged between <24 pmol/l and >1500 pmol/l (median 197 pmol/l) in affected cats. The concentrations were significantly higher in affected cats compared to healthy cats (p=0.008).

Conclusion: The value of genetic tests for detecting HCM is low in the cats of this study. The mutations analysed appear to have a low penetrance. NT-proBNP seems a promising cardiac biomarker in HCM, but more samples are needed.

Key words: Maine Coon cats; Genetic Tests; HCM; NT-proBNP.

Introduction

Hypertrophic cardiomyopathy (HCM) is the most common cardiomyopathy in cats, with a prevalence of 14.7% in a large population of apparently healthy cats (1,2). Many cats with HCM remain asymptomatic for prolonged periods of time. Clinical findings such as heart murmurs are sometimes difficult to hear and variably present(2,3). However, in some cases feline HCM is associated with considerable morbidity and mortality (3,4). HCM is characterised by a concentric hypertrophy of the left ventricle (LV) wall (either regional thickening or concentric hypertrophy), without signs of an underlying cause such as pressure overload (5).

In both cats and humans HCM has been shown to be a familial disease(6-8). Familial HCM in the Maine Coon cat has first been described in a large animal model where a research colony was formed and it was reported that HCM in the Maine Coon cat was inherited as an autosomal dominant trait with 100% penetrance (8). Later, A31P was identified in this colony as a causative mutation: a single base pair change (Guanine to Cytosine) in the myosin protein binding C (*MYBPC3*) gene,

leading to an altered protein conformation and function(9,10). The *MYBPC3* gene encodes for sarcomeric proteins and such a mutation within this gene may lead to the development of the HCM phenotype by affecting the protein conformation and function(9). Two years later, R820W was identified in Ragdoll cats with HCM: a single base change (Cytosine to Thymine) in the *MYBPC3* gene (11). A third single nucleotide polymorphism *(*SNP) in the *MYBPC3* gene, A74T (Guanine to Adenine), has been suggested as another causative mutation, although later disproven (12,13). The mutations appear to be breed specific and have not been described in other breeds of cats with HCM (14). Several studies have demonstrated that A31P is a common mutation in the Maine Coon breed, with prevalence estimates between 22% and 46% (10,12,14,15).

Breed screening is becoming increasingly popular in high-risk cat breeds such as the Maine Coon cat. Echocardiography is the gold standard in diagnosing the disease (3,5). However, this has its limitations: it enables only late state diagnosis and is ambiguous when mild disease is present, has a limited availability, is operator dependent, and costs are relatively high. Ideally, there would be a test that enables early diagnosis, is unambiguous, is easy to do, and is less costly. Blood-based testing for HCM is attractive, because of its quantitative measurement, minimal invasive nature, and widespread availability(16).

At first glance, genotyping would be the ideal screening test as a diagnosis of HCM could be made at a very young age, prior to breeding. Several commercial laboratories provide genetic tests for the above-mentioned mutations. However, the amount of studies about the relationship between genotype and the mutations are minimal and results so far suspect a low penetrance of the mutations analysed(12,13).

N-terminal pro-brain natriuretic peptide (NT-proBNP) measurement seems promising as a cardiac biomarker for HCM in cats. It has been shown to increase in cats with congestive heart failure as well as cats with HCM without heart failure(16,17). Even so, results so far are not unanimous. Whilst some studies show that it is only useful in detecting severe HCM, others show that it can also detect subclinical HCM (16-18).

Abbreviations:

2D	Two dimensional
DVLOTO	Dynamic left ventricular outflow tract obstruction
HCM	Hypertrophic cardiomyopathy
IVSd	End-diastolic thickness of the interventricular septum
LA	Left atrium
LPWVd	End-diastolic thickness of the left ventricular free wall
LV	Left ventricle
LVOT	Left ventricular outflow tract
MYBPC3	Myosin protein binding C
NT-proBNP	N-terminal pro-brain natriuretic peptide
SAM	Systolic anterior motion
SNP	Single nucleotide polymorphism

Therefore, the aim of this study was to fully phenotype Maine Coon cats from the Dutch population, and to evaluate the genotype-phenotype correlation between the *MYBPC3*-A31P mutation, the *MYBPC3*-A74T mutation, and the *MYBPC3*-R820W mutation and HCM in Maine Coon cats. We also aimed to measure NT-proBNP to determine the significance of this cardiac biomarker in the diagnosis of HCM and compare this to the use of echocardiography.

Methods

Animals and study design

Maine Coon cats living in the Netherlands with a pedigree were invited to participate in this prospective pilot study over a period of five months. A total of 33 owners responded to the invitation. Pedigrees were used to determine the origin of the cats. The cats were classified into the groups 'healthy', 'equivocal' or 'HCM', according to echocardiographic results.

Exclusion criteria consisted of Maine Coon cats with hyperthyroidism, Maine Coon cats with hypertension (≥150 mmHg) and Maine Coon cats with chronic kidney disease (IRIS Stage >II) (19,20). Cats in the control group had to be a minimum age of eight years, based on the fact that it is unlikely to still develop HCM at a later age (2,8).

Three cats had to be excluded from the echocardiographic results in this study, as they

were below the age of eight and proved healthy during echocardiography. Cats were considered genotype positive for the A31P, A74T or R820W mutation if at least one mutated allele was detected. Cats showing only the wild-type allele were considered genotype negative. A written informed consent was given to and signed by each owner prior to examination (Appendix I).

Clinical Examination

Bodyweight, sex, neuter status, and chip number were noted from each cat. A routine clinical examination of the cardiovascular system was performed at the University Clinic of Companion Animals of Utrecht University by one researcher (NH) and by a cardiologist (MD). Blood pressure measurement using a Doppler technique was done in all cats ≥8 years of age prior to the consecutive clinical examination. All findings were documented and any heart murmur auscultated, was graded on the Levine scale of I-VI and the point of maximum intensity was determined (21). Basal thyroid hormone was determined in all cats with a hypertrophied LV wall on echocardiography [a]. Blood samples were obtained by venepuncture and collected in glass tubes containing EDTA and glass tubes containing no anticoagulant for NT-proBNP measurement. Samples were centrifuged and supernatant was stored in -70 C˚ until shipment and batch analysis.

NT-proBNP

NT-proBNP values were determined using the Cardiopet proBNP test for all 33 cats by a commercial veterinary laboratory [b] (22). NT-proBNP measurement of ≥ 100 pmol/l was classified as elevated.

Echocardiography

All cats underwent two dimensional (2D) and M-mode echocardiography using GE Logiq 8 (General Electric systems), a transducer with nominal frequency range 4 to 12 MHz with harmonic imaging and simultaneous ECG recording. Echocardiographic examinations were performed by one single board certified cardiologist (MD), who was unaware of the disease status of the cat. The cats were unsedated and gently restrained in right and left lateral recumbency. Standard right and left parasternal long axis and short axis views were used. M-mode was used in order to get a good assessment and measurement of the LV wall. However, in order to diminish the chance of underestimation of wall thickness by missing a regional hypertrophy, 2-D mode was used to measure regional areas of hypertrophy. A right parasternal short axis view was used to measure the diameter of the left atrium (LA) and aorta (Ao) and LA enlargement was considered when the LA/Ao ratio >1.5. The diagnosis of HCM was based on the measurement of maximal end-diastolic wall thickness. Measurements were taken as an average of three cardiac cycles, with ECG monitoring for timing of the measurement in the cardiac cycle. Measurement of the septal wall was done using the leading-edge-to-leading-edge technique and thereby using the same convention as used for M-mode. Tissue Doppler echocardiography was used to measure the velocity in the myocardium during systole and diastole. Colour flow Doppler was used to detect turbulent blood flow and cats with turbulent blood flow in the LV were evaluated for Systolic Anterior Motion (SAM) of the anterior septal mitral valve leaflet. Continuous wave and pulsed wave Doppler was used to determine the maximal systolic velocity of the aortic flow and early (E) and late (A) velocities of the mitral inflow. Definite diagnosis of HCM was based on echocardiographic view of a hypertrophied LV, where a region of the LV wall or the entire wall was hypertrophied and hyperthyroidism and hypertension were excluded. Cats with a body weight ≤5 kg with a LV diameter ≥5.0 mm were classified as HCM positive. Cats with a body weight >5 - <8 kg with a LV wall diameter between 5 – 6 mm were classified as equivocal. Cats with a bodyweight > 8 kg with a LV wall diameter of < 5.5 mm were classified as healthy and with a wall diameter ≥6 mm were classified as HCM(3,23-25).

Genotyping

All 30 cats were genotyped, as well as 14 Maine Coon cats from our DNA database that were previously classified as either healthy or HCM according to the same criteria we used.

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[a] University Veterinary Diagnostics Laboratory, Yalelaan 104, 3584 CM, Utrecht, The Netherlands.

[b] IDEXX laboratories, Mörikestraße 28/3, 71636 Ludwigsburg, Germany.

DNA was extracted from EDTA-stabilised blood samples with the MagCore HF16 Plus [c] according to the manufacturer's instructions. The quantity of the DNA was assessed by photometric measurement. Standard PCR amplification was carried out using a Platinum Taq PCR Master Mix for the A31P and A74T SNPs, with 35 cycles on a ABI9700 thermocycler (Applied Biosystems), using 55 C˚ as an annealing temperature. The forward primer used was 5' AATTGCATCTGTCTCATAGACC 3' and the reversed primer used was 3' CCAAAGCAAAGGCGAGACAG 5'. PCR amplification for R820W was carried out using the Q5HotStart PCR MasterMix, with 35 cycles on a ABI9700 thermocycler, using 55 C˚ as an annealing temperature. The forward primer used was 5' CAGCAATGTGGGTGAGGAC 3' and the reversed primer used was 3' CTGACCAGGGAGGGTGTG 5'. The PCR products were visualised by gel electrophoresis and sequenced using BigDye technology on a 3500xl Genetic Analyzer, using the forward primers.

Statistics

Means are presented as mean ± 1 SD and p-values of <0.05 were considered statistically significant. Skewed variables are presented as a median and range. Tests of normality were done using the Shapiro-Wilk *W* test. Differences in magnitude means were compared using a one-way ANOVA analysis and significant outcomes were confirmed using a Bonferroni post hoc test. Differences in parameter medians were compared using the Kruskal-Wallis H test and confirmed using Mann-Whitney U tests. The Fisher's exact test was used to compare allele frequencies of the phenotype groups 'HCM' and 'healthy'. The validity of the genetic tests was evaluated by calculating the sensitivity and specificity. A receiver operating characteristic curve (ROC) was derived for NT-proBNP and the area under the curve (AUC) was calculated. ROC analysis was used to determine the optimal cut-off value to distinguish healthy cats from cats with HCM. Echocardiographic 2-D measurements were used in the statistical analysis if they exceeded the M-mode measurements. Commercially available software programs were used for statistical analysis (SPSS Statistics version 25 and RStudio version 1.1.463).

Results

Clinical Findings

Four of the cats from our study population (n=30) were already known with HCM (3 males, 1 female). All cats were asymptomatic and none of the cats from our study population had any history with or signs of other systemic diseases. Of the 30 cats, 18 were female (17 neutered) and 12 were male (11 neutered). The age of the cats varied between 3 years and 15 years of age (mean 9.3 ± 3.1 years of age). The bodyweight in the female cats varied between 3.9 and 8.5 kilograms (mean 6.2 ± 1.6 kilograms) and of the male cats between 5.1 and 10.3 kilograms (mean 7.4 ± 1.5 kilograms).

One of the 22 cats that presented as healthy (4.5%) and three of the eight cats (37.5%) that presented with HCM on echocardiographic examination had a systolic heart murmur on clinical examination, all of intensity II/VI with the point of maximum intensity left parasternal. One cat (male, 8 years of age) presented with an irregular heart rhythm.

Echocardiographic results

According to the criteria specified above, eight Maine Coon cats (26.7%; 5 males, 3 females) were diagnosed with HCM (Table 1). The age at the time of diagnosis varied between 2 years and 11 years of age (median 8 years of age). Three cats (1 male, 3 years of age, and 2 females, 10 and 13 years of age) had an asymmetrical hypertrophy of the LV wall. The male cat had an end-diastolic thickness of the left ventricular free wall (LPVWd) of 7 mm and an end-diastolic thickness of the interventricular septum (IVSd) of 6 mm and was one of the cats that presented with a heart murmur that was, on echocardiographic examination, caused by SAM of the mitral valve causing a dynamic left ventricular outflow tract obstruction (DLVOTO) plus moderate mitral regurgitation.

[c] LAB MARK, Pod Cihelnou 23, 161 00 Praha 6-Ruznyě, Czech republic.

[d] Eurogenerics, Esplanade Heysel 22/B, 1020 Brussel, Belgium.

Variable	HCM (n=8)	Equivocal (n=3)	Healthy (n=19)	*p*-Value
Age (years)	8 (2-11) [a]	8 (3-11)	10 (8-13)	0.010
Body weight (kg)	6.8 ± 1.5	7.2 ± 0.2	6.0 ± 1.7	0.398
IVSd (mm)	6.5 (6.0-7.6)	5.2 (5.2-5.5)	4.4 (3.5-5.4)	0.010
LVIDd (mm)	16.8 ± 2.1	16.8 ± 1.2	16.2 ± 2.4	0.818
LPVWd (mm)	6.9 (4.9-8.4)	5.3 (5.2-5.4)	4.3 (3.7-5.3)	<0.001
IVSs (mm)	9.6 ± 1.1	7.4 ± 0.5	7.4 ± 1.4	0.090
LPVWs (mm)	10.0 ± 1.6	9.6 ± 0.5	8.0 ± 1.0	0.059
FS (%)	46 ± 14	51 ± 5	53 ± 13	0.911
AO 2D (mm)	12.1 ± 2.0	9.9 ± 0.6	10.9 ± 1.3	0.517
LA 2D (mm)	14.4 ± 2.5	15.0 ± 2.4	13.2 ± 1.5	0.123
LA/AO 2D	1.1 (1.0-1.7)	1.4 (1.4-1.7)	1.2 (1.1-1.5)	<0.001
AO/LA 2D	0.9 (0.6-1.0)	0.7 (0.6-0.7)	0.8 (0.7-0.9)	0.001

Table 1 Overview of echocardiographic variables per group.
Normally distributed variables are presented as mean ± 1 standard deviation and skewed variables are presented as median (range). IVSd = end-diastolic thickness of the interventricular septum, LVIDd= left ventricular internal diameter, LPVWd = end-diastolic thickness of the left ventricular free wall, IVSs = end-systolic thickness of the interventricular septum, LPVWs = end-systolic thickness of the left ventricular free wall.
[a] Age at the time of diagnosis.

He had been treated with atenolol (q12h 6.25 mg) since the murmur was first detected by its own veterinarian. The female cat of 10 years of age had a local hypertrophy of the IVSd (7.5 mm) at the left ventricular outflow tract (LVOT). The female cat of 13 years of age had a local hypertrophy of the IVSd (6.1 mm), a moderately dilated left atrium and a heart murmur that was, on echocardiographic examination, caused by turbulence in the LV. This cat was diagnosed with HCM at the age of 2. The other six cats were diagnosed with symmetrical HCM (4 males, 10 and 10 and 11 and 15 years of age, and 2 females 9 and 10 years of age). The male cat of 10 years of age also had a heart murmur that was, on echocardiographic examination, caused by turbulence in the LV and was diagnosed with HCM at the age of 8. The male cat of 15 years of age had a moderately dilated left atrium and moderate mitral regurgitation. This cat was diagnosed with HCM at the age of five.

Three cats (10%) (1 female, 11 years of age, and 2 males, 3 and 8 years of age) had a wall dimension greater than 5 mm but less than 6 mm and were classified as equivocal according to the criteria specified above (Table 1). The body weight of the female cat was 7.1 kg and of the two male cats respectively 7.4 and 7 kg. The male cat of 8 years of age was diagnosed with HCM by its own veterinarian at the age of three. It had an irregular heart rhythm and lead II electrocardiography during echocardiography showed atrial fibrillation with a right ventricular branch block. The LA was dilated and the right ventricle and right atrium were also moderately dilated. It had a moderate tricuspid regurgitation and slight mitral valve regurgitation. This cat had been treated with atenolol (q24h 12.5 mg) since its diagnosis.

The other 19 cats (63.3%) were classified as healthy and had an IVSd that ranged from 3.5 to 5.4 mm (median 4.4 mm) and an LPVWd that ranged from 3.7 to 5.3 mm (median 4.3 mm)(Table 1). One of the healthy cats (female, 11 years of age) presented with a systolic heart murmur, but no cause was found during echocardiography.

Two of the healthy cats, (males; 9 and 8 years of age) had wall dimensions greater than 5 mm and were classified as healthy because they had a bodyweight of 8 and 8.7 kg respectively. They had an IVSd of 4.9 and 5.4 mm and an LPVWd of 5.1 and 5.3 mm respectively.

The IVSs, IVSd, LPVWs, and LPVWd were significantly higher in cats with HCM (p<0.001) compared to healthy cats. When the cats that were classified as equivocal were compared to the healthy cats, the IVSd and LPVWd were also significantly higher (p=0.009 and p=0.008 respectively). The LPVWd was also significantly correlated with bodyweight (p=0.009).

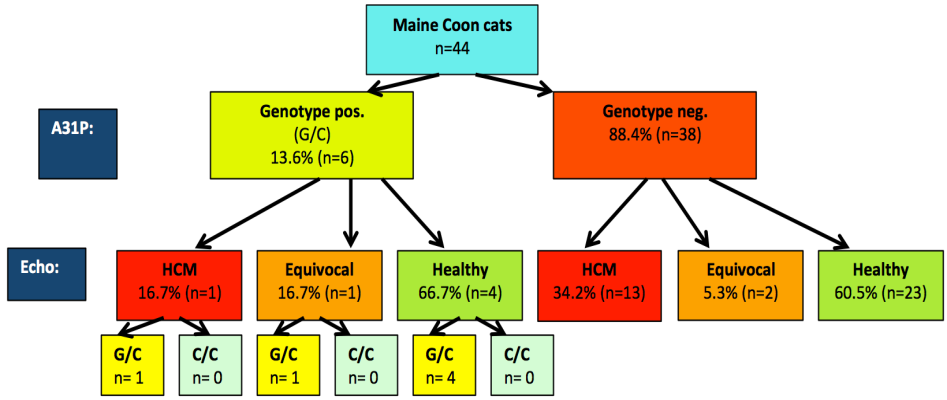

Figure 1 Genotype results and phenotype classifications of the A31P SNP in Maine Coon cats. G/C represents the heterozygous variant and C/C represents the homozygous variant.

The phenotype-genotype correlation

A total of 44 samples were genotyped. The prevalence of HCM in these cats was 31.8 % (14/44).

All 44 samples (100%) sequenced for R820W were of the wild type (C/C) variant.

A31P was found in 6 of the 44 samples (13.6%), but only as a heterozygous G/C variant (Figure 1). Four of the genotype-positive cats were classified as healthy (66.7%), one was classified as equivocal (16.7%) and one was classified as HCM (16.7%) on echocardiographic examination. Of the 44 cats, 38 cats were genotype-negative (86.4%). Of those 38 cats, thirteen were classified as HCM (34.2%) and two as equivocal (5.3%). The minor allele frequency was 0.06 for the C-allele.

The A74T SNP was found in 15 of the 44 samples (34.1%)(Figure 2). Three of the genotype-positive cats were classified as HCM (20.0%) and twelve as healthy (80.0%). Two samples (4.5%) were of the homozygous variant A/A for the A74T SNP, of which one was classified as HCM (50.0%). The other thirteen samples (29.5%) were of the heterozygous variant G/A, two of which were classified as HCM (15.4%). Of the 44 cats, 29 cats were genotype-negative (65.9%). Of those 29 cats, eleven were classified as HCM (37.9%) and three as equivocal (10.3%). The minor allele frequency was 0.23 for the A-allele. None of the cats heterozygous for A31P, were hetero- or homozygous for the A74T SNP and vice versa. There was no statistically significant difference in allele frequencies between cats with HCM and healthy cats for both SNPs (p=0.64 for A31P; p=0.54 for A74T). The sensitivity was very low (5% for A31P; 32% for A74T) for both genetic tests in the study population (Tables 2 and 3).

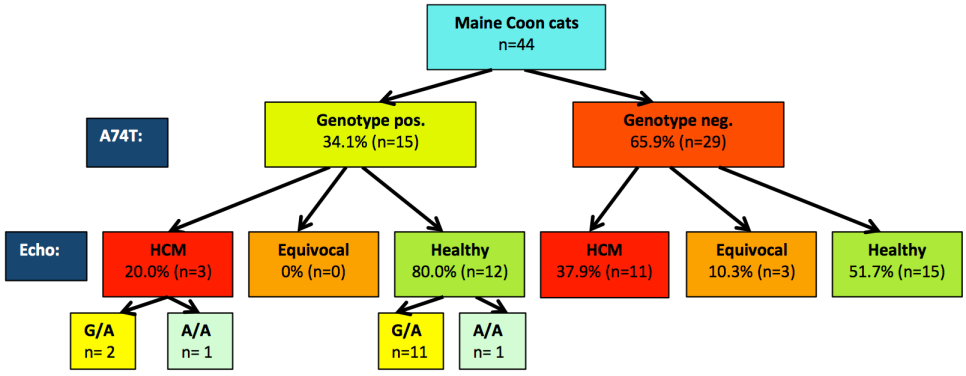

Figure 2 Genotype results and phenotype classifications of the A74T SNP in Maine Coon cats (n=44). G/A represents the heterozygous variant and A/A represents the homozygous variant.

		Genotype positive (=G/C)
A31P		95% CI
Sensitivity	0.05	0.01-0.26
Specificity	0.85	0.66-0.96

Table 2 Sensitivity and specificity of A31P SNP in cats with HCM. 95% CI = 95% Confidence Interval.

	Genotype positive = G/A + A/A		Genotype positive = A/A
A74T		95% CI	95% CI
Sensitivity	0.21	0.05-0.51	0.02-0.34
Specificity	0.56	0.35-0.75	0.81-0.99

Table 3 Sensitivity and specificity of A74T SNP in cats with HCM. On the left genotype positive for G/A and A/A and on the right genotype positive for A/A. 95% CI = 95% Confidence Interval.

NT-proBNP measurement

NT-proBNP was measured of the 30 cats that participated in the study as well as the three cats that had to be excluded from the echocardiographic, clinical en genotyping results because they were below the age of eight and proved healthy during echocardiography.

Measurements of the cats with HCM (n=8) varied between <24 pmol/l and >1500 pmol/l (Figure 3). Six of the cats had values that all exceeded the reference range of 100 pmol/l and varied between 117 pmol/l and >1500 pmol/l (median 197 pmol/l). The two outliers (female, 10 years of age, and female, 13 years of age) both had NT-proBNP values <24 pmol/l. They both had an asymmetrical hypertrophy of the LV and an IVSd of 7.5 and 6.1 mm and an LPVWd of 5.1 and 4.9 mm respectively.

The three cats (2 males, 3 and 8 years of age, and 1 female, 11 years of age) that were diagnosed as equivocal had NT-proBNP values of <24 pmol/l, 287 pmol/l and 38 pmol/l respectively.

NT-proBNP measurement of the healthy cats (n=22) ranged from <24 pmol/l to 278 pmol/l. Twenty cats had NT-proBNP values within the reference range of <100 pmol/l and these values ranged from <24 pmol/l to 93 pmol/l (median 31 pmol/l). The two outliers (male, 13 years of age, and female, 9 years of age) had NT-proBNP values of 278 pmol/l and 212 pmol/l respectively. They had an IVSd of 4.4 and 4.1 mm and an LPVWd of 4.1 and 4.2 mm respectively. The three cats that were below the age of eight and proved healthy during echocardiography (females, 1, 4, and 7 years of age) had NT-proBNP values of respectively <24 pmol/l, 80 pmol/l and <24 pmol/l.

Sensitivity and specificity of NT-proBNP measurement as a screening test for HCM was calculated using ROC analysis (Figure 3). The AUC of the ROC-plot was .735. Using a cut-off value of >50 pmol/l, sensitivity was 75% (95% CI: 0.35-0.97) and specificity was 59.09% (95% CI: 0.37-0.79). Using a cut-off value of >100 pmol/l, sensitivity was 75% (95% CI: 0.35-0.97) and specificity was 91% (95% CI: 0.71-0.91) (Table 4).

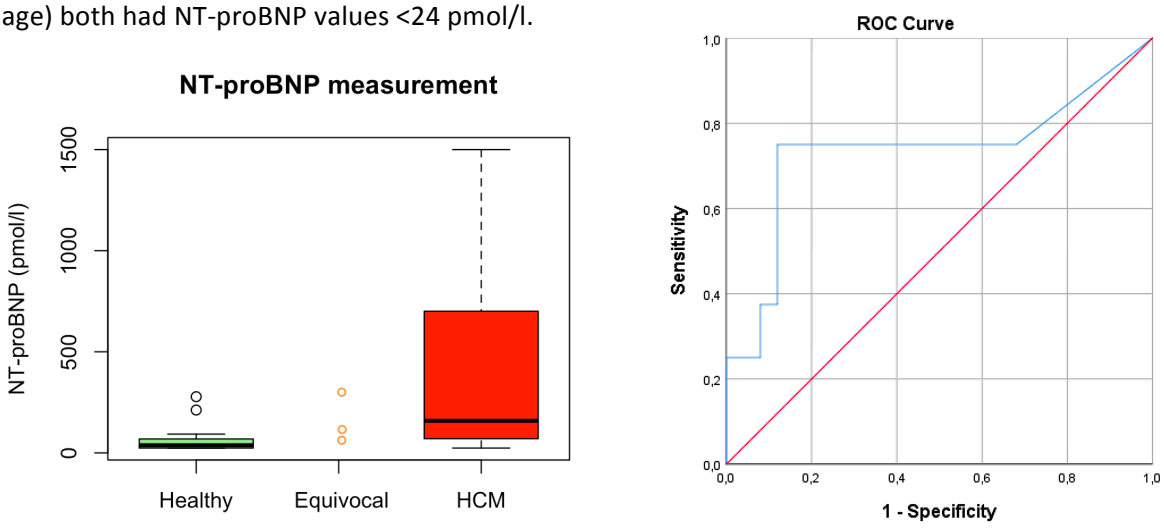

Figure 3. Left: Boxplot of plasma N-terminal pro-brain natriuretic peptide (NT-proBNP) concentrations of 33 cats. Boxes represent the interquartile range, the whiskers indicate the range and the horizontal line in the middle of the boxes represent the median. Black circles indicate the outliers in the healthy group. Orange circles represent the tree equivocal cats. **Right:** Receiver operating characteristic (ROC) curve illustrating the sensitivity and specificity of NT-proBNP measurement in distinguishing cats with HCM from healthy cats. The area under the ROC is 0.735.

NT-proBNP		95% CI
Sensitivity	0.75	0.35-0.97
Specificity	0.91	0.71-0.99

Table 4 Sensitivity and specificity of NT-proBNP measurement in cats with HCM. 95% = 95% Confidence Interval.

NT-proBNP values were significantly elevated in cats with HCM compared to healthy cats (p=0.008). NT-proBNP values were not significantly correlated with any of the mutations.

Discussion

In this study, we aimed to evaluate the genotype-phenotype correlation between the genetic mutations in feline *MYBPC3* at loci A31P, A74T, and R820W and HCM in Maine Coon cats in The Netherlands. We also aimed to determine the significance of NT-proBNP in the diagnosis of HCM and compare this to the use of echocardiography. The tested population consisted of 30 Maine Coon cats, of which eight cats (26.7%) had HCM. Three other Maine Coon cats had to be excluded from the study, but were included in the NT-proBNP results, resulting in 33 Maine Coon cats. Besides the 30 Maine Coon cats from the tested population, 14 other Maine Coon cats from our DNA database were genotyped. Even though there are several studies describing the genotype-phenotype correlation of HCM in the Maine Coon breed, this is to our knowledge the first study describing the genotype-phenotype correlation of a Dutch Maine Coon population and combining it with NT-proBNP measurements.

The main finding of this study was that there was no statistically significant correlation between a mutation in the *MYBPC3* gene and HCM (p=0.64 for A31P; p=0.54 for A74T) in our study group. Furthermore, NT-proBNP seems promising as a cardiac biomarker to exclude HCM in healthy individuals, with a high specificity of 91% and a sensitivity of 75% (p=0.008).

Males were overrepresented in this study (62.5%), similar to previously published male predilection rates of between 63-79% (2,4,8,15,26-29). All of the cats in this study were asymptomatic at the time of diagnosis. This is in contrast to previous reported numbers of 77%, 47%, and 33% in the reports by Trehiou-Sechi *et al.*, Payne *et al.* and Rush *et al.* respectively(2,4,29). This discrepancy may be explained by the specific recruitment of Maine Coon cats for this study. Owners of symptomatic cats with HCM were most likely reluctant to travel to our clinic for research purposes.

Most cats with HCM in the present study were middle-aged animals (mean age 9.9 years of age, mean age at the time of diagnosis 8 years of age), confirming previously reported results(2,4,29). Also, the age range in this study was wide and thus highlighted HCM in very young (2 years old) and older (11 years old) cats, as also previously described(2,4,29). Three cats in this study with HCM presented with a heart murmur (37.5%). This number is somewhat higher compared to the previously published CatScan study by Payne *et al.*, where only 9.9% of the young adult cats and only 29.4% of the senior cats with HCM presented with a heart murmur(2). However, our number of cats with HCM (n=8) is too low to draw conclusions regarding heart murmurs and HCM.

The R820W mutation was not found in any of the cats. Previously, it has already been described that this mutation is breed specific so it was expected that we would not find this SNP in our tested population (13,14). We did not find any correlation between the heterozygous mutated A31P Maine Coon cats and HCM. Only one Maine Coon cat with HCM was heterozygous for the SNP, the other thirteen Maine Coon cats with HCM had a wild-type variant. This finding is supported by previous studies that have already reported an incomplete penetrance for heterozygotes, at least at young age, and no association between a heterozygous mutation and a significant odds ratio for HCM (10,13). These and other studies have also reported a correlation between a homozygous genotypic variant of A31P and the risk of development of HCM (10,13,15,26,30). We cannot support this correlation, because we have not found any Maine Coon cat with a homozygous genotypic variant. What we can say however is that the allele frequency between affected and healthy cats was not significantly different. The lack of homozygous cats may be explained by the small sample size of affected

Maine Coon cats (n=14) in this study, although previous studies that did report a correlation between homozygotes and HCM also had relatively small sample sizes of affected Maine Coon cats (n=18; n=12 respectively) (10,15). Similar to other studies, this study has also shown that the disease occurs quite frequently in cats with a wild-type genotype, making it likely that at least one more mutation associated with HCM in the breed is likely to occur (10,12,15,26).

The A74T SNP of the homozygous-genotype was found in two cats and the heterozygous genotype was found in thirteen cats. However, this was not significantly correlated with HCM, strongly suggesting that it is a polymorphism, not a causative mutation. This has also been described previously (12,15). None of the evaluated genetic tests were able to provide useful predictive information of disease outcome, as has also been reported by Wess *et al.* (12).

NT-proBNP values were determined for the 30 cats in our study as well as three cats that were excluded from our study due to being classified as healthy and being below the age of eight, thus still risking developing HCM (2,8). However, for our purpose of validating NT-proBNP as an early biomarker for detecting HCM, these three cats were of value because they might have the underlying disease and not yet express this on echocardiography. We used a cut-off value of >100 pmol/l, because this has the highest specificity and a similar sensitivity compared to a cut-off value of >50 pmol/l (sensitivity of 75.0% and 75.0%; specificity of 91.0% and 59.09% respectively). This cut-off value of >100 pmol/l has also been used in the study by Wess *et al.* (>50 pmol/l: sensitivity of 97.8%, specificity of 66.7%; >100 pmol/l: sensitivity of 92.4%, specificity of 93.9%)(16). Our results are comparable to those of Wess *et al.:* NT-proBNP values of cats with HCM are significantly elevated compared to healthy cats (p=0.008) and especially the specificity is high (91%). These results are in contrast to previous studies, where NT-proBNP measurement failed to identify subclinical HCM and was only helpful in detecting severe HCM (LV wall thickness >7 mm in the study of Hsu *et al.*) (17,18). The study of Hsu *et al.* used a different assay compared to our study and the study of Wess *et al.* and used a research colony from the University of California, Davis. This research colony may not have represented the Maine Coon population.

We did however find four outliers, where NT-proBNP values were inconsistent with the phenotype on echocardiography. Two cats had NT-proBNP values below the reference range (both <24 pmol/l) and were classified as HCM and two cats had elevated NT-proBNP values (278 pmol/l and 212 pmol/l) and were classified as healthy. One possible explanation for the outliers with HCM and NT-proBNP values below reference is that NT-proBNP is indeed only useful in detecting severe HCM as suggested in previous studies (17,18). Even so, this will only explain the low values, not the high values. A second explanation could be that the samples have been mixed-up. However, samples were stored in batches from each screening day and none of the cats screened on the same day as the outliers with lower and higher values than expected, had values >100 pmol/l whilst being classified as healthy and values <100 pmol/l whilst being classified as having HCM respectively. Even so, one cat classified as equivocal with an NT-proBNP value <24 pmol/l was screened on the same day as the cat classified as healthy with an NT-proBNP value of 212 pmol/l. It cannot be excluded that these two samples have been mixed up. Retesting of these cats could either confirm or reject this hypothesis. A third possible explanation might be that the assigning of a phenotype according to echocardiography was not done accurately. However, IVSd measurements of the cats with HCM were rather convincing (7.5 and 6.1 mm). Even so, it should be noted that both cats had asymmetrical hypertrophy of the LV. The cats classified as healthy had IVSd and LPVWd measurements <4.4 mm and so were also rather convincing. A fourth explanation might be that the NT-proBNP test is not yet optimised. However, we retested two of the outliers; one cat with HCM and a value <24 pmol/l and one healthy cat with a value of 278 pmol/l and retesting gave almost similar results (<24 pmol/l and 261 pmol/l respectively). A fifth explanation might be that the samples were not stored correctly. The samples were stored in -70 C˚ until batch analysis. Samples were shipped on

dry ice in contrast to the study by Wess *et al.*, where NT-proBNP analysis was performed on site (12). However, all samples were shipped at once, so this cannot explain the four outliers. None of the cats had hypertension, so this cannot be the cause of the elevated values either (31). Hyperthyroidism is not excluded in the healthy cats, as we only tested in the cats with HCM. Renal failure is also not excluded, although both healthy cats had no signs of disease at the clinical examination. One of the healthy cats with the elevated NT-proBNP value of 278 pmol/l died however two weeks after the clinical examination due to unknown cause.

A limitation to this study is the relatively small sample size. Another limitation of this study was that the control group might have had underlying disease or myocardial damage at cellular level, which we did not detect by echocardiography. Additionally, we did not exclude hyperthyroidism in the healthy cats. Another limitation is that this study is not a longitudinal study and thus the progression of the disease and NT-proBNP values will remain unknown. The results have been valuable in providing insights in the diagnostic tools we aimed to study but our results are only preliminary data and should be verified by large studies.

Conclusion

In conclusion, the genetic tests for HCM are not useful in detecting HCM. NT-proBNP seems promising, but the amount of outliers is too high in our study and more cases need to be tested. For now, echocardiography remains the gold standard in diagnosing HCM when performed by a skilled cardiologist. Future aims are to increase our sample size, look for other possible biomarkers and sequence twelve of the cats with HCM without the A31P mutation in order to find another causative mutation.

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Appendix I

Universiteit Utrecht: onderzoek Hypertrofische Cardiomyopathie Maine Coon
Toestemmingsverklaring

Datum: _____

Gegevens kat

Label

Onderzoek

Hypertrofische Cardiomyopathie (HCM) is een veel voorkomende hartspierziekte bij de Maine Coon. Er is reeds een genetische test beschikbaar voor de Maine Coon waarmee getest kan worden of uw Maine Coon een mutatie heeft die HCM veroorzaakt. Echter zijn er ook Maine Coons met HCM maar zonder deze mutatie en Maine Coons met de mutatie maar zonder HCM. Dit onderzoek heeft als doel te onderzoeken hoe vaak HCM voorkomt binnen de Nederlandse populatie Maine Coons en hoeveel van deze Maine Coons de bekende mutatie hebben, welke bevindingen in het bloed en via een echo passen bij Maine Coons met HCM, en om wellicht een andere mutatie te vinden die ook HCM veroorzaakt bij de Maine Coon.

Wat houdt het meedoen aan deze studie in voor u en uw kat? Uw kat zal een keer grondig lichamelijk onderzocht worden. Daarnaast wordt een kleine hoeveelheid bloed afgenomen om te kijken of er in het bloed aanwijzingen zijn voor een hartziekte en om het DNA van uw kat te verkrijgen. Tot slot wordt er een echo van het hart gemaakt door drs. Mark Dirven, cardioloog. Hiervoor zal er een klein beetje vacht weggeschoren worden aan beide zijden van de borstholte. Tijdens de echo zal er tevens wat urine worden afgenomen. Mochten er tijdens één van de onderzoeken aanwijzingen gevonden worden voor een hartziekte bij uw kat, dan wordt u hier uiteraard over geïnformeerd. Aan dit onderzoek zitten voor u geen kosten verbonden. De persoonsgegevens van u of uw kat zullen niet met derden worden gedeeld zonder uw toestemming.

Hierbij verklaar ik dat:

- Ik de eigenaar ben van bovengenoemde kat.
- De onderzoeker me het doel en het verloop van het onderzoek heeft uitgelegd.
- Ik begrijp dat er in dit onderzoek gekeken wordt naar HCM bij de Maine Coon, zodat hier meer over bekend zal worden.
- Ik erin toestem dat er bij mijn kat aan beide zijden van de borstholte een kleine plek kaal geschoren wordt om de hartecho uit te kunnen voeren.
- Ik erin toestem dat er een kleine hoeveelheid bloed en urine zal worden afgenomen ten behoeve van het onderzoek.
- Ik erin toestem dat het DNA van mijn kat zal worden verkregen en gebruikt mag worden in onderzoek naar erfelijke gebreken.
- Ik de onderzoekers toestemming verleen om alle medische gegevens van mijn kat ten behoeve van dit onderzoek in te zien, op te slaan en te gebruiken voor het onderzoek.
- Ik begrijp dat alle gegevens van mij en mijn kat enkel geanonimiseerd worden gebruikt.

- Ik begrijp dat ik door ondertekening toestemming verleen om mijn kat deel te laten nemen aan dit onderzoek, maar ook dat ik te allen tijde het recht heb om mijn toestemming in te trekken.

Handtekening eigenaar

_____

Handtekening onderzoeker

_____