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Anatomopathological staging of feline hypertrophic cardiomyopathy through quantitative evaluation based on morphometric and histopathological data.

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Abstract

Diagnosis of feline hypertrophic cardiomyopathy (HCM) is both clinical and anatomopathological. Since standardized echocardiographic parameters have previously been established for its diagnosis and classification, the aim of the present study is to provide an original, complete and repeatable quantitative anatomopathological evaluation of this myocardial disease. Since ES-HCM is a clearly defined clinicopathological entity of feline HCM, the present study also aims to investigate its temporal evolution. The hearts of 21 cats with previous diagnosis or suspicion of HCM and 6 control animals were submitted for morphometric and histopathological investigations. The proposed quantitative assessment of gross and histopathological features of HCM appears to be original and repeatable. Correlations between morphometric data allow to establish that the progression to the end-stage phenotypes, primarily characterized by increase in left ventricular fibrous tissue deposition, is accompanied by dilation of left ventricular lumen (P=0.0004) and left atrium (P=0.0017) and increase in intramural coronary arteriosclerosis (P=0.0293).

Keywords: feline, heart, myocardial disease, ventricular hypertrophy
Feline hypertrophic cardiomyopathy (HCM) is a primary myocardial disease characterized by increased cardiac mass associated with a non-dilated and hypertrophic left ventricle (Fox, 2003). It represents the most common myocardial disease in cats (Ferasin et al., 2003; Riesen et al., 2007). Idiopathic HCM occurs in the absence of other diseases potentially contributing to or producing cardiac abnormalities such as hypertension, hyperthyroidism, mitral dysplasia and acromegaly (Ferasin, 2012). Male and middle-aged cats are the most affected patients (Migliorini et al., 2012), with a breed preference for Maine Coon, Ragdoll, Persian, British Shorthair (Migliorini et al., 2012) and Domestic Shorthair (Smith et Dukes-McEvan, 2012). A genetic predisposition has been demonstrated in Maine Coons and Ragdolls only by identification of a causative mutation in the sarcomeric gene for the cardiac myosin binding protein C (MYBPC3) (Meurs et al., 2005; Meurs et al., 2007). A wide phenotypic variability suggests the usefulness of gross examination, since HCM can cause diffuse, asymmetrical or segmental patterns of left ventricular hypertrophy (Fox, 2003). Furthermore, the left ventricular cavity is reduced, papillary muscles are hypertrophied and left atrium is moderately to severely dilated. Pulmonary edema and arterial thromboembolism (ATE) can also occur in a varying percentage of cases. Myofiber disorientation (“disarray”) is considered a distinctive histopathological finding in HCM. It appears in the form of peculiar and disorganized cellular architecture. Additional common findings are intramural coronary arteriosclerosis and increased interstitial matrix or myocardial replacement fibrosis (Fox, 2003). End-stage hypertrophic cardiomyopathy (ES-HCM) is the dilated phase of HCM. It is characterized by relative dilation of the ventricular chambers, relative thinning of the ventricular walls, marked left atrial dilation and variable myocardial scarring, with consequent decreased ventricular contractility often closely followed by congestive heart failure (Baty et al., 2001; Cesta et al., 2005).
Echocardiography is the standard method to diagnose and classify feline HCM. In minimal HCM, the thicknesses of the left ventricular free wall and/or the interventricular septum range between 6.0 and 6.5 mm and LA/AO (Left Atrial/Aortic Root ratio) is less than 1.5. In mild patterns, the thicknesses of the left ventricular free wall and/or the interventricular septum range between 6.5 and 7.0 mm and LA/AO is less than 1.8. Finally, in severe HCM the thicknesses of the left ventricular free wall and/or the interventricular septum are greater than 7.0 mm and LA/AO is greater than 1.8 (Migliorini et al., 2012).

Contrary to the standardized echocardiographic guidelines used for feline HCM, in veterinary pathology the quantitative parameters which allow the characterization of this myocardial disease are limited. Similarly to the quantitative assessment of left ventricular hypertrophy (Kershaw et al., 2012), myofiber disarray (Kershaw et al., 2012) and myocardial fibrosis (Kershaw et al., 2012; Khor et al., 2014) proposed for feline HCM, the aim of the present study is to provide an original, complete and repeatable quantitative anatomopathological evaluation of feline HCM features based on morphometric and histopathological data. Since ES-HCM is a clearly defined clinicopathological entity of this myocardial disease (Baty et al., 2001; Cesta et al., 2005), the present study also evaluates the possibility of staging its temporal evolution by a morphological differentiation of the phenotypes in the initial/middle phase of the pathology from the end-stage ones based on anatomopathological findings only.

Material and methods

Animals

Twenty-seven cats were included in the present study. Twenty-one animals showed acute onset or chronic development of cardiac disorders, whereas the remaining six had no heart diseases and were used as controls. After spontaneous death or euthanasia, all cats were referred to the Department of Veterinary Science of University of Turin for postmortem examination between 2010 and 2014. A
diagnosis of HCM was made by a combination of anamnesis, clinical signs, radiographic and echocardiographic findings and gross and histological features previously reported by Fox (Fox, 2003). Cases in which clinicopathological investigations indicated an underlying cause of left ventricular hypertrophy, such as systemic hypertension, hyperthyroidism, subaortic stenosis, mitral dysplasia and acromegaly, were excluded (Ferasin, 2012). Since blood pressure measurements were not available, systemic hypertension was ruled out on the basis of post mortem findings. Target organ damages, such as retinopathy/choroidopathy, hypertensive encephalopathy or nephrosclerosis and medial thickening of renal arterioles (Japsen, 2011), were not detected during gross and histopathological examination. Cats also showed no chronic kidney disease, which is the most common cause of feline systemic hypertension (Japsen, 2011). Since idiopathic hypertension occurs in the minority of hypertensive cats (Japsen, 2011), it was considered unlikely and consequently ruled out. Hyperthyroidism was excluded because there was no clinical suspicion and no thyroid lesions were identified by gross and histopathological examination (Peterson, 2014). Finally, acromegaly was ruled out because there was no clinical suspicion and no skeletal abnormalities and pituitary adenomas were detected at post mortem examination (Greco, 2012).

**Gross and histopathological examination**

A complete necropsy was performed in all the 27 cats. All thoracic and abdominal organs were collected and fixed in 10% buffered formalin solution. The fixed heart was externally examined and opened following the inflow and outflow tracts after transverse sectioning at the level of the middle third of the ventricles in order to evaluate all the cardiac structures. Heart transverse sectioning was chosen according to previous anatomopathological studies (Liu et al., 1981; Cesta et al., 2005; Kershaw et al., 2012; Khor et al., 2014). Transverse sections of myocardium involving the left ventricular (LV) free wall, the interventricular septum and the right ventricular (RV) free wall and other organs samples were routinely embedded in paraffin wax blocks. Sections were cut at 5 µm thickness, mounted on glass slides, stained with Haematoxylin & Eosin (HE) and Masson’s
Trichrome (MT) and examined by light microscopy. The transverse myocardial section corresponding to the cut surface was examined with an optical microscope provided with an “Object Marker”. Areas of disarray within the LV myocardium were marked at 100x magnification and successively counted. On the same transverse myocardial section, pathological intramural coronary arteries of the entire LV myocardium, characterized by medial and intimal thickening and narrowed lumen, were identified at 200x magnification and manually counted. Both qualitative and quantitative evaluation of disarray and intramural coronary arteriosclerosis were double-blind assessed by two authors (IB and MT). On the transverse myocardial section stained with MT the presence of interstitial, replacement and endocardial fibrosis of the left ventricle was also evaluated.

**Morphometric analysis**

Morphometric analysis using the Image®-Pro Plus software was used to evaluate the thicknesses of the LV free wall and the interventricular septum, the surface area of the LV lumen and left auricle and the percentage of LV fibrous tissue. The thickness of the LV free wall and of the interventricular septum were measured by applying the “Measurement” function to the photograph of the transverse section at the level of the papillary muscles. The same photograph was used to measure the surface area of the LV lumen with “Count / Size” function. This function was also applied to evaluate the surface area of the left auricle on the photograph of the auricular face. The use of a marker of known size in every photograph allowed calibration. To obtain the percentage of LV fibrous tissue, the transverse section of the left ventricle stained with MT was photographed and the picture was divided in four quadrants. The “Count / Size” function was applied to each image, exploiting the chromatic difference between fibrous tissue and myocardium highlighted by MT stain. Definitive LV fibrous tissue percentages were calculated as the mean result of the four quadrants.

**Statistical analysis**
GraphPad Prism® software was used to perform statistical analysis. Shapiro-Wilk’s test was used to establish normality or non-normality of distribution. In order to evaluate the statistical repeatability of the morphometric measurements, ten cases were randomly selected and every gross and histopathological feature evaluated by morphometry was measured three times. Coefficient of Variation (CV) was evaluated in the three series of measurements for every parameter considered. Comparisons between data were tested using Student’s t test and Mann-Whitney U test, while Spearman’s rank correlation coefficient was used for correlations. P values < 0.05 were considered statistically significant. Normally distributed data were expressed as mean ± SD, while non-normally distributed data were expressed as median and IQR (25%-75%).

Results

Animals

All the 21 cats with cardiac disorders were diagnosed with HCM. In 57% (12/21) of them, HCM was previously diagnosed by echocardiographic examination as indicated by Migliorini et al. (2012). In the remaining 43% (9/21) the diagnosis of HCM was highly suspected on the basis of clinical signs (dyspnea, ATE and/or sudden death) and radiographic findings (cardiomegaly, pulmonary edema and/or pleural effusion). Of all cats with HCM, 57% (12/21) were male, whereas the remaining 43% (9/21) was female. Breeds represented were Domestic Shorthair (13/21, 62%), Persian (3/21, 14%), Ragdoll (2/21, 9%), Maine Coon (1/21, 5%), Devon Rex (1/21, 5%) and British Shorthair (1/21, 5%). Age of cats ranged from 1 to 16 years (7.55 ± 4.16 years). Of all control cats, 67% (4/6) were male, while the remaining 33% (2/6) was female. Breeds represented were Domestic Shorthair (4/6, 66%), Siamese (1/6, 17%) and Sphynx (1/6, 17%). Age of cats ranged from 2 to 14 years (8.00 ± 4.73 years).

Gross and histopathological examination
Histopathological data related to the evaluation of disarray, intramural coronary arteriosclerosis and LV fibrous tissue deposition are summarized in Table 1. All gross and histopathological features of HCM were identified in the affected cats. All cats presented severe and diffuse myocyte hypertrophy, characterized by thick myofibers with oval and vesicular nuclei with prominent nucleoli. Myofiber disarray (Fig. 1A-B) was identified in 95% (20/21) of the cats, while intramural coronary arteriosclerosis (Fig. 1C-D) was detected in 81% (17/21) of them. LV fibrous tissue deposition was observed in 67% (14/21) of the cats. There was myocardial replacement fibrosis in 24% (5/21) of them, myocardial interstitial fibrosis in 24% (5/21) and endocardial fibrosis in 18% (4/21). Control cats showed no myocyte hypertrophy, disarray, intramural coronary arteriosclerosis and LV fibrous tissue deposition.

**Morphometric investigation**

Morphometric data related to the evaluation of the LV free wall, the interventricular septum, the LV lumen, the left auricle (Fig. 2A-B) and LV fibrous tissue (Fig. 3A-B) are summarized in Table 1. In all the cats with HCM there was no statistically significant difference (Student’s t test, $P > 0.05$) between the thicknesses of the LV free wall (7.37 ± 1.38 mm) and the interventricular septum (7.34 ± 1.45 mm). No statistically significant correlation was found between the percentage of LV fibrous tissue and the thicknesses of the LV free wall (Spearman’s rank correlation coefficient, $r = -0.1257$, $P > 0.05$) and the interventricular septum (Spearman’s rank correlation coefficient, $r = -0.3334$, $P > 0.05$), respectively. On the contrary, there was a statistically significant positive correlation between the percentage of LV fibrous tissue and the surface area of the LV lumen (Spearman’s rank correlation coefficient, $r = 0.6985$, $P = 0.0004$) and the left auricle (Spearman’s rank correlation coefficient, $r = 0.6561$, $P = 0.0017$), respectively (Fig. 4-5). Furthermore, a statistically significant positive correlation was found between the percentage of LV fibrous tissue and the number of pathological intramural coronary arteries within the LV myocardium (Spearman’s rank correlation coefficient, $r = 0.4756$, $P = 0.0293$). Finally, there was no statistically significant correlation
between the percentage of LV fibrous tissue and the number of the areas of disarray within the LV myocardium (Spearman’s rank correlation coefficient, P > 0.05). The thicknesses of the LV free wall (6.10 ± 0.89 mm) and the interventricular septum (5.82 ± 0.59 mm) were significantly less (Mann-Whitney U test, P = 0.0473 and P = 0.0182) in control cats. On the contrary, the surface areas of the LV lumen and the left auricle were not significantly different (Mann-Whitney U test, P > 0.05) between control and affected animals.

Repeatability

Coefficient of variation (CV) of the measurements series of the thicknesses of the LV free wall and the interventricular septum, the surface areas of the LV lumen and the left auricle and the percentage of the LV fibrous tissue was 1.27, 1.85, 1.92, 4.66 and 1.47%, respectively.

Discussion

Despite several anatomopathological studies on feline HCM (Liu et al., 1975; Tilley et al., 1977; Liu et Tilley, 1980; Van Vleet et al., 1980; Liu et al., 1981; Liu et al., 1993; Fox, 2003; Cesta et al., 2005; Kershaw et al., 2012; Khor et al., 2014), the quantitative parameters which allow the characterization of this myocardial disease in veterinary pathology are limited. Similarly to the standardized echocardiographic parameters previously established for diagnosis and classification of feline HCM (Migliorini et al., 2012), the present study aims to propose an original, complete and repeatable quantitative anatomopathological evaluation of gross and histopathological features of this myocardial disease.

Morphometry plays a key role in the quantitative evaluation of feline HCM proposed in the present study. Kershaw et al. (2012) evaluated the possibility of identifying diagnostically relevant morphometric criteria that clearly distinguish feline HCM from normal hearts, based on the analysis of transversal myocardial sections, myocyte hypertrophy, myofiber disarray and myocardial fibrosis. Only gross morphometric analyses proved useful in distinguishing pathological from
normal hearts, while histological ones failed to identify any significant differences. On the contrary, the present study proposes an original use of both gross and histological morphometry in feline hearts with HCM, while also presenting evidence of its reliability as measurement tool. The low CV found in the three measurements series for the thicknesses of the LV free wall and the interventricular septum, the surface area of the LV lumen and the left auricle and the percentage of LV fibrous tissue suggests that morphometry is a repeatable measurement technique to quantify gross and histopathological features of HCM.

In the cats with HCM of the present study population, the mean thickness of the LV free wall is less than the values reported in previous anatomopathological studies (8.3 (Van Vleet et al., 1980), 8.1 (Liu et Tilley, 1980) and 8.0 mm (Liu et al., 1981)). Similarly, in the present study population, the mean thickness of the interventricular septum is less than the values of 8.8 (Van Vleet et al., 1980; Liu et Tilley, 1980) and 8.0 mm (Liu et al., 1981) identified in the same studies. These differences could be explained by the use of morphometry, which is a more accurate measurement tool than a simple ruler. The mean thicknesses of the LV free wall and the interventricular septum in the control cats of the present study population were similar to the values (6.0 ± 0.3 mm and 5.0 ± 0.2 mm) previously reported by Fox (2003). Comparisons between control and affected cats suggest that the thicknesses of the LV walls could be reliable indicators of LV hypertrophy.

Contrary to the thicknesses of the LV free wall and the interventricular septum, systematic measurements of the surface areas of the LV lumen and the left auricle in feline hearts have never been performed, except for the semi-quantitative scoring system of cardiac chamber enlargement proposed for nonhypertrophied RCM (Fox et al., 2014) and the single values of 24 mm related to severely dilated left atria reported for HCM (Baty et al., 2001; Cesta et al., 2005).

A quantitative evaluation of myocardial fibrosis has already been described for feline HCM (Kershaw et al., 2012; Khor et al., 2014), but without evaluation of the repeatability of the measurement technique.
In veterinary pathology there are no reference guidelines about the optimal myocardial section for disarray evaluation, but in human HCM cross sections of interventricular septum are mandatory in demonstrating it (Burke et Tavora, 2011). Myofiber disarray is observed in almost all cats with HCM of the present study, with a greater prevalence than previously reported (27% (Liu et al., 1993) and 30% (Liu et al., 1981)). This result confirms that myofiber disarray constitutes a distinctive histopathological finding in HCM (Fox, 2003). Intramural coronary arteriosclerosis was identified with a greater prevalence than previously reported (74% (Liu et al., 1993; Fox, 2003)). Despite the fact that quantitative (Liu et al., 1981; Kershaw et al., 2012) and semi-quantitative (Fox et al., 2014) disarray evaluations have already been performed in feline hearts with HCM and nonhypertrophied RCM, respectively, no quantitative assessment of intramural coronary arteriosclerosis has been previously described. Both disarray and intramural coronary arteriosclerosis evaluations proposed in the present study can be easily performed using the light microscope, without the use of sophisticated software. Furthermore, the double-blind assessment of the authors proves that these measurement techniques are reliable and repeatable.

As previously reported, ES-HCM is a clearly defined clinicopathological entity of HCM, characterized by relative dilation of the ventricular chamber, relative thinning of the ventricular wall, marked left atrial dilation, variable myocardial scarring and congestive heart failure (Baty et al., 2001; Cesta et al., 2005). Cesta et al. (2005) were the first to describe the pathological alterations associated with ES-HCM and investigate its pathogenesis in a family of four cats in which Baty et al. (2001) previously performed serial echocardiograms. The present study aims to deepen this first qualitative approach, staging the temporal evolution of feline HCM by a strictly anatomopathological and quantitative evaluation in a larger study population. All the correlations performed allow investigating the variation of LV free wall, interventricular septum hypertrophy and LV lumen and left auricle dilation on varying LV fibrous tissue deposition. Apart from replacement fibrosis, interstitial and endocardial ones are also considered, since they could probably
alter cardiac contractility. The presence of a positive correlation between the percentage of the LV fibrous tissue and the surface areas of the LV lumen and the left auricle demonstrates that as the LV fibrous tissue deposition increases, the LV lumen and the left atrium (of which left auricle could be a reliable marker) dilate. Therefore, end-stage phenotypes are characterized by dilated LV lumen and left atrium and extended LV fibrous tissue deposition, whereas the initial/middle ones show reduced LV lumen and left atrium and almost absent LV fibrous tissue deposition. The absence of a statistically significant correlation between the percentage of the LV fibrous tissue and the thicknesses of the LV free wall and the interventricular septum suggests that as the LV fibrous tissue deposition increases, the LV free wall and the interventricular septum do not become thinner. However, the identification of a negative trend between them does not rule out that end-stage phenotypes could be characterized by reduced LV free wall and interventricular septum thicknesses, while HCM forms in the initial/middle phase of the pathology could show increases in these values.

The positive correlation between the percentage of the LV fibrous tissue and the number of pathological intramural coronary arteries of the LV myocardium demonstrates the potential involvement of intramural coronary arteriosclerosis in the genesis of ES-HCM. As previously observed in humans and cats with HCM, abnormal coronary vessels could result in alterations of blood flow leading to myocardial ischemia and subsequent replacement fibrosis (Maron et Spirito, 1998; Cesta et al., 2005). Disarray may also predispose to ischemia and consequent myocardial fibrosis (Iida et al., 1998; Varnava et al., 2001; Cesta et al., 2005), since disorganized myocytes promote inefficient contraction and thus an increase in energy demand (Varnava et al., 2001), but no positive correlation between the percentage of the LV fibrous tissue and the areas of disarray within the LV myocardium is observed in the present study. On the basis of the obtained correlations between LV fibrous tissue deposition (which is considered the hallmark histological feature of ES-HCM) and the other gross and histopathological features of HCM, it was possible to separate the initial/middle HCM phenotypes from the end-stage ones. Cats with reduced LV lumen

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and left atrium, minimal LV fibrous tissue deposition and low number of pathological intramural coronary arteries were diagnosed with initial/middle HCM. On the contrary, cats with dilated LV lumen and left atrium, extended LV fibrous tissue deposition and increased number of pathological intramural coronary arteries were considered having ES-HCM. Despite the fact that no serial echocardiograms were performed in the present study, the identification of extended LV fibrous tissue deposition at post mortem examination was considered indicative of a decrease in myocardial contractility (Cesta et al., 2005).

In conclusion, the present study provides an original, complete and repeatable anatomopathological quantitative evaluation of feline HCM based on morphometric and histopathological data. Furthermore, it stages its temporal evolution by the differentiation of the phenotypes in the initial/middle phase of the pathology from the end-stage ones. Further investigations involving a greater number of cats with HCM, secondary LV hypertrophy and normal hearts are suggested to better understand the heterogeneity and the complexity of this myocardial disease.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.
References


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Fig. 1
Fig. 2

Fig. 3
Fig. 4

Fig. 5
Table 1. Summary of the quantitative evaluation obtained by heart examination of all the cats included in the study.

<table>
<thead>
<tr>
<th></th>
<th>HCM (n = 21)</th>
<th>Control (n = 6)</th>
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<tbody>
<tr>
<td><strong>Left ventricular free wall</strong></td>
<td>7.37 ± 1.38 (4.63-9.37)</td>
<td>6.10 ± 0.89 (4.42-7.01)</td>
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<td>(mm)</td>
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<tr>
<td><strong>Interventricular septum</strong></td>
<td>7.34 ± 1.45 (4.76-10.50)</td>
<td>5.82 ± 0.59 (5.06-6.61)</td>
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<tr>
<td>(mm)</td>
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<tr>
<td><strong>Left ventricular lumen</strong></td>
<td>18.34 (8.17-62.55)</td>
<td>22.43 (9.13-34.42)</td>
</tr>
<tr>
<td>(mm²)</td>
<td></td>
<td></td>
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<tr>
<td><strong>Left auricle</strong></td>
<td>229.90 ± 138.40 (59.18-520.10)</td>
<td>155.00 ±19.14 (126.50-183.70)</td>
</tr>
<tr>
<td>(mm²)</td>
<td></td>
<td></td>
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<tr>
<td><strong>Left ventricular fibrous tissue</strong></td>
<td>7.56 (0.00-15.37)</td>
<td>Absent</td>
</tr>
<tr>
<td>(%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Disarray of left ventricular myocardium (n)</strong></td>
<td>3.00 (2.50-6.00)</td>
<td>Absent</td>
</tr>
<tr>
<td><strong>Intramural coronary arteriosclerosis of left ventricular myocardium (n)</strong></td>
<td>3.00 (1.00-4.50)</td>
<td>Absent</td>
</tr>
</tbody>
</table>

Left ventricular free wall thickness, interventricular septum thickness and left auricle surface data are expressed as mean ± SD (min-max), while the remaining data are expressed as median with IQR (25%-75%). n = number of disarray areas or pathological intramural coronary arteries.
Figure legends

**Figure 1.** Histopathological features of feline hearts with HCM. (A) Myofiber disorientation (“disarray”) appears in the form of bizarre and disorganized cellular architecture. Moderate interstitial fibrosis is also observed. Masson’s Trichrome, 20x. (B) Areas of disarray within the left ventricular myocardium are identified by the “Object Marker” tool of the optic microscope. Haematoxylin & Eosin, photograph of the glass slide. (C) Intramural coronary arteriosclerosis appears in the form of abnormal intramural coronary arteries with thickened wall and narrowed lumen. Masson’s Trichrome, 20x. (D) Three pathological intramural coronary arteries (asterisks) are identified. Masson’s Trichrome, 10x.

**Figure 2.** Gross morphometry of feline hearts with HCM initial/middle phenotypes. (A) Measurements of the thicknesses of the left ventricular free wall and the interventricular septum (black bars) and the surface area of the left ventricular lumen (green contour) are made. Image®-Pro Plus software. (B) Measurement of the surface area of the left auricle (red contour) is performed. Image®-Pro Plus software.

**Figure 3.** Histological morphometry of feline hearts with HCM end-stage phenotypes. (A) Left ventricle is divided in four quadrants for successive evaluation of the percentage of the left ventricular fibrous tissue. Masson’s Trichrome, photograph of the glass slide. (B) Assessment of the percentage of myocardium occupied by fibrous tissue (green) and cardiomyocytes (red) is performed on one of the four quadrants (*). Image®-Pro Plus software.
**Figure 4.** A statistically significant positive correlation between the percentage of the left ventricular fibrous tissue and the surface area of the left ventricular lumen is observed. GraphPad Prism® software.

**Figure 5.** A statistically significant positive correlation between the percentage of the left ventricular fibrous tissue and the surface area of the left auricle is observed. GraphPad Prism® software.