Alteration in Left Ventricular Contractile Function Develops in Puppies With Duchenne Muscular Dystrophy

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Abstract

Background Dystrophin-deficient cardiomyopathy is becoming the dominant cause of death in patients with Duchenne muscular dystrophy (DMD) but its developmental process remains elusive. This study aimed to assess the development of left ventricular (LV) dysfunction in golden retriever muscular dystrophy (GRMD) dogs that mimic DMD pathologies. **Methods** Transthoracic echocardiography was sequentially performed in GRMD dogs (n=23) and age-matched healthy littermates (n=7) from 2 to 24 months old. Conventional, tissue Doppler imaging and speckle-tracking echocardiography parameters were analyzed. **Results** At 2 months of age, GRMD dogs showed a pathological decrease in the subendocardial-subepicardial gradient of radial systolic myocardial velocity along with altered LV twist and longitudinal strain, all being aggravated with age (ANOVA, $p<0.001$). Receiver operator characteristic analysis showed good ability to discriminate normal from GRMD dogs. LV ejection fraction was significantly decreased in GRMD dogs starting from 9 months and reached a pathological level (<50%) at 24 months. **Conclusions** The development of cardiomyopathy in GRMD dogs was characterized by subendocardial dysfunction, altered LV twist and reduced longitudinal strain at a very young age to overall LV dysfunction in adult with transmural dysfunction, reduced LV ejection fraction and diastolic abnormalities, and even heart failure. This indicates the necessity to evaluate LV transmural myocardial velocity gradient, twist and longitudinal strain in early childhood of DMD patients.

Key words: canine model of Duchenne muscular dystrophy; dystrophin-deficient cardiomyopathy; echocardiography, myocardial strain; twist; transmural myocardial velocity gradient

Abbreviations: AUC (area under the curve), CoV (coefficient of variation), DMD (Duchenne muscular disease), EF (ejection fraction), $G_{Endo-Epi}$ (transmural myocardial velocity gradient), GRMD (golden retriever muscular dystrophy), LV (left ventricular), ICC (interclass correlation coefficient), PW (posterior wall), ROC (receiver operating characteristic curve), STE (Speckle Tracking Echocardiography), SV_{Endo} (radial subendocardial systolic myocardial velocity), SV_{epi} (radial subepicardial systolic myocardial velocity), TDI (tissue Doppler imaging), 3-, 4- and 2-C (3-, 4- and 2-chamber), Vp (flow propagation velocity)

Introduction

Duchenne muscular dystrophy (DMD) caused by mutations of the dystrophin gene is a lethal X-linked recessive disorder with an estimated incidence of 1/3500-5000 live male births. DMD is characterized by progressive skeletal muscle degeneration and weakness, leading to respiratory failure. With the improvement of care and treatment, the lifespan of DMD patients has been markedly prolonged, which causes a shift of leading cause of death in DMD patients from respiratory failure to heart failure [1,2]. Thus, it is essential to detect early cardiac dysfunction in DMD patients for further improving the care and treatment of dystrophin-deficient cardiomyopathy. Although a decreased left ventricular (LV) ejection fraction (EF) occurs relatively late in most DMD patients [3], studies showed an altered LV longitudinal strain in DMD patients of 3-12 years [4,5], abnormal circumferential strain in young DMD patients (<10 years) [3,6], and reduced systolic radial strain in DMD patients of 3-18 years [4,7]. However, it remains to determine the temporal changes of these LV function indices in DMD patients.

Golden retriever muscular dystrophy (GRMD) dogs present similar symptoms and signs to DMD patients and were used to develop novel therapeutic strategies for the treatment of DMD [8,9]. Besides skeletal muscle alterations, GRMD dogs also develop cardiomyopathy [10-12]. It has been shown that GRMD dogs developed a subendocardial dysfunction at the age of 6 months assessed by tissue Doppler imaging (TDI) [10]. However, as in DMD patients, the temporal process of cardiomyopathy in GRMD dogs is still elusive. Thanks to simplicity and accuracy, different techniques of echocardiography are widely used in clinical practice and research. Therefore, the aim of the present study was to assess the development of left ventricular (LV) dysfunction in GRMD dogs by echocardiography focusing on the deformational indices such as longitudinal, radial and circumferential strain, the subendocardial function and LV twist mechanics that have not been studied in DMD patients

and GRMD dogs, so as to provide information for the effective use of this model in the advance of therapeutic strategies to fight against dystrophin-deficient cardiomyopathy. Accordingly, we longitudinally monitored the evolution of LV function by echocardiography, particularly focusing on the aspects of LV transmural function, rotational mechanics, longitudinal strain and global contractile function in a cohort of GRMD dogs and their healthy littermates from 2 to 24 months of age.

Materials and methods

Animals

Twenty-three GRMD and seven normal control dogs with the same genetic background (which were the littermates of the some GRMD dogs) were provided by the Centre d'Elevage du Domaine des Souches (Mezilles, France) and were handled and cared by veterinarians throughout the study. In addition, six old GRMD dogs aged from 48.5 to 69.0 months (average: 57.0±4.4 months) showing symptoms and signs of heart failure such as dyspnea and ascites were also examined. The GRMD was diagnosed by DNA analysis at 1 month of age. The experimental protocol was approved by the common ethical committee of the ANSES, ENVA and UPEC (approval number: 20/12/12-18) and the experimental procedures were maintained in accordance with Directive 2010/63/EU of the European Union.

Among the twenty-three GRMD dogs, three died of pneumonia or digestive tract disorders between 2 and 4 months of age, three were euthanized around their 6 months for loss of ambulation, three were euthanized between 9 and 12 months of age for severely reduced mobility, bad general conditions or severe bronchopneumonia, three were euthanized due to reduced mobility and respiratory failure between 12 and 18 months of age and one died of heart failure. No death occurred in control dogs.

Echocardiographic study

Echocardiographic examination was longitudinally performed in GRMD dogs and healthy control dogs at 2, 6, 9, 12, 18 and 24 months of age. Echocardiographic data were also obtained in 6 old GRMD dogs with heart failure symptoms and signs.

Echocardiographic data acquisition

Echocardiographic image acquisition and analysis were performed by the same examiner. Due to its physical appearance and walking gait, a GRMD dog is easy to be

recognized, so it was impossible for the sonographer to be blinded in this study. Using a Vivid 7 ultrasound unit (General Electric Medical System, Horten, Norway) under ECG monitoring, echocardiographic images were acquired in awake (i.e. without the use of sedatives or anesthetics) 2-month-old puppies in standing position in the arms of an animal technician using a 7S cardiac sector array probe and in awake dogs of other ages, standing in a sling using 5S or M4S cardiac sector array probe. The echocardiography images were acquired when the heart rate of the animal was stable after a period adaptation of 20-30 min in the sling.

Short-axis TDI images at the mid-papillary muscle level were obtained for calculating radial myocardial velocities of left ventricular (LV) subendocardium and subepicardium. Mitral inflow tracings were obtained at the apical 4-chamber (4-C) view by pulsed-wave Doppler and color M-mode Doppler echocardiography.

High frame rate images (60-119 frame/s) were recorded on parasternal short-axis views at the base, mid-ventricle and apex as well as on the apical 3-C, 4-C and 2-C views. When an adequate 3-C view was difficult to obtain, the apical 5-C view was used. The basal short axis view was recorded at the level of mitral valve leaflets. The mid-ventricle view was recorded at the level of papillary muscles. The apical short axis view was defined by the smallest LV cavity and no papillary muscle visible. For all measurements, at least three consecutive cardiac cycles were stored digitally for blinded offline analysis.

Echocardiographic analysis

Echocardiographic analysis was performed as recommended. LV ejection fraction was calculated by modified biplane Simpson's method using both the apical 4-C and 2-C views [13]. Mitral inflow E and A waves and E/A ratio were calculated using pulsed-wave Doppler mitral inflow tracings, and flow propagation velocity (Vp) was calculated from M-mode color mitral inflow tracings. The onset of the systole was set at 10 ms before the R peak in ECG.

Using video recorded in TDI mode at the mid-ventricle, subendocardial and subepicardial radial systolic velocities of the posterior wall (PW) were calculated as described previously [10]. The transmural myocardial velocity gradient ($G_{Endo-Epi}$) was calculated as the difference between the subendocardial and the subepicardial systolic velocities.

Speckle Tracking Echocardiography (STE) was performed offline using dedicated software (EchoPac 6.0, GE Healthcare). The cardiac cycle length was measured from the start of the systole. After automatically delineating an area of interest covering LV wall and manual adjustments to include all LV wall, the software generated 6 strain curves of 6 segments of the LV wall at each view by tracking the motion of acoustic objects on a frame-by-frame basis, which were averaged to obtain global strain value. LV basal and apical rotation data were derived from a single cardiac cycle from basal and apical short-axis images. After normalizing temporal data of basal and apical rotations with cardiac cycle length [14] and cubic spline interpolation [15] using Winpython 3.6.5 software, peak LV twist and untwisting rate were calculated by subtracting instantaneous basal rotational values from apical rotational values. Longitudinal strain was obtained from apical 3-C (or 5-C), 4-C and 2-C views (an example of analysis of longitudinal strain at the apical 4-C view in a GRMD dogs at 2 and 24 months of age are shown in Suppl Video). Peak systolic radial and circumferential strains were calculated from the parasternal short-axis image at the mid-papillary muscle level. Global radial strain was obtained by averaging the digital values of 6 strain curves using Excel (Microsoft Corp, Seattle, WA).

Statistics

Statistical analysis was performed with the StatView software (Version 5.0, Abacus Concepts Inc). Data were presented as the means ± SEM. One-way analysis of variance (ANOVA) was performed to determine the within-group difference over time. The data from GRMD dogs and healthy control dogs with echocardiography from 2 to 24 months were

analyzed by ANOVA for repeated measurements over time to test the between-group difference. Data obtained in all dogs at each time point were analyzed separately using Student's t-test for independent samples to determine the difference between GRMD and control dogs at each time point. To determine the ability of the techniques to differentiate GRMD dogs from healthy control animal, the receiver operating characteristic curve (ROC) analysis was performed with Microsoft Excel 2016 and the area under the curve (AUC) was calculated using the trapezoid method. A difference was considered statistically significant when $p<0.05$. In addition, interclass correlation coefficient (ICC) was calculated for the 4 key echocardiographic parameters ($G_{Endo-Epi}$, LV twist, longitudinal strain at the apical 4-C view and ejection fraction) using Real Statistics Resource Pack. Coefficient of variation (CoV) for these echocardiographic parameters was calculated as: CoV (%) = standard deviation/mean x 100. The results of ICC and CoV are shown in Suppl Table 1.

To examine the intra-observer variability, a set of images used for the calculation of $G_{Endo-Epi}$, LV twist, longitudinal strain at the apical 4-C view and ejection fraction were taken from 6 randomly selected dogs and analyzed twice by the same sonographer in an interval of 24 h or more (3 heart beats for each image at each time point). The results of the two measurements were used for the calculation of the ICC and CoV for $G_{Endo-Epi}$ (0.928 and 4.5±1.6%, respectively), LV twist (0.961 and 11.4±2.3%, respectively), longitudinal strain at the apical 4-C view (0.925 and -3.0±0.9%, respectively) and ejection fraction (0.855 and 2.2±0.2%, respectively).

Results

Alteration of LV transmural gradient of myocardial contractile function

Figures 1a and b show representative tracings of subendocardial and subepicardial radial velocities of the PW for determining transmural gradient in 2-month-old GRMD and control dogs. The calculated transmural gradient ($G_{Endo-Epi}$) was significantly smaller in GRMD dogs than in control dogs throughout the protocol (ANOVA, $p<0.001$, Figure 1c). The pathological reduction of $G_{Endo-Epi}$ (i.e., < cut-off value of the mean-2SD of the age-matched control dogs) occurred in 52% (12/23), 75% (15/20), 75% (12/16), 92% (12/13), 100% (8/8) and 100% (8/8) in GRMD dogs at 2, 6, 9 12, 18 and 24 months, respectively. This index showed a good discriminative ability as indicated by an AUC value of 0.89 at 2 months and 1 at 12 months and thereafter (Figures 1d and e). This contractile dysfunction was selective to subendocardial layer as subepicardial radial systolic velocities remained similar between the two groups (Table 1).

Alteration of LV twist mechanics in GRMD dogs

LV twist was analyzed as illustrated by representative tracings obtained in 2-month-old GRMD and control dogs (Figures 2a and b). As shown in Figure 2c and resulting from analysis of apical and basal rotations (Table 1), LV twist was significantly reduced in GRMD versus control dogs (ANOVA, $p<0.001$), while control dogs did not show significant changes in LV twist in the study period (Figure 2c). Pathologically altered LV twist (i.e. > -10°) was detected in 43% (10/23), 45% (9/20), 50% (9/20), 62% (8/16), 88% (7/8) and 100% (8/8) at 2, 6, 9, 12, 18 and 24 months old, respectively, indicating the increased incidence of altered LV twist mechanics with age in GRMD dogs. This technique showed a good discriminative ability as indicated by an AUC of 0.80 at 2 months, 0.92 at 12 months (Figure 2d and e) and 1 at 24 months further confirming early detection and increased incidence of altered LV twist mechanics with age in GRMD dogs.

Altered LV myocardial longitudinal strain in GRMD dogs

GRMD dogs had a worse longitudinal strain analyzed from the apical 4-C view than control dogs (ANOVA, $p<0.001$, Figure 3a), and ROC analysis showed an AUC of 0.83 and 0.87 at 2 and 12 months, respectively (Figure 3b and c). The alteration considered as pathological (i.e. > mean value+2SD of age-matched control dogs) occurred in 26% (6/23), 30% (6/20), 31% (5/16), 46% (6/13), 63% (5/8) and 88% (7/8) of GRMD dogs at 2, 6, 9, 12, 18 and 24 months. A similar trend was also observed in 3-C and 2-C views (Table 1) except that the difference between GRMD and control dogs became statistically significant from 9 months (3-C view) or 12 months (2-C view). When a global longitudinal strain was calculated by averaging the values of all the 3 views, GRMD dogs also showed a significantly worse longitudinal strain than healthy control dogs starting from 2 months of age (Table 1). These parameters were further deteriorated in old GRMD dogs (Table 2). In addition, dyskinetic segment was observed in GRMD dogs starting from 6 months old (15%) and the incidence increased with the age (30% at one year, 75% at 24 months and massively in old dogs with heart failure), while no dyskinetic segment was observed in healthy control dogs.

As shown in Table 1, there were no significant differences in radial and circumferential strains measured at short-axis papillary muscle between GRMD and control dogs at different ages. The old GRMD dogs showed significantly smaller radial and circumferential strains than 24-month-old GRMD dogs (Table 2).

Evolution of LV global function in GRMD dogs

GRMD dogs had a lower EF than control dogs (ANOVA, $p<0.001$), which started from 6 months (Figure 4a). However, the percentage of GRMD dogs that had a pathologically low EF (i.e. <50%) was 19% (3/16), 23% (3/13), 50% (4/8) and 100% (8/8) at 9, 12, 18 and 24 months, respectively, and 24-month-old GRMD dogs had an EF of 44±2% (37-49%) while old GRMD dogs had an EF of 33±4% (21-40%), clearly showing an evolution from LV

dysfunction to LV failure. The ROC analysis showed an AUC of 0.66, 0.83 and 1.0 at 6, 12 and 24 months, respectively, indicating a good ability of EF to differentiate LV dysfunction of GRMD dogs from normal LV function of control dogs at these time points (Figure 4b and 4c).

Altered LV diastolic functions in GRMD dogs

The diastolic function was analyzed by 3 parameters: mitral flow E/A ratio, Vp and LV untwisting rate (Table 1). Although there was no significant difference in the E/A ratio between GRMD and control dogs, it was worth noting that over the study period, LV diastolic dysfunction did occur in GRMD dogs. For example, two GRMD dogs had an E/A ratio<1.0 at 2 months (one was euthanized just before the age of 6 months due to loss of ambulation; another had an E/A ratio close to 1.0 at 18 and 24 months); one had a pseudo normal E/A ratio from the age of 6 months; at 9 months old, one GRMD had an E/A ratio>2.0 and died thereafter and another had an E/A ratio<1.0 and evaluated toward pseudo normal thereafter; another GRMD dog had an E/A ratio>2.0 from the age of 12 months. Among old GRMD dogs, two had an E/A ratio<1.0 and three had an E/A ratio>2.0. Thus, it appears that changes in the E/A ratio were not uniform in GRMD dogs from 2 to 24 months. In contrast, a significant decrease in Vp was observed in GRMD dogs from the age of 12 months and the difference with control dogs increased with age (Table 1), indicating the occurrence of diastolic dysfunction in GRMD dogs. This parameter was further decreased in old GRMD dogs (Table 2). Compared with control dogs, GRMD dogs had a decreased LV untwisting rate from 12 months (Table 1), which was further decreased in old GRMD dogs (Table 2).

Discussion

Using different techniques such as ECG, echocardiography, or cardiovascular magnetic resonance, previous studies reported important information about arrhythmia, cardiac dysfunction and myocardial fibrosis in DMD patients [3-7,16-20] and in canine models of DMD [10-12,21,22]. However, the data concerning the evolution of LV function in DMD patients and animal models are still lacking. This longitudinal study examined the developmental course of LV dysfunction in GRMD dogs from a very young age to 2 years old. The alterations in LV transmural myocardial contractile function, LV twist mechanics and LV longitudinal strain occurred in very young GRMD puppies (2 months old) and were all aggravated with age. This is remarkable, cconsidering that the first living year of a golden retriever corresponds approximately to the first 20 years of a human [23], the first 3 months of age of a DMD dog correspond to the first 5 years of a DMD child, and the next 3 months, from 3 to 6 months of age, analogous to 5-10 years of age for a DMD child [24]. The use of EF failed to detect such early LV contractile dysfunction. This parameter progressively decreased and achieved a pathologically low level only starting from 9 months old, which is consistent with our previous results using conventional echocardiography [14] and observations in DMD patients. Considered together, these results evidenced an evolution from a subtle decrease in LV myocardial contractile performance at a young age to a more pronounced overall systolic and diastolic dysfunction and even heart failure in adults.

Using TDI technique, a previous report showed a decreased $G_{Endo-Epi}$ in 6-month-old GRMD dogs with obvious normal global function [10], but younger animals were not investigated in this study. Using the same technique, we found that this change in transmural myocardial function occurred much earlier as more than 50% of 2-month-old GRMD dogs had a decreased $G_{Endo-Epi}$, and this phenomenon rose with age. This technique showed a good

ability to differentiate LV subendocardial function of GRMD dogs from that of control dogs as indicated by AUC values (0.89 and 1.0 at 2 and 12 months, respectively). The change in $G_{Endo-Epi}$ was mainly due to a decrease in the subendocardial systolic velocity (Table 1), indicating LV subendocardial dysfunction in GRMD dogs, which is consistent with previous studies [10,12]. The preferential alteration of the subendocardium compared with the subepicardium is known to occur in various cardiac diseases [25-27], highlighting the functional differences [28], and differences in blood flow distribution [26,29,30], oxygen consumption and effect of stress on the myocardium [31] between subepicardial and subendocardial layers [12,26,27]. This early subendocardial dysfunction may contribute to the slightly decreased overall LV contractile function as indicated by slightly smaller EF observed in young GRMD dogs. Although the change in $G_{Endo-Epi}$ was not evaluated in DMD patients, it is likely to find such alteration in DMD patients because the region where $G_{Endo-Epi}$ was measured corresponds to the most vulnerable region in DMD patients [32,33].

Owing to its independence on Doppler angle of incidence, STE has been developed as a reliable method for objectively quantifying regional myocardial function and LV motion. Indeed, using this technique, we analyzed LV rotational mechanics, another important aspect of LV function, and found for the first time an early alteration in LV twist and that its incidence increased with age from 43% at 2 months to 62% and 100% at 1 and 2 years old. This change in LV rotational mechanics certainly contributed to the development of overall LV dysfunction and heart failure, and these results suggest that evaluating the change in LV twist mechanics may be a means to detect early LV dysfunction in DMD patients.

In addition, we found that GRMD dogs had a decreased longitudinal strain, which could be detected at 2 months old at the 4-C view and when all 3 views were considered together. This alteration in LV longitudinal strain is consistent with the clinical studies showing an early alteration of myocardial strain in young DMD patients (3-12 years) [4,5],

further supporting the use of LV longitudinal strain for the detection of early LV dysfunction in DMD patients. Moreover, longitudinal strain analysis revealed an increased number of dyskinetic segments with age in GRMD dogs, especially for dogs with heart failure. Reduced longitudinal strain and increased dyskinetic segments partly explain the decline in LV contractile function in GRMD dogs.

Previous studies found the presence of abnormal circumferential strain in young DMD patients (<10 years) [3,6], which was worsen in DMD patients aged more then 10 years [3]. However, such alteration was not found in GRMD dogs until 18 months. The reason for this remains unclear.

A previous study in rats showed that radial strain abnormality is associated with histological changes in the myocardium [34]. Previous studies found significant reduction of radial strain localized at inferolateral and anterolateral walls in DMD patients (3-12 years) [4] or at the posterior wall in DMD patients (age: 14.8 +/- 3.1 years) [7]. In the present study, when the global radial strain was calculated, there was no significant difference between GRMD and control dogs over the study period. We speculate that this preserved global radial strain may serve as a compensatory mechanism to maintain LV contractile function in GRMD dogs.

Otherwise, it appeared that the occurrence of LV diastolic dysfunction was relatively later in GRMD dogs when accessed by Vp and LV untwisting rate. Although isolated abnormal mitral flow E/A ratio could be observed in GRMD dogs, the different types of E/A ratio representing different degrees of LV diastolic dysfunction (for example in old GRMD dogs) resulted in confusing average values.

Limitations

The person making the measurements was not blinded to the condition of the animal (GRMD or control) or the time point of the measurement.

The GRMD is highly complex model with great variability between animals. This variability was evaluated by the examination of coefficients of variation (Suppl Table 1). In addition, this parameter allows us to know whether the change in a parameter of LV function was due to the alteration in a certain number of animals or it reflected the trend of the entire population.

This study was designed to characterize the temporal evolution of LV function in GRMD dogs and does not provide additional cellular and molecular mechanisms to explain the early myocardial function changes noted. Clearly, further studies are required to determine the mechanisms involved in the temporal changes of LV function in GRMD dogs, although it is known that myocardial fibrosis and edema [21], structural and functional changes in sarcomeres of cardiomyocytes due to the loss of dystrophin [35], endothelial dysfunction [36], altered expression of endothelial and neuronal nitric oxide synthases [12], and changed expression of sarcomeric protein genes could all be involved in this process [12]. In addition, this study did not assess whether the early changes in LV transmural function, twist and strain parameters predict a decrease in LV ejection fraction within a certain time period. However, since the alterations of these LV functional parameters occur earlier than the decrease in LV ejection fraction, it may allow us to reasonably think that the alterations of these parameters contribute to the decrease in LV ejection fraction.

Conclusions

This longitudinal study demonstrated that LV systolic dysfunction in GRMD dogs occurs much earlier than previously considered, which is characterized by significantly altered LV transmural function, impaired LV twist mechanics and longitudinal strain in very young GRMD dogs. These results strongly indicate the necessity to assess the alterations of LV transmural function and myocardial strain mechanics in early childhood of DMD patients.

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Conflicts of Interest: None

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Figure Legends

Figure 1 Evolution of left ventricular (LV) transmural gradient of radial myocardial systolic velocity ($G_{Endo-Epi}$) in GRMD and control dogs

Panels (a) and (b) showed examples of radial subendocardial (yellow curve) and subepicardial (cyan curve) velocities of a 2-month-old control dog and a 2-month-old GRMD dog, respectively. (c) Mean values (±SEM) of $G_{Endo-Epi}$ obtained by subtracting the subepicardial systolic velocity from the subendocardial systolic velocity; (d) ROC curve of $G_{Endo-Epi}$ of 2-month-old GRMD and control dogs; (e) ROC curve of $G_{Endo-Epi}$ of 12-month-old GRMD and GRMD dogs. † $p<0.01$ versus control dogs of the same age. $ indicates a within-group difference over time of (ANOVA, $p<0.001$). n = 23, 20, 16, 13, 8 and 8 for GRMD dogs, and 7, 7, 4, 4, 4 and 4 for control dogs at 2, 6, 9, 12, 18 and 24 months of age.

Figure 2 Evolution of LV twist analyzed by STE in GRMD and control dogs

(a) Plot of LV apical rotation, basal rotation and twist curves of a 2-month-old control dog (LV twist curve was obtained by subtracting simultaneous basal rotation from the apical rotation); (b) plot of LV apical rotation curve, basal rotation curve and twist of a 2-month-old GRMD dog; (c) Mean values (±SEM) of LV twist obtained in GRMD and control dogs at different ages; (d) ROC curve of LV twist of 2-month-old GRMD and control dogs; (e) ROC curve of LV twist of 12-month-old GRMD and GRMD dogs. * $p<0.05$ and † $p< 0.01$ versus age-matched control dogs. $ indicates a within-group difference over age (ANOVA, $p<0.001$). n = 23, 20, 16, 13, 8 and 8 for GRMD dogs, and 7, 7, 4, 4, 4 and 4 for control dogs at the age of 2, 6, 9, 12, 18 and 24 months, respectively.

Figure 3 Evolution of longitudinal strain analyzed by speckle tracking echocardiography in GRMD and control dogs

(a) Mean values (±SEM) of left ventricular (LV) global strains obtained at 4-chamber view; (b) ROC curve of LV global strain at 4-chamber view of 2-month-old GRMD and control dogs; (c) ROC curve of LV global strain at 4-chamber view of 12-month-old GRMD and control dogs. * $p<0.05$ and † $p<0.01$ versus control dogs of the same age. $ indicates a within-group difference over age (ANOVA, $p<0.001$). n=23, 20, 16, 13, 8 and 8 for GRMD dogs, and 7, 7, 4, 4, 4 and 4 for control dogs at 2, 6, 9, 12, 18 and 24 months of age, respectively.

Figure 4 Evolution of left ventricular (LV) ejection fraction (EF) in GRMD and control dogs

(a) Mean values (±SEM) of EF obtained in GRMD and control dogs at different ages; (b). ROC curve of EF of 6-month-old GRMD and control dogs; (c) ROC curve of EF of 12-month-old GRMD and control dogs. AUC: area under curve. * $p<0.05$ and † $p<0.01$ compared to age-matched control dogs at the same age. $ indicates a within-group difference over age (ANOVA, $p<0.001$). n=23, 20, 16, 13, 8 and 8 for GRMD dogs, and 7, 7, 4, 4, 4 and 4 for control dogs at 2, 6, 9, 12, 18 and 24 months of age, respectively. The dashed line indicates the level of 50% EF.

Supplementary Video 1 Speckle–tracking analysis of left ventricular longitudinal strain at the apical 4-chamber of view from one 2-month-old GRMD dog

Supplementary Video 2 Speckle–tracking analysis of left ventricular longitudinal strain at the apical 4-chamber of view from the same GRMD dog at 24 months of age

Table 1 Echocardiographic parameters measured in GRMD and control dogs

Parameter	Group	Age, months						ANOVA *p* value	
		2	6	9	12	18	24	Within group	Between groups
Ejection fraction (%)	control	58.8±1.4	61.9±1.8	64.3±2.8	59.1±2.1	63.1±2.4	58.8±0.5	0.168	0.001
	GRMD	57.4±1.1	57.4±1.1*	52.9±1.2†	52.8±1.4†	50.0±2.0†	43.8±1.6†	0.0001	
SV_{Endo} (cm/s)	control	7.9±0.3	8.8±0.2	8.8±0.7	8.5±0.3	8.8±0.6	7.9±0.4	0.295	0.001
	GRMD	6.9±0.1†	7.0±0.2†	7.0±0.2†	6.9±0.3*	6.7±0.4†	5.9±0.3†	0.091	
SV_{Epi} (cm/s)	control	4.1±0.2	4.6±0.3	5.2±0.7	4.6±0.1	4.6±0.5	4.8±0.3	0.374	0.413
	GRMD	4.0±0.2	4.5±0.2	4.5±0.2	4.5±0.4	4.5±0.3	4.2±0.4	0.374	
$G_{Endo-Epi}$ (cm/s)	control	3.8±0.2	4.0±0.2	3.6±0.2	3.9±0.2	4.6±0.2	4.8±0.1	0.015	0.001
	GRMD	2.9±0.1†	2.4±0.1†	2.5±0.1†	2.4±0.1†	2.2±0.2†	1.7±0.2†	0.0001	
Apical rotation (°)	control	12.0±1.0	10.2±1.2	10.6±1.5	8.9±1.7	10.1±0.8	9.7±1.0	0.617	0.020
	GRMD	9.5±0.5*	8.2±0.6	7.9±0.6	7.1±0.7	6.5±0.9	5.0±0.7*	0.003	
Basal rotation (°)	control	-4.2±0.6	-6.1±0.5	-6.2±2.0	-7.4±0.5	-6.1±0.8	-4.6±1.0	0.077	0.121
	GRMD	-4.7±0.5	-4.5±0.5	-4.6±0.5	-4.5±0.6*	-3.7±0.4	-1.1±0.4*	0.002	
Twist (°)	control	13.1±0.6	13.0±0.6	12.4±0.9	12.1±0.5	13.0±1.2	12.2±0.9	0.796	0.001
	GRMD	10.8±0.5*	10.1±0.5†	9.6±0.6*	9.1±0.6*	7.8±0.8†	5.7±0.5†	0.0001	
Untwisting rate (°/s)	control	-145±11	-134±23	-116±15	-149±17	-117±18	-154±24	0.354	0.257
	GRMD	-111±15	-129±11	-112±11	-86±11*	-84±14	-68±9*	0.040	
Longitudinal strain (3-C view, %)	control	-20.9±0.5	-20.2±0.4	-21.8±0.9	-22.1±1.4	-19.7±1.6	-19.0±1.3	0.318	0.002
	GRMD	-20.3±0.5	-19.0±0.6	-19.2±0.5*	-18.8±0.5†	-17.1±0.4*	-15.0±0.9*	0.0001	
Longitudinal strain (4-C view, %)	control	-23.0±0.8	-22.0±0.6	-21.0±1.4	-21.8±1.3	-20.3±1.5	-18.4±0.8	0.0319	0.001
	GRMD	-20.3±0.3†	-20.0±0.4†	-19.5±0.5	-18.5±0.6*	-18.1±0.9	-14.6±0.8*	0.0001	
Longitudinal strain (2-C view, %)	control	-21.9±0.2	-21.1±0.4	-21.2±1.3	-21.8±1.3	-19.7±1.9	-18.4±0.8	0.053	0.002
	GRMD	-20.3±0.5	-19.9±0.4	-19.5±0.6	-17.4±0.8*	-16.0±0.5*	-15.7±0.8*	0.0001	
Global longitudinal strain (average of 3 views, %)	control	-21.9±0.3	-21.1±0.4	-21.3±0.4	-21.9±0.6	-19.9±1.4	-18.7±0.6	0.006	0.001
	GRMD	-20.3±0.3†	-19.6±0.3*	-19.4±0.4*	-18.3±0.5†	-17.1±0.4*	-15.1±0.6†	0.0001	
Radial strain (%)	control	65.3±3.8	49.5±5.2	48.1±4.5	46.4±1.4	47.2±4.6	47.0±6.1	0.015	0.097
	GRMD	54.8±2.7	49.7±3.0	49.9±3.7	42.3±2.7	41.3±3.4	37.8±3.9	0.003	
Circumferential strain (%)	control	-20.5±0.5	-20.0±0.8	-20.5±1.0	-19.5±1.6	-21.8±2.1	-18.9±1.5	0.654	0.078
	GRMD	-19.1±0.7	-20.8±0.8	-20.3±0.6	-19.7±0.8	-19.2±1.5	-15.7±1.1	0.008	
E/A ratio	control	1.38±0.18	1.38±0.11	1.32±0.06	1.44±0.01	1.45±0.12	1.45±0.13	0.592	0.966
	GRMD	1.38±0.10	1.27±0.05	1.29±0.08	1.42±0.20	1.55±0.17	1.54±0.18	0.389	
Vp (cm/s)	control	74.0±3.8	76.9±3.2	74.7±7.1	78.5±3.1	80.6±1.9	75.4±5.4	0.849	0.001
	GRMD	72.1±1.8	69.3±2.4	62.9±2.8	57.1±3.0†	48.2±2.7†	48.3±4.7†	0.0001	

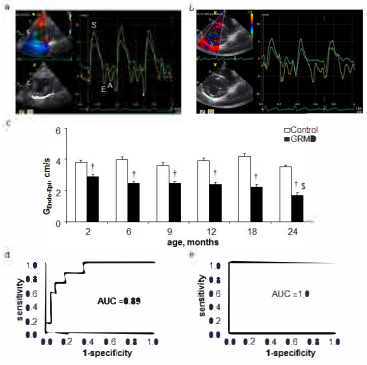
Data were expressed as mean±SEM. One-way ANOVA was performed to test the within-group difference. ANOVA for repeated measurements over time was performed on data from 8 GRMD (golden retriever

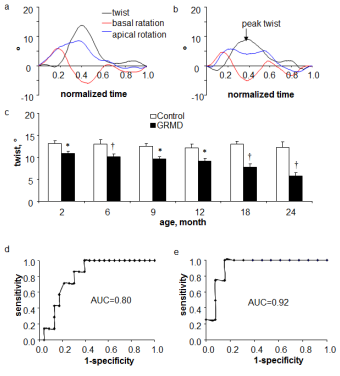
muscular dystrophy) dogs and 4 healthy control dogs with echocardiography from 2 to 24 months to test the between-group difference. Student's t-test for independent samples was performed on data obtained in all dogs at each time point to determine the difference between GRMD and control dogs at each time point. n=23, 20, 16, 13, 8 and 8 for GRMD dogs, and 7, 7, 4, 4, 4 and 4 for control dogs at 2, 6, 9, 12, 18 and 24 months of age, respectively. * $p<0.05$ and † $p<0.01$ versus control dogs at the same age. $G_{Endo-Epi}$: transmural myocardial velocity gradient between the subendocardium and the subepicadium; SV_{Endo}: radial subendocardial systolic myocardial velocity; SV_{epi}: radial subendocardial systolic myocardial velocity; Vp: flow propagation velocity; 3-C, 4-C and 2-C: 3-chamber, 4-chamber and 2-chamber.

Table 2 Comparison of the main parameters of old GRMD with 2-year-old GRMD

Parameter	GRMD (2 years old, n=8)	Old GRMD (>4 years old, n=6)
Ejection fraction (%)	43.8±1.6	32.7±3.6†
SV_{Endo} (cm/s)	5.9±0.3	4.9±0.3*
SV_{Epi} (cm/s)	4.2±0.4	3.7±0.2
$G_{Endo-Epi}$ (cm/s)	1.7±0.2	1.1±0.3
Apical rotation (°)	5.0±0.7	4.1±1.4
Basal rotation (°)	-1.1±0.4	-3.5±0.6*
Twist (°)	5.7±0.8	4.4±0.5
Untwisting rate (°/s)	-68.2±8.7	-34.2±6.8*
Longitudinal strain (3-C view, %)	-15.0±0.9	-9.2±2.9*
Longitudinal strain (4-C view, %)	-14.6±1.1	-10.8±1.3*
Longitudinal strain (2-C view, %)	-15.7±0.8	-10.0±2.0†
Global longitudinal strain (average of 3 views, %)	-15.1±0.6	-10.0±1.6*
Radial strain (%)	37.8±3.9	23.5 ±3.4*
Circumferential strain (%)	-15.7±1.1	-9.4±1.1†
E/A ratio	1.54±0.18	1.48±0.24
Vp (cm/s)	48.3±4.7	31.8±5.3*

Data were expressed as mean±SEM. Student's t-test for independent samples was performed. * $p<0.05$ and † $p<0.01$ versus 2-year-old GRMD (golden retriever muscular dystrophy) dogs. $G_{Endo-Epi}$: transmural myocardial velocity gradient between the subendocardium and the subepicadium; SV_{Endo}: radial subendocardial systolic myocardial velocity; SV_{epi}: radial subendocardial systolic myocardial velocity; Vp: flow propagation velocity; 3-C, 2C, and 4-C: 3-chamber, 2-chamber and 4-chamber.


a
S
E
A
b
c
Control
GRMD
6
4
2
0
2
6
9
12
18
24
age, months
d
sensitivity
1-specificity
AUC =
e
sensitivity
AUC =1
1-specificity


a
b
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e
twist
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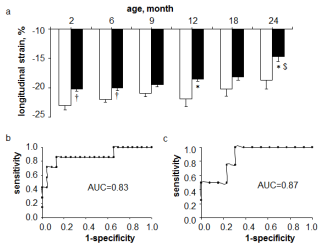
apical rotation
peak twist
normalized time
Control
GRMD
twist, °
age, month
sensitivity
1-specificity
AUC=0.80
AUC=0.92


a
age, month
2
6
9
12
18
24
longitudinal strain, %
-10
-15
-20
-25
†
*
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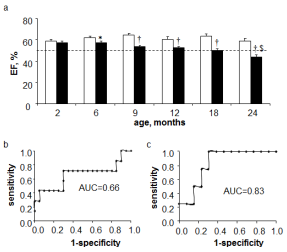
b
sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
AUC=0.83
0.0
0.2
0.4
0.6
0.8
1.0
1-specificity
c
sensitivity
AUC=0.87
1-specificity


a
80
60
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20
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EF, %
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24
age, months
*
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b
1.0
0.8
0.6
0.4
0.2
0.0
sensitivity
AUC=0.66
0.0 0.2 0.4 0.6 0.8 1.0
1-specificity

c
1.0
0.8
0.6
0.4
0.2
0.0
sensitivity
AUC=0.83
0.0 0.2 0.4 0.6 0.8 1.0
1-specificity

Supplementary Table 1 Interclass correlation coefficient and coefficient of variation of key echocardiographic parameters measured at different time points in GRMD and control dogs

Age (months)	Coefficient	Echocardiographic parameters							
		$G_{Endo-Epi}$		Twist		Longitudinal strain		Ejection fraction	
		control	GRMD	control	GRMD	control	GRMD	control	GRMD
2	ICC	0.978	0.919	0.981	0.905	0.989	0.989	0.997	0.986
	CoV	11.9	21.5	11.1	23.5	-9.8	-8.0	6.0	9.1
6	ICC	0.975	0.907	0.986	0.927	0.993	0.988	0.992	0.988
	CoV	13.1	23.3	10.9	23.0	-7.2	-8.5	7.1	8.2
9	ICC	0.999	0.958	0.983	0.918	0.997	0.985	0.993	0.985
	CoV	10.1	15.4	11.9	22.5	-12.3	-9.2	7.5	9.0
12	ICC	0.993	0.924	0.993	0.910	0.989	0.980	0.997	0.985
	CoV	8.9	21.2	7.4	24.1	-12.0	-10.7	6.1	9.3
18	ICC	0.995	0.903	0.970	0.899	0.997	0.969	0.994	0.982
	CoV	7.3	24.9	16.2	27.3	-14.2	-13.7	6.6	10.4
24	ICC	0.998	0.903	0.998	0.913	0.999	0.958	0.999	0.984
	CoV	7.6	28.8	13.5	25.1	-4.7	-19.6	1.5	9.8

Interclass correlation coefficient (ICC) was calculated using Real Statistics Resource Pack with the individual values of GRMD (golden retriever muscular dystrophy) dogs and healthy control animals at different time points. Coefficient of variation (CoV) was calculated using the formula of CoV (%) = standard deviation/mean x 100 with mean values and standard deviations of GRMD (golden retriever muscular dystrophy) dogs and healthy control animals at different time points. Higher coefficient of variation in LV $G_{Endo-Epi}$, twist, longitudinal strain and ejection fraction in GRMD dogs than in healthy control dogs reflected the heterogeneous alterations in LV function of GRMD dogs, which is a characteristic of GRMD model. n = 23, 20, 16, 13, 8 and 8 for GRMD dogs, and 7, 7, 4, 4, 4 and 4 for control dogs at 2, 6, 9, 12, 18 and 24 months of age, respectively. Longitudinal strain was analyzed at the apical 4-chamber

view. $G_{Endo-Epi}$: transmural myocardial velocity gradient between the subendocardium and the subepicadium.