Mitral valve transcriptome analysis in thirty-four age-matched Cavalier King Charles Spaniels with or without myxomatous mitral valve disease

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Abstract

We here report the results of a mitral valve transcriptome study designed to identify genes and molecular pathways involved in development of myxomatous mitral valve disease (MMVD) in dogs. The study is focused on a cohort of elderly age-matched dogs (n=34, age ~10 years) from a single breed – Cavalier King Charles Spaniels – with a high incidence of MMVD. The cohort comprises 19 dogs (10, 9) without MMVD, or with early stages of MMVD, and 15 dogs (6, 9) with congestive heart failure caused by MMVD. I.e. we compare gene expression in breed and age matched groups of dogs, which only differ with respect to severity of disease. We identify 56 genes, which are differentially expressed between the two groups. In this list of genes, we confirm an enrichment of genes related to the TNFβ signaling pathway, extracellular matrix organization, vascular development, and endothelium damage, which also have been identified in previous studies. However, the genes with the greatest difference in expression between the two groups are CNTN3 and MYH1. Both genes encode proteins, which are predicted to have an effect on the contractile activity of myocardial cells, which in turn may have an effect on valvular performance and hemodynamics across the mitral valve. This may result in shear forces with impact on MMVD progression.

Introduction

Myxomatous mitral valve disease (MMVD) is the most common heart disease in dogs. It is especially common in small size dog breeds but the Cavalier King Charles Spaniel (CKCS) stands out as a breed with an extraordinary high prevalence of this disease (Darke 1987; Häggström et al. 1992).

MMVD has been intensively studied over the last decades and several excellent reviews have been published (e.g. Aupperle and Disatian 2012; Borgarelli and Buchanan 2012; Borgarelli and Haggstrom 2010; Burchell and Schoeman 2014; Fox 2012; O’Brien et al. 2021). The disease is characterized by morphological changes in the mitral valve leaflets, chordae tendineae and chordal-papillary muscle. The changes include elongation and thickening of the valves in combination with derangement of connective tissue, reduction in connective tissue density, damages to the basement membrane and endothelium, damages to the valve collagen matrix and accumulation of immature collagens, laminin, proteoglycans and glycosaminoglycans in the extracellular matrix (ECM) of the mitral valves (Aupperle et al. 2009a; Buchanan 1977; Corcoran et al. 2004; Falk et al. 2010; Hadian et al. 2010; Han et al. 2010; Han et al. 2013a; Han et al. 2013b). The morphological changes result in inadequate biomechanical properties of the heart valves, insufficient closure and subsequent regurgitation of blood from the left ventricle to the left atrium (Sargent et al. 2015). In severe cases, consequences of MMVD include congestive heart failure (CHF) resulting in reduced expected lifespan and reduced life quality for both dog (Boswood et al. 2018; Haggstrom et al. 2013) and owner (Clements et al. 2003). Lifelong treatment is usually required (Borgarelli et al. 2012; Borgarelli et al. 2008; Boswood et al. 2018; Haggstrom et al. 2013; Keene et al. 2019). Alternatively, heart valve surgery is also a possibility for dogs now (Aoki et al. 2022; Griffiths et al. 2004; Liu et al. 2020; Matsuura et al. 2022; Mizuno et al. 2013; Uechi et al. 2012; Yoshida et al. 2022).
It has been demonstrated that genetics play an important role for MMVD (Olsen et al. 1999; Stern et al. 2015; Swenson et al. 1996) and selective breeding can reduce MMVD prevalence in CKCS (Birkegard et al. 2015). Numerous studies have been performed to identify and elucidate the genetic and molecular mechanisms underlying MMVD. This includes genome-wide association studies (French et al. 2012; Madsen et al. 2011; Stern et al. 2015), whole genome sequencing studies (Axelsson et al. 2021; Williams et al. 2021), proteome analysis (Locatelli et al. 2017), micro-RNA studies (Jung and Bohan 2018; Li et al. 2015b; Yang et al. 2017; Yang et al. 2018), gene expression / transcriptome studies (Li et al. 2015a; Lu et al. 2015; Markby et al. 2020b; Markby et al. 2017b; Oyama and Chittur 2006; Zheng et al. 2009), and other genomic studies (Bionda et al. 2020). While previous gene expression studies have included several dog breeds of different age and multiple stages of MMVD, the present study focus exclusively on CKCS and, more specifically, on two large age-matched groups of CKCS with no/early MMVD and CKCS with CHF caused by MMVD. Hence, the objective of the present study is to identify differentially expressed (DE) genes, which may explain the difference in MMVD progression in two groups of elderly dogs, which resemble each other very closely except for their MMVD status.

Materials And Methods

Animals and sample collection

Privately owned CKCS with no MMVD or different stages of MMVD were recruited at time for elective euthanasia at Department of Veterinary and Animal Sciences, University of Copenhagen. The dogs were collected from August 2008 to August 2020. The study was approved by the Danish Animal Experiments Inspectorate (licenses no. 2006/561−1145, 2011/561−71 and 2016-15-0201-01074). Dogs with cardiac disease other than MMVD were excluded. Some of the dogs have previously been included in other studies with other research aims (Christiansen et al. 2021; Cremer et al. 2015; Cremer et al. 2014; Madsen et al. 2011; Moesgaard et al. 2014; Moesgaard et al. 2012; Rasmussen et al. 2012; Reimann et al. 2021; Reimann et al. 2017; Reimann et al. 2014a; Reimann et al. 2016; Reimann et al. 2014b; Spiljak Pakkanen et al. 2012; Zois et al. 2013; Zois et al. 2012). Prior to euthanasia and upon written owner consent, all dogs underwent a clinical examination using a standardized protocol including owner interview, physical examination, auscultation and echocardiography as previously described (Reimann et al. 2021). Mitral regurgitation murmur intensity was graded on a scale of 1−6 (Gompf 1988). The diagnosis of MMVD was based on auscultation, echocardiography and presence of clinical signs of congestive heart failure due to MMVD, according to the American College of Veterinary Internal Medicine (ACVIM) MMVD classification guidelines (Keene et al. 2019). Echocardiographic assessment and ACVIM staging was performed as described elsewhere (Reimann et al. 2021). The dogs were divided in two groups based on severity of MMVD symptoms. One group contained a dog without MMVD (ACVIM stage A) plus dogs with early stage MMVD (ACVIM stage B). In this study, we will collectively term this group of dogs ‘no/early MMVD’. The other group of dogs, which we in this study will term ‘severe MMVD’, were diagnosed with CHF due to MMVD (ACVIM stage C). CHF diagnosis was based on a history of MMVD, previous or current clinical signs of CHF (e.g., cough, dyspnea, tachypnea, nocturnal restlessness, and exercise intolerance),
echocardiographic changes compatible with severe MMVD and response to diuretic treatment. Dogs were euthanized using pentobarbitale (200–400 mg/kg or until effect, IV) after sedation and pain relief with butorphanol (0.1 mg/kg, IM) and dexmedetomidine (0.02 mg/kg, IM). The heart was collected and the mitral valves were excised within 60 minutes after euthanasia and stored in RNAlater (Merck KGaA, Darmstadt, Germany).

RNA isolation and sequencing

RNA was isolated from mitral valves using the Qiagen RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instruction including DNase treatment. RNA quantity was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and RNA quality, expressed as an RNA Integrity Number (RIN), was determined for each sample using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Subsequently, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase, and then RNaseH was used to degrade the RNA. Second strand cDNA synthesis was subsequently performed using DNA polymerase I and dNTP. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, adaptors with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 370 ~ 420 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). After PCR amplification, the PCR product was purified by AMPure XP beads, and the sequencing library was finally obtained. Libraries were constructed at E-GENE, Shenzhen, China and sequenced by an Illumina NovaSeq 6000 platform at Novogene, Beijing, China. Paired-end sequencing reads of 150bp were generated.

RNA sequence quality was ascertained using FastQC (Andrews 2010) and reads were mapped to the canFam4/UU_Cfam_GSD_1.0 dog genome assembly using the RNA sequence aligner STAR (Dobin et al. 2013). Subsequently, a feature annotation file was compiled based on the canFam4 NCBI-RefSeq annotation. This set of features was augmented by a set of putative genes extracted among canFam4 aligned human XenoRefSeq genes from the UCSC genome browser repository. These step captured the presence of protein coding genes, which may not have been properly annotated in the canine genome. Mapped RNA sequence reads were concomitantly assigned to genomic features and counted using featureCounts (Liao et al. 2014). Lowly expressed genes, i.e., features with a sum of less than 100 counts across all samples, were filtered out and excluded from further analysis.

Analyses of gene expression

The BioLayout-3.4 network analysis tool (Theocharidis et al. 2009) was used to cluster samples based on similarity of overall gene expression pattern and visualize the results in a three-dimensional network where nodes represent samples and the distance in space between nodes represent the correlation in gene expression between two samples. When cases and controls are marked up in the network, any
clustering according to MMVD status will be observable. The tool was used to calculate Pearson correlation coefficients based on gene expression data, and the advanced graph layout Fruchterman-Rheingold algorithm was used to visualize the results. In order to minimize noise and to emphasize biologically meaningful results, very abundant features (i.e. essential household genes), with over 10,000 reads per feature per sample on average, were filtered out before the BioLayout analysis. A Pearson correlation coefficient cut-off of 0.93 was used for clustering. This was the highest cut-off that allowed a combined cluster of all samples.

An analysis of differences in gene expression between dogs with no/early MMVD and dogs with severe MMVD was performed using the R package DESeq2 (Love et al. 2014). Analyses were performed on the raw un-normalized feature count data in accordance with the DESeq2 documentation and guidelines. Sex was included as a fixed effect. A volcano plot illustrating the results was created using the R package ggplot2 (Wickham 2016). A Benjamini-Hochberg false discovery rate (FDR) threshold of 0.1 was used to identify significant DE-genes.

**STRING network analysis**

A STRING network and enrichment analysis was performed using Cytoscape (Jensen et al. 2009; Shannon et al. 2003). The analysis was performed using gene names for all DE genes with a FDR < 0.1. Two analyses were performed, one using the STRING default confidence score of 0.4 and one using a stringent confidence score of 0.9. Furthermore, up to 10 additional interactors were allowed in both analyses. The default confidence network provided an overview of the functional networks of which the identified DE genes play a role. The stringent confidence network enabled identification of the functions for which the dataset provided the strongest evidence. Inclusion of additional interactors allowed STRING to build a network even though specific components (genes) could be missing in the dataset.

**Results**

Thirty-four dogs were included in the study. Nineteen dogs (10 females and 9 males) had no/early MMVD. These included one dog with ACVIM stage A, 10 dogs with ACVIM stage B1, and eight dogs with ACVIM stage B2. Furthermore, 15 dogs (6 females and 9 males) had severe MMVD and were diagnosed with CHF due to MMVD indicated by ACVIM stage C. Age and body weight did not differ significantly between the two groups of dogs (Table 1, Fig. 1).
Table 1: Descriptive statistics for dogs with no/early (n=19) or severe (n=15) MMVD. sd = standard deviation. Min = minimum. Max = maximum. LAAo ratio = the ratio of diameters for the left atrium and aorta. LAAo ratio was missing for one dog with asymptomatic MMVD. LVIDDN = Left ventricular end diastolic diameter normalized for body weight. Three dogs (two B2 and one C) underwent the full clinical examinations, auscultation and echocardiography 4 months before euthanasia. None of them had developed clinical signs that would indicate a change of MMVD status on the day of euthanization. Tissue collection was delayed for one dog (in RNAlater 90 minutes after euthanasia). This had no observable effect on gene expression results.

RNA isolation resulted in 34 samples with a RIN number > 8.6 (average 9.23, sd = 0.37) and sequencing resulted in 22.4–35.6 M reads per sample with an average of 27.7 M reads per sample remaining after the quality check.

The result of the BioLayout gene expression correlation analysis is illustrated in a three-dimensional sample-to-sample weighted network graph in Fig. 2. Dogs with no/early MMVD are marked with blue spheres and dogs with severe MMVD are marked using red spheres. A clear clustering of dogs with severe MMVD is observed. This indicates that the advanced stage of MMVD was associated with a distinct gene expression pattern. However, it is also evident that several dogs with no/early MMVD had expression patterns that overall resembled the pattern observed in dogs with severe disease.
A more detailed gene-by-gene analysis of differences in gene expression between dogs with no/early MMVD and dogs with severe MMVD was performed using the DESeq2 software package. We used a significance threshold of 0.1 for the p-adj value calculated by DESeq2. This value is a Benjamini-Hochberg FDR, which effectively corrects for multiple testing. The analysis revealed a set of 56 genes, which were significantly differentially expressed between the two groups (Table 2, Fig. 3).
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Table 2: Results of the DESeq2 analysis. baseMean = Average of normalized feature counts across all samples. lfcSE = standard error of log2FoldChange. pvalue = unadjusted p-value. FDR = Benjamini-Hochberg false discovery rate.

Compared to dogs with severe MMVD, five genes were down regulated with a log2FoldChange < -1 and fifteen were up regulated with a log2FoldChange > 1 in CKCS with no/early MMVD. Among these DE-genes, two genes, MYH1 and LOC102724058, were up regulated with a log2FoldChange > 3. The most down regulated gene was CNTN3 with a log2FoldChange of -2.62.

A STRING network analysis for significant DE genes allowing for ten additional interactors and a stringent confidence level of 0.9 revealed a functional network incorporating 20 genes (13 DE genes plus 7 additional interactors) (Fig. 4) and a small network of three genes (2 DE genes, ALDH1A2, RDH10 plus 1 additional interactor, CYP26A1). A functional enrichment analysis of the large network revealed a predominance of genes involved in TGFβ signaling and ECM organization, whereas the three genes in the small network all were involved in retinol metabolism. Additionally, genes in the large network were involved in focal adhesion, laminin interaction, circulatory system development and more (see Supplementary Table 1). We furthermore used the full set of DE genes to build networks using the STRING default confidence level of 0.4. This resulted in a network comprising 37 genes of which 27 were DE genes and the remaining 10 genes were additional interactors (Fig. 5). A functional enrichment analysis on the default confidence network revealed significant enrichment of genes involved in e.g. ‘regulation of cell population proliferation’, ‘estrogen-dependent nuclear events downstream to ESR-membrane signaling’ and ‘fluid shear stress and atherosclerosis’ (Supplementary Table 2).

Four of the 13 DE genes in the high confidence large network had an observed |Log2FoldChange| > 1. Ten of the 27 DE genes in the default confidence large network had a |Log2FoldChange| > 1. It is worth noticing that the genes with the greatest difference in gene expression between CKCS with no/early MMVD and CKCS with severe MMVD (|Log2FoldChange| > 2) were not part of the identified networks.

Discussion

We here report the results of a mitral valve transcriptome analysis performed in a cohort of 34 age-matches CKCS dogs with no/early MMVD (n = 19) or severe MMVD (n = 15).

We identified 56 genes, which were differentially expressed between the two groups. Among these, we identified genes, which previously have been associated with heart disease, and gene networks, which previously have been associated with MMVD development. Furthermore, we identified a small number of genes of importance for sarcomere assembly and function, which we hypothesize, may have a close connection with the genetic causes of MMVD.
It should be noted, that we do not strictly compare healthy and affected dogs. Rather, the control group (no/early MMVD) contained dogs, which were not as severely affected by MMVD as the case dogs (severe MMVD), even though they had the same age. The majority of the control dogs had a clear heart murmur due to mitral regurgitation and they were classified as ACVIM stages B1 or B2. The cases – severe MMVD – on the other hand were classified as ACVIM stage C. Age of dogs in both groups were around 10 years, with a slightly higher average age in the no/early MMVD group. I.e. we only looked at elderly dogs. We looked at one group of dogs, which at this age had developed a more advanced and critical stage of MMVD compared to another group of dogs with less severe disease. It would have been preferable to have a control group of CKCS dogs without any sign of MMVD. Unfortunately, as we aimed to perform this study on age-matched groups of dogs, such dogs were not available. The majority of elderly CKCS dogs have some degree of murmur due to regurgitation (Beardow and Buchanan 1993).

Previous MMVD-focused transcriptome studies have typically been performed on 3–6 MMVD cases and a similar number of controls. Most often there has been an uneven distribution of breeds between cases and controls and a significant difference in age between the two groups (Li et al. 2015a; Lu et al. 2015; Markby et al. 2017a; Markby et al. 2020a; Markby et al. 2020b; Oyama and Chittur 2006; Zheng et al. 2009). Significant strengths in the present study are that we included more individuals and that the two groups of dogs only differed in one parameter, i.e. severity of MMVD. Thus, the number of possible confounding factors was smaller compared to previous studies. In the present study, we searched for alterations in gene expression, which could explain severity in MMVD development in ~ 10 years old CKCS and/or patterns in gene expression, which demonstrated compensatory mechanisms explaining the difference in resilience to development of severe disease at this age.

Compared to several of the above-mentioned transcriptome studies, the present study found a relatively low number of DE genes. This was probably due to the close similarity between the case and control groups and the absolute minimization of confounding factors in the present study. Furthermore, the larger sample size improved the power to avoid spurious differences in feature counts between the two groups. I.e., it reduced the risk of false positive results in the DE analysis. The benefit of this is that the observed differences in gene expression can more reliably be assigned to disease status.

In the functional enrichment analysis, we identified some of the same pathways as have been identified in previous studies. This included the TNFβ signaling pathway, pathways related to ECM organization, and pathways related to vascular development, endothelium damage, and metallopeptidases (Aupperle et al. 2009b; Li et al. 2015a; Lu et al. 2015; Markby et al. 2020a; Markby et al. 2020b; Moesgaard et al. 2014; Oyama and Chittur 2006; Zheng et al. 2009).

Among DE-genes detected in the present study, CRIP1 and SERPINE1 were also identified as DE genes by Markby et al. (2020b). In a more detailed comparison of our results with this study, we noticed that many of the DE genes detected in the two studies are members of the same gene families. For example, several ADAMTS variants, collagen genes, laminins and different myosin genes were detected in both studies.
Some of the DE genes identified in the present study have previously been directly linked to cardiac diseases including valve disease. ADAMTS19 has been associated with progressive non-syndromic heart valve disease (Wünnemann et al. 2020). ALDH1A2 has been linked to human congenital heart disease (Pavan et al. 2009). BMPER regulates cardiomyocyte size and changes in the mitral valve have been detected in BMPER knockout mice (Willis et al. 2013). COL17A1 has been linked to mitral regurgitation and mitral valve prolapse in humans (Uysal et al. 2022). CRIP1 expression has been associated with cardiac hypertrophy and an increased risk of stroke (Zeller et al. 2017). A partial deletion of CYP21A2 has been found in patients with mitral valve prolapse (Chen et al. 2009). MAOA can play a role in the pathogenesis of heart failure (Kaludercic et al. 2011). MYH1 is one of many genes important for the morphogenesis of the heart (England and Loughna 2013; Henderson et al. 2017) and MYHAS encodes an antisense RNA, which regulate expression of myosin heavy chain genes including MYH1 (Haddad et al. 2003).

We primarily found a number of genes upregulated in dogs with no/early MMVD and only a few genes down regulated in this group. Many of the upregulated genes play a central role in mechanisms, which can be considered beneficial for heart maintenance. Hence, a relevant question is, why some dogs (the no/early MMVD dogs) could institute an appropriate response to whatever caused the disease, and why some dogs (the severe MMVD dogs) did not do this?

Among the DE genes identified in the present study, ALDH1A2 and RDH10 formed a small high-confidence functional network together with the additional interactor CYP26A1. The network is closely connected with retinoic acid (RA) biosynthesis, i.e., the oxidation of retinol (vitamin A) to retinaldehyde and the subsequent irreversible conversion of retinaldehyde to RA. RDH10 is the primary enzyme responsible for the first step in this reaction (Farjo et al. 2011), while the later step is catalyzed by retinaldehyde dehydrogenases (RALDHs) among which, the aldehyde dehydrogenase 1A2 (ALDH1A2) is the major form involved in cardiac development (Moss et al. 1998; Niederreither et al. 1997). A number of variations in ALDH1A2 has been described in human patients with congenital heart disease (CHD) but none of them could be confirmed as significant modifiers of the risk of CHD in humans (Pavan et al. 2009). Genes involved in RA biosynthesis have previously been associated with MMVD in dogs. A retinoic acid receptor responder (RARRES3) was associated with MMVD in a microarray gene expression study performed in 10 CKCS dogs with MMVD, Whitney grade ≥ 3 and 6 dogs without signs of MMVD (Lu et al. 2015). Furthermore, RA signaling and the ALDH1A2 gene has been linked to cardiac repair mechanisms in mice (Da Silva et al. 2021). Hence, we suggest that the observed changes in expression of ALDH1A2 and RDH10 may be a compensatory reaction to MMVD rather than a cause of disease. The interpretation of our results is thus that dogs, which upregulate expression of these two genes, have a better chance of not developing CHF due to MMVD. Why some dogs had an appropriate up regulation of these genes and why some did not, needs to be investigated further.

Overall, it must be expected that compensatory mechanisms to disease, including MMVD, are established and managed in an orchestrated way, which in a transcriptome analysis will appear as networks of functionally related genes expressed in a coordinated manner. Hence, it is not surprising that many of the
genes in the identified larger networks are involved in mechanisms such as TGFβ-signaling and ECM organization. Abundance of myxomatous effector proteins has previously been shown to increase in response to increased tensile strain on the heart valves (Lacerda et al. 2012; Orton et al. 2012). TGFβ-signaling and disturbances in ECM organization have also been suggested as primary causes of MMVD (reviewed by Tang et al. 2022). However, the present results, i.e., the apparent well-orchestrated expression of genes related to these pathways, encourage us to suggest that these pathways were upregulated as a well-regulated compensatory mechanism to MMVD in ~10 years old dogs with no/early MMVD. This up-regulation resulted in a more benign development of the disease, i.e., a disease that did not progress into severe MMVD with CHF.

On the other hand, the genes with the greatest difference in expression between no/early MMVD and severe MMVD were three genes, \textit{MYH1}, LOC102724058 and \textit{CNTN3}, which were not part of the identified gene networks. I.e., they were not part of an orchestrated response to disease but might instead be possible causative agents of disease.

Of these three genes, LOC102724058 is a human gene that aligns to a 17 kb region of canine chromosome 36 with 91% identity. It was one of the features that were identified by augmenting the canine reference annotation with homologous human genes (see Material & Methods section). The gene is a long non-coding RNA gene with unknown function. The other two genes, \textit{CNTN3} and \textit{MYH1}, encodes contactin 3 and myosin heavy chain 1, respectively. Contactin 3 has among other things been associated with heart rate recovery after exercise (Verweij et al. 2018). \textit{MYH1} expression was significantly upregulated in mice with cardiomyopathy (Szema et al. 2013). Both of these genes relate to the contractile activity of the heart, the coordination of this activity, and consequently the hemodynamics across the mitral valves. This may explain the observed DE of genes in the default-confidence network related to ‘fluid shear stress and vascular changes’, which furthermore corroborate the observed arteriosclerotic changes in dogs with MMVD (Falk et al. 2006). Comparatively, a vast number of different human myopathies including cardiomyopathies are caused by mutations in one of the many cytoskeletal sarcomeric proteins, of which \textit{MYH1} is one (reviewed by Henderson et al. 2017). Mitral valve function relies on proper biomechanical performance of several structural components including the left atrial, ventricular and papillary myocardium (Fox 2012; Richards et al. 2012; Schoen 2008). A disturbance in the hearts contractile activity due to suboptimal coordination of sarcomere assembly and function may explain the compensatory responses illustrated by the TGFβ-signaling and ECM organization related gene networks described here. Mitral regurgitation secondary to myocardial dysfunction is well known in human patients (Asgar et al. 2015). Interestingly, myocardial bers are present in the proximal third of the mitral valve in dogs (Buchanan 1977; Fox 2012). Hence, it is possible that the changes in valvular gene expression, relevant for myocardial function, affect valvular performance and integrity. Based on these observations, we suggest that the primary cause of MMVD in CKCS may be found in a gene coding for one of the heart muscle proteins. However, the conclusion based on the present results is that an appropriate upregulation of \textit{MYH1} and downregulation of \textit{CNTN3}, as a response to a so far unknown causative factor, can protect a dog with MMVD from developing CHF. An alternative conclusion is that an
inappropriate downregulation and upregulation of MYH1 and CNTN3, respectively, in CKCS with MMVD may lead to development of CHF.

**Statements And Declarations**

The authors declare that the research was conducted and all the results are reported here in the absence of any commercial, financial or non-financial relationships that could be construed as a conflict of interest.

**Author Contributions**

MJR contributed to clinical examination including echocardiography, euthanasia and sample collection and performed echocardiographic assessment. SiCR contributed to euthanasia and sample collection. LC contributed to euthanasia and sample collection. EI contributed to differential gene expression analyses. FG performed RNA sequencing. SuCi performed RNA isolation and contributed to interpretation of results. MF contributed with analyses of results and final interpretation. LHO contributed to clinical examination including echocardiography, euthanasia, and sample collection and was in charge of diagnostic procedures and sample collection. PKM conceived the study, performed data analyses, performed analyses of results and final interpretation, and drafted the manuscript. All authors read, commented and approved the final manuscript.

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**Data Availability**

All data are made available in the Gene Expression Omnibus repository. GEO accession: GSE217750

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**Figures**

**Figure 1**

Split-violin plots and box-plots illustrating descriptive statistics and key diagnostic parameters for dogs with no/early MMVD (n=19) and dogs with severe MMVD (n=15). LVIDDN = left ventricular end-diastolic diameter normalized for body weight. LAAo-ratio = the ratio of diameters for the left atrium and aorta.
Based on the box-plots, two dogs with no/early MMVD were identified as outliers (grey dots): One with regard to body weight and another dog with regard to LAAo ratio and LVIDDN. Both were kept in the dataset based on an overall evaluation of their status.

Figure 2

Three-dimensional illustration of the results of the BioLayout gene expression correlation analysis. Blue = dogs with no/early MMVD; Red = dogs with severe MMVD. The distances between dots corresponds to the correlations in overall gene expression between individuals.
Figure 3

Volcano plot of DESeq2 analysis results. The log2FoldChange is on the X-axis and the negative logarithm of the false discovery rate (FDR = padj) is on the Y-axis. The FDR threshold of 0.1 is indicated by a horizontal line. Vertical lines indicate log2FoldChanges of -1 and +1
Figure 4

High confidence large functional network constructed using STRING. Marked with light blue: Genes involved in TGFβ signaling. Marked with dark blue: Genes involved in extracellular matrix organization.
Figure 5

Default confidence large functional network constructed using STRING. Genes associated with the KEGG pathway “Fluid shear stress and atherosclerosis” are marked with pink circles

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- SupplTab1enrichmenttable.xlsx
- SupplTab2enrichmenttable.xlsx