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### Gene network and canonical pathway analysis in canine myxomatous mitral valve disease

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Abstract: Myxomatous mitral valve disease (MMVD) is the single most common acquired heart disease of the dog and is particularly common in small pedigree breed dogs such as the cavalier king Charles spaniels (CKCS). There are limited data on the mitral valve transcriptome and the aim of this study was to use the microarray technology in conjunction with bioinformatics platforms to analyse transcript changes in MMVD in CKCS compared to normal dogs (non-CKCS). Differentially expressed genes (n = 5397) were identified using cut-off settings of fold change, false discovery rate (FDR) and  $P < 0.05$ . In total, 4002 genes were annotated to a specific transcript in the Affymetrix canine database, and after further filtering 591 annotated canine genes were identified: 322 (55%) were up-regulated and 269 (45%) were down-regulated. Canine microRNAs (cfa-miR; n = 59) were also identified.

Gene ontology and network analysis platforms identified between 6 and 10 significantly different biological function clusters from which the following were selected as relevant to MMVD: inflammation, cell movement, cardiovascular development, extracellular matrix organisation and epithelial-to-mesenchymal (EMT) transition. Ingenuity pathway analysis identified three canonical pathways relevant to MMVD: caveolar-mediated endocytosis, remodelling of epithelial adherens junctions, and endothelin-1 signalling. Considering the biological relevance to MMVD, the gene families of importance with significant difference between groups included collagens, ADAMTS peptidases, proteoglycans, matrix metalloproteinases (MMPs) and their inhibitors, basement membrane components, cathepsin S, integrins, tight junction cell adhesion proteins, cadherins, other matrix-associated proteins, and members of the serotonin (5-HT)/transforming growth factor - $\beta$  signalling pathway.

1 **Gene network and canonical pathway analysis in canine myxomatous mitral valve**  
2 **disease: A microarray study**

3

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15

16

17 **Abstract**

18 Myxomatous mitral valve disease (MMVD) is the single most common acquired heart  
19 disease of the dog and is particularly common in small pedigree breed dogs such as the  
20 cavalier king Charles spaniels (CKCS). There are limited data on the mitral valve  
21 transcriptome and the aim of this study was to use the microarray technology in conjunction  
22 with bioinformatics platforms to analyse transcript changes in MMVD in CKCS compared to  
23 normal dogs (non-CKCS). Differentially expressed genes ( $n = 5397$ ) were identified using  
24 cut-off settings of fold change, false discovery rate (FDR) and  $P < 0.05$ . In total, 4002 genes  
25 were annotated to a specific transcript in the Affymetrix canine database, and after further  
26 filtering 591 annotated canine genes were identified: 322 (55%) were up-regulated and 269  
27 (45%) were down-regulated. Canine microRNAs (cfa-miR;  $n = 59$ ) were also identified.

28

29 Gene ontology and network analysis platforms identified between six and 10  
30 significantly different biological function clusters from which the following were selected as  
31 relevant to MMVD: inflammation, cell movement, cardiovascular development, extracellular  
32 matrix organisation and epithelial-to-mesenchymal (EMT) transition. Ingenuity pathway  
33 analysis identified three canonical pathways relevant to MMVD: caveolar-mediated  
34 endocytosis, remodelling of epithelial adherens junctions, and endothelin-1 signalling.  
35 Considering the biological relevance to MMVD, the gene families of importance with  
36 significant difference between groups included collagens, ADAMTS peptidases,  
37 proteoglycans, matrix metalloproteinases (MMPs) and their inhibitors, basement membrane  
38 components, cathepsin S, integrins, tight junction cell adhesion proteins, cadherins, other  
39 matrix-associated proteins, and members of the serotonin (5-HT)/transforming growth factor  
40  $-\beta$  signalling pathway.

41

42 *Keywords:* Myxomatous mitral valve disease; Canine; Transcriptome; Pathway analysis;

43 Microarray; Cavalier king Charles spaniel.

44

## 45 **Introduction**

46 Myxomatous mitral valve disease (MMVD) is the single most common acquired heart  
47 disease of the dog and is characterised by endothelial damage, stromal matrix degeneration,  
48 interstitial cell proliferation in the sub-endothelial zone and interstitial cell phenotypic  
49 changes (Buchanan, 1977; Beardow and Buchanan, 1993; Corcoran et al., 2004; Black et al.,  
50 2005; Disatian et al., 2008; Han et al., 2008; Lacerda et al., 2009; Borgarelli and Buchanan,  
51 2012). The end-stage disease results in significant mitral regurgitation which can lead to left-  
52 sided congestive heart failure (Hägström et al., 2009). While much is known about the  
53 structural and cellular changes in canine MMVD, less is known about the molecular  
54 mechanisms and biochemical changes (Richards et al., 2012). Limited proteomic and  
55 transcriptomic data are available and provided interesting insights into disease pathogenesis,  
56 for example, the role of serotonin (5-hydroxytryptamine or 5-HT) in MMVD (Oyama and  
57 Chittur, 2006; Lacerda et al., 2009).

58  
59 The only genomics study of canine MMVD to date used the Affymetrix Canine Gene  
60 1.0 array covering approximately 23,000 gene transcripts and looked at a cohort of mixed  
61 breed dogs (Oyama and Chittur, 2006). Since that study, there have been significant advances  
62 in the quality of canine gene microarrays with much improved transcript annotation (27,681  
63 genes), transcript sensitivity (590,097 probes, 24 probes/gene) and bioinformatics tools. The  
64 study by Oyama and Chittur (2006) identified 229 probe sets that were differentially  
65 expressed (at least two-fold change; 70% up-regulated and 30% down-regulated) of which  
66 166 could be assigned to recognised genes. The main functional classes attributed to the  
67 affected genes, in descending order of importance, included cell signalling, metabolism,  
68 extracellular matrix (ECM), inflammation, cell defence, immunity, cell transport and cell  
69 structure (Oyama and Chittur, 2006). Of particular note was the ~four-fold increase in the 5-

70 *HT<sub>2B</sub> receptor* gene, which fits in with the serotonin hypothesis of MMVD pathogenesis.  
71 Limited numbers of gene expression studies have also been undertaken in human MMVD  
72 with a reported number of differentially expressed genes of around 400 ( Hulin et al., 2012;  
73 Sainger et al., 2012). These studies demonstrated informative changes in metallothioneins  
74 and ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin Motifs), and  
75 transforming growth factor  $\beta$ -2 (TGF $\beta$ -2) and bone morphogenic protein 4 (BMP-4 of the  
76 TGF- $\beta$  signalling superfamily), proteoglycans, collagens, SOX-9 and CRTAC1 (cartilage  
77 acidic protein 1), genes that are implicated in ECM production and remodelling.

78

79         However, the number of reported differentially expressed genes in any transcriptomic  
80 study is affected by the calculated false discovery rates (FDRs), fold change thresholds  
81 (correction for signal intensity) and batch effects. Additionally, there have been major  
82 developments in bioinformatics, canine annotated databases and analysis platforms. The aim  
83 of this study, therefore, was to use more stringent threshold criteria and quality control, in  
84 line with current recommended protocols and with an affected group consisting of the same  
85 breed (Cavalier King Charles spaniel, CKCS) in order to improve the reported differential  
86 expression of genes in canine MMVD, and to identify novel signalling pathways that might  
87 contribute to the pathogenesis of MMVD.

88

## 89 **Materials and methods**

### 90 *Tissue sample*

91         Myxomatous mitral valve leaflets (anterior) ( $n = 10$ , Whitney grade  $\geq 3$ ) were  
92 collected from CKCS dogs presented to the Hospital for Small Animals, Royal (Dick) School  
93 of Veterinary Studies, the University of Edinburgh. All dogs previously had been clinically  
94 confirmed to have MMVD and were on a range of treatments for congestive heart failure.

95 Control normal anterior mitral valve leaflets ( $n = 6$ ) were collected from young adult dogs  
96 euthanased for reasons other than cardiac disease. All samples were collected within 10 min  
97 of death and Whitney classified by at least two investigators (CCL, MML, BMC). The valve  
98 leaflets were immediately placed in RNAlater (Invitrogen) and stored at  $-20^{\circ}\text{C}$ . All tissue  
99 samples were collected with full owner informed consent and the study conformed to national  
100 (UK) and institutional ethical guidelines for the use of animals in research.

101

### 102 *RNA extraction and quality assessment*

103 Tissue (100 mg) was minced, snap-frozen in liquid nitrogen and pulverised for 1 min  
104 at 2000 oscillations/min in a liquid nitrogen cooled dismembrator (Braun Mikro-  
105 Dismembrator Vessel, Braun Biotech International). Phenol/guanidine HCl reagents (1 mL;  
106 TriReagent, Sigma) was added to the powdered sample and 200  $\mu\text{L}$  of chloroform was added  
107 to the microcentrifuge tubes prior to centrifugation at 12,000  $g$  for 10 min. RNA extraction  
108 from the aqueous phase and DNA digestions were carried out using commercially available  
109 kits (RNeasy Mini Kit and RNase-Free DNase Set; Qiagen). RNA purity was analysed by  
110 spectrophotometer (Thermo Scientific NanoDrop 1000, 260/280 ratio  $\approx 2$ ) and RNA quality  
111 and integrity and RNA integrity number ( $\text{RIN} \geq 7$ ) determined by electropherogram (Agilent  
112 2100 Bioanalyser). Four normal and four diseased samples reached the required quantity and  
113 quality of RNA and were stored at  $-80^{\circ}\text{C}$  prior to reverse transcription.

114

### 115 *Microarray hybridisation*

116 The Affymetrix GeneChip WT Terminal Labelling Kit and the Ambion WT  
117 Expression Kit were used to generate amplified and biotinylated sense-strand DNA targets  
118 for the Affymetrix Canine Gene 1.0 ST Array. Each ST (Sense Target) array selected probes  
119 were distributed along the entire length of each transcript. Briefly, total RNA was reverse-



120 transcribed to single-stranded cDNA, and then converted to double-stranded cDNA. The  
121 double-stranded cDNA then underwent in vitro transcription to generate unlabelled cRNA.  
122 The cRNA was cleaned for any antisense RNA, reverse-transcribed to the dUTP incorporated  
123 second single-stranded cDNA and fragmented with uracil DNA glycosylase and  
124 apurinic/aprimidinic endonuclease 1. The fragmented DNA was labelled by terminal  
125 deoxynucleotidyl transferase (TdT) with the Affymetrix proprietary DNA Labeling Reagent,  
126 covalently linked to biotin, and then hybridised to the array. The arrays were washed, stained  
127 and scanned. The two cycle RNA-cDNA amplification, hybridisation, and chip scanning  
128 were performed by ARK Genomics (Roslin Institute, UK).

129

130 For differential gene expression analysis, all the raw Affymetrix CEL files, which  
131 contained probe-sets ID and calculated pixel intensity value for each array, were imported  
132 into Partek Genomic Suite 6.6 (PGS). Normalized signal intensity data for whole probe sets  
133 were also produced by Affymetrix Expression Console. Robust Multi-array Average (RMA)  
134 was used for data normalisation and final summarisation. QC metrics combined with  
135 principal component analysis (PCA) were generated for post-import quality assessment, and  
136 two potential outliers were identified and excluded. Analysis of variance (ANOVA) was used  
137 to identify differentially expressed genes between the two groups. Hierarchical clustering was  
138 based on the significant genes, and the original gene list was used to generate a Volcano plot  
139 (Fig. 1) for total unadjusted gene expression pattern. Finally, a list of genes of interest was  
140 generated according to the cut-off of fold-changes, FDR, and signal intensity.

141

#### 142 *Multi-platform in silico categorisation and network analysis*

143 Gene categorisation and pathway analysis were conducted using a combination of  
144 ToppFun (gene list enrichment tool), DAVID 6.7 and the Ingenuity Pathways Analysis (IPA)

145 database by uploading the differentially expressed gene list to the respective online servers.  
146 Functional analysis of a gene network identified the biological functions that were most  
147 attributable to the genes in that network (Chen et al., 2009; Huang et al., 2009).

148

#### 149 *Quantitative reverse-transcription polymerase chain reaction (RT-PCR)*

150 Assays for 13 canine genes and one reference gene (Table 1) were designed using  
151 methods previously reported (Clements et al., 2006). Primers (MWG Biotech) and probes  
152 (Roche Diagnostics) were synthesised using locked nucleic acid with 5'-end labelled with a  
153 reporter fluorescein dye (FAM, 6-carboxy fluorescein) and 3'-end labelled with a dark  
154 quencher dye. In brief, quantitative RT-PCR assays were performed in triplicate in 96-well  
155 plates (LightCycler 480 system using Probes Masters; Roche Diagnostics) with one no-  
156 template control for each sample with total reaction volume of 5  $\mu$ L per well. The  
157 amplification was performed according to a standard protocol. Quantitative RT-PCR data  
158 were analysed using LightCycler 480 Basic Software (Roche Diagnostics). The reference  
159 genes was used to normalise target gene relative expression level and to calculate the  $-\Delta\Delta C_t$   
160 values.

161

## 162 **Results**

### 163 *General description*

164 RNA of sufficient quality and quantity for transcriptomic analysis was obtained from  
165 four dogs from each group (RIN > 7). There was one outlier in each group and when these  
166 were excluded 5397 differentially expressed genes were identified fulfilling the cut-off  
167 settings of fold change (> 1.5), FDR < 0.05, and  $P < 0.05$ . In total, 4002 genes were annotated  
168 and 1395 probes were not assigned to a specific transcript in the Affymetrix canine database  
169 and their sequences were tracked and matched using the NCBI nucleotide BLAST and

170 miRBase BLAST tools. Only 139 probes were successfully identified as transcript homologs  
171 to other species and 59 of these were matched with canine microRNA family members (cfa-  
172 mir-RNA) (details to be included in a further report). The gene and the data sets were further  
173 filtered by individual signal intensity value and a final total of 635 differentially expressed  
174 probe sets, representing 591 annotated canine genes, were identified, 322 (55%) were up-  
175 regulated and 269 (45%) genes were down-regulated (Appendix; Supplemental file 1).

176

### 177 *Quantitative RT-PCR*

178 For data validation between different platforms, differential expression of 13 genes  
179 were examined and 12 (*LAMA2*, *ENG*, *COL6A3*, *HTR2B*, *MYH11*, *MMP12*, *BMP6*,  
180 *ANGPT1*, *CHAD*, *ADAMTS19*, *ACTG2*, and *KERA*) were found to be in agreement with the  
181 microarray data, while one (*SOX-9*) was not (Appendix; Supplemental file 2).

182

### 183 *Gene ontology, and network and canonical pathways*

184 Using ToppFun, 28 significant terms for gene ontology (GO) and biological processes  
185 (BP) were identified. Six functional subgroups were further identified based on the biological  
186 functions of each gene ontology term (GO: BP), and included inflammation and immune  
187 response, cellular adhesion and movement, cardiovascular development, ECM, osteogenesis,  
188 and epithelial cell proliferation. In each subgroup, GO: BP terms deemed biologically  
189 relevant to MMVD were selected and identified genes that are involved in response to  
190 wounding, biological adhesion, positive regulation of cell migration, cardiovascular system  
191 development, ECM organization, ossification, and regulation of epithelial cell proliferation  
192 (Appendix; Supplemental file 3).

193

194 DAVID identified 16 biological categories based on gene functions (Table 2).  
195 Functional annotation clustering analysis after data enrichment identified 10 clusters based  
196 around similar biological function (Table 3) and the specific named genes are in  
197 Supplementary file 4 (Appendix). Analysis using IPA identified 11 significant biological  
198 functions and diseases relevant to the gene set, including endocrine system disorders, cellular  
199 movement, connective tissue disorders, inflammatory disease, immune cell trafficking,  
200 cancer, haematological system development, cardiovascular disease, humoral immune  
201 response, cardiovascular system development and cell death and survival. IPA also identified  
202 33 canonical pathways with three functions applicable to MMVD, including caveolar-  
203 mediated endocytosis signalling, remodelling of epithelial adherens junctions, and  
204 endothelin-1 signalling, were identified (Appendix; Supplementary file 5). Gene networking  
205 analysis identified three significant and biologically relevant networks for MMVD, including  
206 cardiovascular system development and function, cellular movement and cell-to-cell  
207 signalling (Fig. 2). By overlaying these data with the disease-functions-canonical pathway  
208 analysis, specific genes of interest were identified (Table 4). Analysis by IPA also identified  
209 the top 10 upstream regulators: *IL-1B*, *IL-13*, *TGFβ1*, *DYSF*, *IFN-γ*, *TNF*, *LDL*, *CSF2*, *NFκB*,  
210 and *NFE2L2*. Of these, LDL activation appears to be the most directly relevant to MMVD.

211

212 By combining analysis from the three platforms, the following relevant functional and  
213 disease categories can be derived for the MMVD transcriptome: inflammatory response, cell  
214 movement, cardiovascular development, ECM organisation and epithelial-to-mesenchymal  
215 transition.

216

217 *Individual gene signal intensity*

218 Using the data from annotation clustering analysis, genes deemed biologically  
219 relevant to MMVD from the five selected categories were examined on the basis of their  
220 signal intensity and the details are shown in Table 5. The gene families of particular interest  
221 were: collagens, ADAMTS peptidases, proteoglycans, matrix metalloproteinases (MMPs)  
222 and their inhibitors (TIMPs), basement membrane components (nidogen1 and laminin2),  
223 cathepsin S, integrins, tight junction cell adhesion proteins (claudin, occludin), cadherins, and  
224 other matrix-associated proteins (fibrillin, fibronectin, periostin, fibulin, HAS2). Other genes  
225 that showed reasonably high expression, but were not necessarily significantly changed  
226 between the two groups, included: elastin, NOS, adhesion molecules (PECAM, VCAM),  
227 NOTCH, Snai1,  $\beta$ -catenin, members of the TGF- $\beta$  superfamily (TGF $\beta$ -1, endoglin, BMP9,  
228 BMPR1B) and cytoskeleton proteins (actin, myosin, SM22).

229

## 230 **Discussion**

231 Transcriptional profiling for this study was carried out using the Affymetrix Canine  
232 Gene 1.0 ST Array. In total, 590,097 probes against 27,681 genes (both annotated and  
233 predicted canine genes) were included according to the genome information from canFam2.  
234 The design of 26 unique 25-mer probes for each transcript allowed for the highest coverage  
235 yet and provided a more accurate detection of transcribed genes and a higher resolution than  
236 3'-biased microarrays. The only previous report of the canine mitral valve transcriptome used  
237 the first generation array Affymetrix GeneChip Canine Genome 1.0 (Oyama and Chittur,  
238 2006). The current study confirmed the previous findings of up-regulation of *5-HTR<sub>2B</sub>*,  
239 *endoglin* and *BMP-6*, but did not find differences in the expression of *TGF- $\beta$*  or *TGF- $\beta$*   
240 *receptors*.

241

242 In human MMVD, the two published transcriptomic studies also reveal an  
243 orchestrated regulation of *TGF-β* and *BMP-6*, and in functional studies, control of ECM  
244 production and valve interstitial cell activation by BMP-4 (Hulin et al., 2012; Sainger et al.,  
245 2012). However, there are no reports of up-regulation of *5-HTR<sub>2B</sub>* in any of the human valve  
246 studies. The findings of interest in the human mitral valve transcriptome are down-regulation  
247 of metallothioneins-1 and -2 (MT1/2) and members of the ADAMTS family of proteases, and  
248 increased expression of genes encoding for ECM components, including collagens,  
249 proteoglycans and MMPs. In contrast, in the dog, ECM genes were generally down-regulated  
250 or unchanged. While canine and human MMVD share many similar features, the diseases  
251 appear to be different at least at the transcriptomic level.

252

253 The possible involvement of the TGF-β signalling superfamily in MMVD is not  
254 surprising as it has important roles in cancer, fibrosis, and calcification (Geirsson et al.,  
255 2012). TGF-β signalling through SMAD pathways triggers myofibroblastic differentiation of  
256 valve interstitial cells and increases expression of SMAD-targeted genes associated with  
257 ECM such as COL1A, 3A1, 6A1, 6A3, elastin and TIMP1 (Verrecchia et al., 2001; Walker et  
258 al., 2004). However, there was no differential expression of the TGF-βs and their receptors or  
259 downstream target genes between the normal and affected dogs, although high signal  
260 intensity for various members indicated their role in valve matrix homeostasis. Increased  
261 TGFβ-1 and TGFβ-3, but not TGFβ-2, expression has been shown using  
262 immunohistochemistry in canine valves (Aupperle et al., 2008). These differences between  
263 gene and protein expression may be due to post-transcriptional modification. This contrasts  
264 with human myxomatous mitral valves where there is higher transcriptional level of all  
265 isoforms of TGF- β and the downstream genes *COL1A1*, *COL3A1* and *elastin*, suggesting  
266 activation of fibrotic mechanisms (Geirsson et al., 2012).

267           The identification of LDL activation and its positive association with 17/23  
268 downstream genes using IPA was an unexpected but interesting finding for MMVD. LDL  
269 signalling through low-density lipoprotein receptor-related protein-5 (Lrp5) has been shown  
270 to play a crucial role in calcific degeneration in human aortic and mitral valves (Neufeld et al.  
271 2014). There is an association between LDL and aging in dogs which is coincidental with the  
272 natural history of MMVD, and LDL is worth considering as a potential contributing factor to  
273 MMVD in this species (Buchanan, 1977; Beardow and Buchanan, 1993; Osorio, 2009;  
274 Borgarelli and Buchanan, 2012).

275

276           Increased expression of inflammation-associated cytokine genes in the diseased mitral  
277 valve appears to be a consistent finding, but is not reported in the human mitral valve (Oyama  
278 and Chittur, 2006; Hulin et al., 2012; Sainger et al., 2012). IPA identified, central to the  
279 inflammation network, up-regulation of toll-like receptor 4 (*TLR4*) and interleukin 18 (*IL-*  
280 *18*), as well as *IL-6*, *TLR1* and *TLR8*. Since there is no evidence of inflammatory cell  
281 contribution to MMVD pathogenesis, ECM degeneration and remodelling might be  
282 triggering TLR receptor signalling which could further contribute to ECM changes. Heat  
283 shock protein 70, fibronectin, hyaluronic acid, heparan sulfate, and hyaluronan, all important  
284 components of ECM remodelling in MMVD, can act as endogenous ligands for TLRs (Chao,  
285 2009). Finally, IL-6 can trigger endothelial-mesenchymal transformation (EndoMT), an  
286 important mechanism during valve development, and potential contributor to the  
287 pathogenesis of MMVD (Mahler et al., 2013).

288

289           Matrix gene expression changes have been found in canine and human MMVD, and  
290 included genes encoding for ECM and basement membrane (BM) proteins. The BM is  
291 important for maintaining endothelial integrity, and endothelial damage, activation and cell

292 loss are features of canine MMVD (Corcoran et al., 2004; Han et al., 2013). Gene network  
293 analysis identified down-regulation of laminin beta 1 (*LAMBI*) and alpha 2 (*LAMA2*),  
294 nidogen-1 (*NIDI*) and *COL6A3*, and increased expression of the protease cathepsin S (*CTSS*),  
295 all of which would reflect endothelial damage or BM dismantling as part of EndoMT  
296 (Lakatta and Levy, 2003; Li and Bertram, 2010). Breakdown of NID1, LAMB, COL and  
297 elastin by CTSS has been shown to impair BM integrity and stability (Sage et al., 2012; Turk  
298 et al., 2012). Increased expression of CTSK and CTSS has been reported in human MMVD,  
299 and cyclic strain increases CTSK expression in sheep mitral valve myofibroblasts (Rabkin et  
300 al., 2001; Aikawa et al., 2006; Lacerda et al., 2012).

301

302         Regarding ECM proteins, there were not surprisingly high intensity signals for many  
303 collagen genes and these tended to be lower in the disease group (*COL1*, *COL2*, and *COL4*),  
304 but only statistically different for *COL6A3*. There is a marginal and localised reduction in  
305 collagen expression in MMVD, at least in mild to moderately affected dogs, and this would  
306 be reflected by these gene expression changes (Hadian et al., 2010). The reduction in  
307 *COL6A3* is important because its role in BM production and force-resistant collagen bundle  
308 formation suggests a clear contribution to the pathogenesis of MMVD (Klewer et al., 1998;  
309 Kruithof et al., 2007). Collagen maturation from procollagen relies on the ADAMTS family  
310 of metalloproteases, and *ADAMTS2*, *ADAMTS9*, and *ADAMTSL4* were all significantly  
311 down-regulated in the diseased canine mitral valves. Lower expression of *ADAMTS2* and  
312 *ADAMTS9* suggests an inactive collagen turnover state exists, and this has been shown in  
313 *ADAMT9*-deficient mice, and in canine MMVD using X-ray diffraction and HPLC (Hadian  
314 et al., 2010; Kern et al., 2010). *ADAMTS4* also has a regulatory role on fibrillin-1 and low  
315 expression will decrease elastin fibre formation in MMVD in a manner similar to that seen in  
316 Marfan syndrome (Chandra et al., 2012).



317

318           Gene expression for a limited number of MMPs and their tissue inhibitors (TIMPs)  
319 has been previously reported using a combination of microarray and PCR, and expression at  
320 the protein level has also been reported using immunohistochemistry. In the current study, 25  
321 MMPs and 4 TIMPs were identified, but in general the data contradicted previous reports in  
322 particular for *MMP1*, which had a low intensity signal, but has been previously reported as  
323 increased using PCR and immunohistochemistry (Oyama and Chittur, 2006; Disatian et al.,  
324 2008; Aupperle et al., 2009, 2012). Remodelling activity at the time of sampling are likely to  
325 have effects on global MMPs and TIMP expression profiles and changes in expression need  
326 to be interpreted with caution (Rabkin et al., 2001; Rabkin-Aikawa et al., 2004).

327

328           Overall gene ontology analysis for all studies reported to date show similar biological  
329 functions in the differentially expressed gene sets, including genes involved in cell signalling,  
330 inflammation, extracellular matrix, immunity, cell defence, and metabolism. In the current  
331 study, additional functional categories of cellular movement and epithelial-to-mesenchymal  
332 transition were identified. Three canonical pathways were selected that would appear most  
333 relevant to the pathogenesis of MMVD: caveolae-mediated endocytosis, which controls  
334 endothelial cell growth, cell migration and can affect TGF-  $\beta$  signalling-induced fibroblast  
335 activation; endothelin signalling, which has a variety of functions including ECM  
336 remodelling; and remodelling of epithelial adherens junction, which with vascular endothelial  
337 (VE)-cadherin can affect cell proliferation and migration (Mow and Pedersen, 1999;  
338 Salanueva et al., 2007; Galdo and Lisanti, 2008; Sowa, 2012).

339

340           For all ECM products, proteoglycan (PG) genes had the highest signal intensity with  
341 lumican, versican, and biglycan being the three strongest, but only chondroadherin (*CHAD*)

342 and keratocan (*KERA*) were significantly down-regulated in the CKCSs. *KERA* is a small  
343 leucine rich PG and plays a pivotal role in ECM assembly in the cornea to maintain  
344 translucency, and *CHAD* is an anchor to the matrix by binding tightly to collagens I, II, and  
345 VI in cartilage (Liu et al., 2003; Hesse et al., 2013). Nevertheless, the role of *CHAD* and  
346 *KERA* in the mitral valve is unknown. In a canine MMVD proteomic study, decorin and  
347 biglycan were found to be up-regulated in the early-stage MMVD, but down-regulated in the  
348 late-stage MMVD (Lacerda et al., 2009). In contrast in human MMVD, biglycan (protein),  
349 decorin (both mRNA and protein), and versican (protein) were found more abundantly  
350 expressed compared with normal mitral valves (Radermecker et al., 2003; Gupta et al., 2009).  
351 In our study, unchanged expression of the major PGs (e.g. lumican, versican, decorin, and  
352 biglycan) between diseased and normal mitral valves suggests that cellular and structural  
353 changes in end-stage MMVD had no direct effect on PG gene expression. The variation of  
354 PG expression in different studies may suggest post-transcriptional and translational  
355 modification of PG mRNA and proteins.

356

357         There were no changes in the endothelium adhesion molecules *VCAM-1*, *ICAM1* or  
358 *PECAMI*, but there was over-expression of *E-selectin* and *TLRs*. Strong staining for E-  
359 selectin and *VCAM-1* in human myxomatous mitral valves, without morphological evidence  
360 of inflammation, has been previously reported. (Müller et al., 2000) The closure and opening  
361 of the cell-cell endothelial adherens junctions, cell motility, and maintaining vascular  
362 permeability and integrity is controlled by VE-cadherin (*CDH5*), and there was high signal  
363 intensity for a range of *CDHs* (2, 11 and 13) in the microarray (Dejana and Orsenigo, 2013).  
364 High expression of the cadherins promotes cell migration and proliferation through the  
365 ERK1/2 pathway (*CDH13*), and mesenchymal cell (*CDH11*) and myofibroblast  
366 differentiation (*CDH2*) (Ivanov et al., 2004). *CDH5* had the lowest signal intensity and was

367 significantly reduced in the CKCS group, and down-regulation of CDH5 through NOTCH-  
368 Snai1 or TGF- $\beta$  signalling permits endothelial migration and EndoMT (Armstrong and  
369 Bischoff, 2004). Down-regulation of CDH5 was matched by up-regulation of Snai1 in  
370 affected valves, but NOTCH was also down-regulated, suggesting the possible presence of a  
371 NOTCH-independent Snai1 signalling pathway in canine MMVD. The pattern of cadherins  
372 expression also suggests a proliferative and migratory phenotype, with minimal osteogenic  
373 activity, and the contribution of these adhesion proteins to disease pathogenesis needs further  
374 investigation.

375

376 Certain expression data suggested the presence of EndoMT, such as differential  
377 expression of genes associated with BM components, mesenchymal differentiation and  
378 NOTCH signalling pathways. There is evidence of transition into a mesenchymal phenotype  
379 in MMVD, represented by increased expression of mesenchymal markers such as  $\alpha$ -smooth  
380 muscle actin ( $\alpha$ -SMA, ACTA2), SM22 (TAGLN), and  $\gamma$ -SMA (ACTG2). This mesenchymal-  
381 transition could be initiated by the up-regulation of the TGF- $\beta$  superfamily members BMP6  
382 and BMPRI1, increased NOTCH-Snai1 signalling, down-regulating the expression of CDH5,  
383 decreased expression of the BM components NID1 and LAMA2, and increased expression of  
384 the BM lytic enzyme CTSS. Lastly, the increased expression of hyaluronic acid synthase 2  
385 (Has2) in the CKCS could provide the hyaluronic acid rich subendothelial matrix necessary  
386 for transition of endothelial cells (Bakkers et al., 2004; Camenisch et al., 2001; Lagendijk et  
387 al., 2013). These changes in the valve transcriptome are reminiscent of the changes needed in  
388 valve development to allow the valve to form from the endocardial cushion through the  
389 migration of endothelial cells into the hyaluran-rich embryonic stroma, where they  
390 differentiate into valve interstitial cells which then generate the valve matrix (Camenisch et

391 al., 2001; Hinton and Yutzey, 2011; de Vlaming et al., 2012). The data from the current study  
392 suggest that EndoMT contributes to MMVD pathogenesis and this area needs further study.

393

394 The main limitations of this study were the small sample size, the lack of age- and  
395 breed-matched controls, the extraction of RNA from a mixed tissue type, sampling at a single  
396 disease end-point and differences in in silico analysis platforms. The difficulties in getting  
397 sufficient RNA of adequate quality from valves are illustrated by rejection of 6/10 CKCS  
398 samples, but the statistical analysis confirmed three vs. three was sufficient for credible  
399 comparison. MMVD research is hampered by the lack of suitable age- and breed-matched  
400 controls, because of the ubiquity of the disease in aged dogs and the limited sample pool  
401 when having to use family pets. This limitation cannot be overcome, but at least in this study  
402 the test group were all from the same breed reducing variability to some extent. In silico  
403 analysis show differences among different platforms and network clustering and gene-gene  
404 interaction analysis are based on several different tissue and cell types and may not  
405 necessarily be directly applicable to the mitral valve. However the use of three platforms  
406 resulted in somewhat consistent conclusions as to the major gene categories in MMVD and  
407 identified two novel categories not previously reported. Network analysis did allow for the  
408 identification of potential pathogenesis pathways that are worthy of further investigation.  
409 Furthermore, with continual updates of annotations and increased accuracy of canine  
410 genomics databases, data set reanalysis will be possible in the future.

411

## 412 **Conclusions**

413 The characterization of the MMVD transcriptome identified differentially expressed  
414 genes associated with inflammation, cell movement, development, and extracellular matrix  
415 organization and EndoMT. Signal intensity analysis identified genes important in ECM,

416 EndoMT and valve development, with patterns of gene expression suggesting decreased  
417 collagen turnover, ECM weakening, BM disruption, increased cell migration, active  
418 endothelial and myofibroblast differentiation in MMVD affected dogs.

419

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424

## 425 **Appendix .Supplementary material**

426 Supplementary data associated with this article can be found in the online version, at  
427 doi

428

## 429 **Conflict of interest statement**

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

432

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607 Table 1.

608 Primer Sequences for Microarray Data Validation by Q-PCR are shown.

Gene Name	Forward Primer	Reverse Primer	Probe Number
<i>LAMA2</i>	5'CCGCATTGAGCTGACAGT AG3'	5'CAAACCTGGTTGAGGCCATCT3 ,	38
<i>ENG</i>	5'GGTGCTCAAGAAAGACCT CATC3'	5'GCAGGACAAACTGGTCATCT C3'	30
<i>COL6A3</i>	5'AAGGAAGCTTCAGCACAA AGA3'	5'TGAACTAGAAGCCAACCTTG C3'	39
<i>HTR2B</i>	5'GGTCTGGATTACAAACAG AATCG3'	5'TCCCTGTTGCTCACCAGTCT3'	6
<i>MYH11</i>	5'ATGCAGCTGGCCAAGAAG 3'	5'TCTGAGCAATTTTCATCATCGA G3'	81
<i>MMP12</i>	5'CCAATTTGAGTTTTGATGC TGT3'	5'CACTGTTCTTTGGACTCTCTG GA3'	22
<i>BMP6</i>	5'TCTCCAGCGCCTCAGATTA C3'	5'TGGAAGCTCACATACAGCTC A3'	4
<i>ANGPT1</i>	5'AGGAAACGAAAAGCAGA ACTACA3'	5'ATCAGCACCATGTAAGATCA GG3'	18
<i>CHAD</i>	5'CCAGTCTTTCGGCAGATAC C3'	5'ACATGTTTCAGTGTGGTCAC G3'	20
<i>ADAMT S19</i>	5'TCAACCCTGCAATGAGAA GA3'	5'CGTATCACTCGGCAGTACAC A3'	14
<i>ACTG2</i>	5'GGTCATCACCATTGGCAA C3'	5'TGAATCCCAGCAGACTCCAT3 ,	11
<i>Sox-9</i>	5' CCAACGCCATCTTCAAGG3'	5' GGAGTGCACCTCGCTCAT3'	63
<i>KERA</i>	5' GACTATGCATGACTTTGACT GTCC3'	5'TTTCACAGTATAAAGCAGTA GGGAAA3'	29
<i>MRPS25</i>	5'TCTTGGGGAAGAACAAGG AA3'	5'AGTGGGCTGGGTGAGAAAG3 ,	15

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Table 2.  
Gene ontology chart showing the differentially expressed genes in canine mitral valves using the gene analysis platform DAVID 6.7.

Gene Ontology Term	Gene Counts	Gene %	p-value
Response to wounding	43	8.5	2.10E-08
Inflammatory response	31	6.2	9.50E-08
Immune response	42	8.3	4.70E-05
Cell adhesion	41	8.1	1.40E-04
Biological adhesion	41	8.1	1.40E-04
Defence response	37	7.3	1.80E-04
Vasculature development	20	4	3.10E-04
Positive regulation of cytoskeleton organization	8	1.6	4.30E-04
Blood vessel development	19	3.8	6.30E-04
Regulation of cell proliferation	42	8.3	7.50E-04
Blood vessel morphogenesis	17	3.4	8.90E-04
Regulation of cytoskeleton organization	13	2.6	1.10E-03
Innate immune response	13	2.6	1.20E-03
Response to oxygen levels	13	2.6	1.40E-03
Collagen metabolic process	6	1.2	1.50E-03
Epithelial to mesenchymal transition	5	1	2.00E-03

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620 Table 3.  
 621 Functional annotation clustering using DAVID 6.7, listing the ten clusters and the relative  
 622 gene density.  
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Annotation Clusters	Enrichment Score	Gene Count	p-value
Vasculature development	3.25	20	3.10E-04
Collagen metabolic process	2.57	6	1.50E-03
Protein processing	2.41	11	2.50E-03
Vacuole	2.36	18	2.20E-03
Positive regulation of cytoskeleton organization	2.24	10	4.30E-04
Regulation of cell motion	1.91	14	7.10E-03
Regulation of bone mineralization	1.71	5	1.30E-02
Positive regulation of cell motion	1.71	9	1.10E-02
Cysteine-type endopeptidase activity	1.61	9	5.40E-03
Epithelial to mesenchymal transition	1.52	5	2.00E-03

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626 Table 4.

627 Gene networking analysis using the gene analysis platform IPA.

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629 A. Cardiovascular system development

<b>Diseases and functions</b>	<b>Genes</b>
Abnormal morphology of mitral valve	<i>NFATC1</i>
Abnormal morphology of cardiovascular system	<i>NFATC1, PPDX1, RBPJ, NOTCH1, ZMIZ1</i>
Abnormal morphology of cardiac valve	<i>NFATC1, ZMIZ1</i>
Morphogenesis of endocardium	<i>RBPJ</i>
Morphogenesis of atrioventricular valve	<i>NOTCH1</i>
Morphogenesis of heart	<i>NOTCH1, RBPJ, ZMIZ1</i>
Looping morphogenesis of heart	<i>NOTCH1, RBPJ</i>
Differentiation of endocardial cells	<i>NFATC1</i>
Differentiation of endothelial cells	<i>NFATC1, RBPJ, NOTCH1</i>
Development of mesenchymal cells	<i>NOTCH1</i>
<b>Canonical Pathway</b>	<b>Genes</b>
Regulation of the epithelial-mesenchymal transition pathway	<i>Secretase <math>\gamma</math>, RBPJ, NF<math>\kappa</math>B1, NOTCH1, CSL-HIF-1A</i>
NOTCH signalling	<i>Secretase <math>\gamma</math>, RBPJ, NOTCH1, CSL-HIF-1A</i>

630 B. Cellular movement and connective tissue development

<b>Diseases and functions</b>	<b>Gene</b>
Cell movement of fibroblast	<i>SKAP2, WASF2, ANGPT1, ENPP2</i>
Familial thoracic aortic aneurism	<i>MYH11</i>
Endothelial cell development	<i>ANGPTL1, ANGPT1</i>
Cell movement of endothelial cells	<i>ANGPT1, ENPP2</i>
Injury of endothelial cells	<i>ANGPT1</i>
<b>Canonical Pathway</b>	<b>Gene</b>
Integrin Signalling	<i>Arp2/3, Talin, calpain, Akt, integrin, JINK1/2, Lfa-1</i>

631 C. Cell-to-cell signalling and tissue development

<b>Diseases and functions</b>	<b>Gene</b>
Adhesion of fibroblast cell lines	<i>TNMD, TENC1</i>
Adhesion of vascular endothelial cells	<i>ITGB1, SELE</i>
Association of extracellular matrix	<i>ITGB1</i>
Attachment of smooth muscle cells	<i>ITGB1</i>
Binding of vascular endothelial cells	<i>ITGB1, SELE</i>
Morphogenesis of endothelial tube	<i>PDPN</i>
Basement membrane disruption	<i>NID1, Laminin</i>

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633

634  
635 Table 5.  
636 Examples of single gene intensity changes are shown.  
637  
638

Category	Genes
Collagen	<i>COL6A3</i> ↓(H)
ADAMTS	<i>ADAMTS2</i> ↓(M), <i>ADAMTS19</i> ↓(M), <i>ADAMTSL4</i> ↓(M)
PGs and GAGs	<i>KERA</i> ↓(H), <i>CHAD</i> ↓(H)
MMP and TIMP	<i>MMP12</i> ↑(M), <i>MMP14</i> ↓(M), <i>MMP16</i> ↓(M)
Basement membrane	<i>NIDI</i> ↓(H), <i>LAMA2</i> ↓(L), <i>CTSS</i> ↑(H)
Cathepsin	<i>CTSC</i> ↑(M), <i>CTSS</i> ↑(H)
Integrin	<i>ITFG1</i> ↑(H), <i>ITGA</i> ↑(M), <i>ITGA8</i> ↑(M), <i>ITGB1</i> ↑(H), <i>ITGB4</i> ↓(M), <i>ITGBL1</i> ↑(M)
Claudin and occludin	<i>CLDN1</i> ↑(M), <i>CLDN11</i> ↓(H)
Cadherin	<i>CDH5</i> ↓(H)
Others	<i>CILP</i> ↓(H), <i>Has2</i> ↑(M), <i>HAPLN1</i> ↓(H)
Caveolin, PECAM, ICAM, SELE, and VCAM	<i>SELE</i> ↑(L)
NOTCH and SNAI1	<i>NOTCH1</i> ↓(L), <i>RBPJ</i> ↑(H)
Catenin, VEGF, and NFATc	<i>NFATc1</i> ↓(M)
TGF-β and superfamily	<i>ENG</i> ↓(H), <i>BMPRI1B</i> ↑(L), <i>BMP6</i> ↑(M)
Actin, myosin, and SM22	<i>ACTA2</i> ↑(H), <i>ACTC1</i> ↓(H), <i>ACTG2</i> ↑(H), <i>TAGLN</i> ↑(H)

639  
640 Statistically significantly different genes comparing cavalier king Charles spaniels (CKCSs)  
641 and normal dogs, selected on the basis of their likely biological relevance to myxomatous  
642 mitral valvular disease (MMVD), and using the five selected categories derived from  
643 annotation clustering analysis (ToppFunn, DAVID and IPA). Genes were also examined on

644 the basis of their signal intensity; H, high signal intensity; M, medium signal intensity; L, low  
645 signal intensity. ↑ up-regulated, ↓ down-regulated.  
646



647 **Figures Legends**

648

649 **Figure 1.** Volcano plot demonstrating the overall gene expression pattern based on the X-axis  
650 (fold-change value) and Y-axis ( $P$ ). Each dot represents one gene. Two vertical (fold-change  
651 value at 1.5 and -1.5) cut-off lines and one horizontal ( $P = 0.05$ ) cut-off line are shown. In  
652 general, the plot is equally distributed. The significantly down-regulated genes in zone 3 were  
653 more diffusely distributed compared with the significantly up-regulated genes in zone 4.  
654 Unchanged genes in zone 1 ( $P > 0.05$ ) and zone 2 ( $P < 0.05$ ) had the highest plot intensity.

655

656 **Figure 2.**

657 Gene networking analysis using Ingenuity pathway analysis (IPA) identified three significant  
658 and biologically relevant networks for myxomatous mitral valvular disease (MMVD),  
659 including network (1) cardiovascular system development and function, network (17) cellular  
660 movement and Network 23) cell-to-cell signalling. Green is down-regulated and red is up-  
661 regulated, with fold changes shown beneath each gene.

662

Figure 1  
[Click here to download high resolution image](#)

Volcano plot CKCS vs control

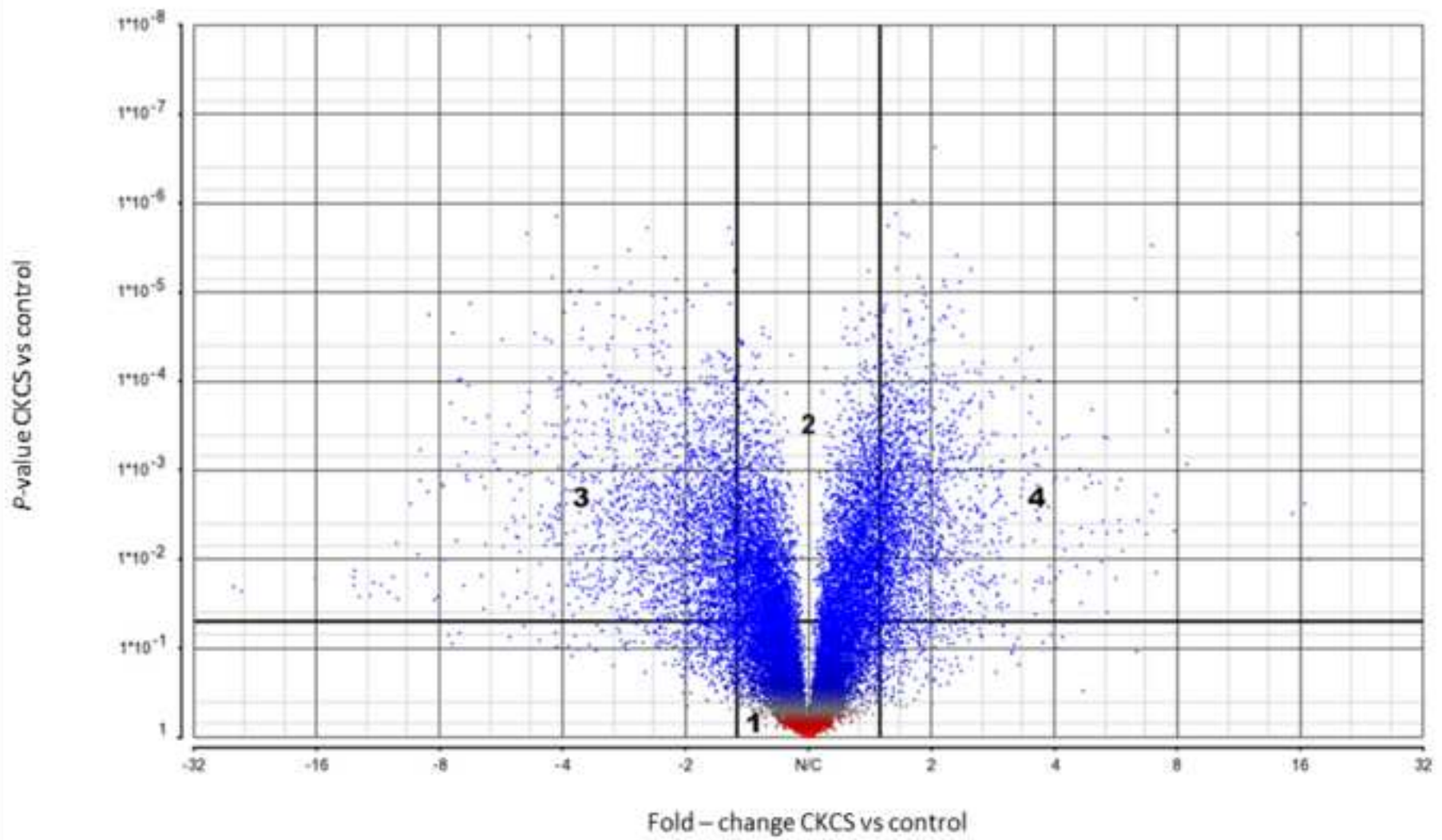


Figure 2A  
[Click here to download high resolution image](#)

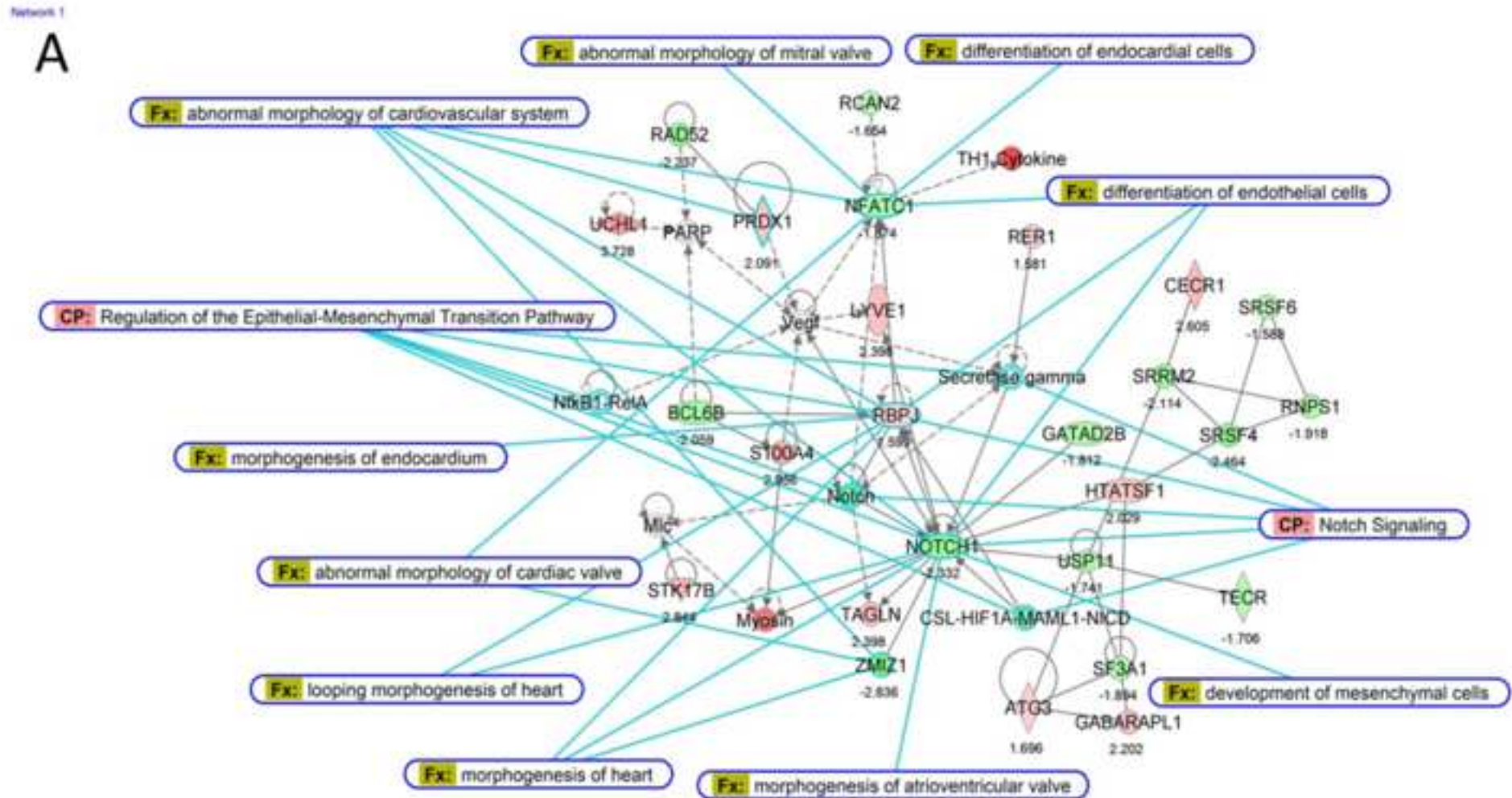


Figure 2B  
[Click here to download high resolution image](#)

Network 17

B

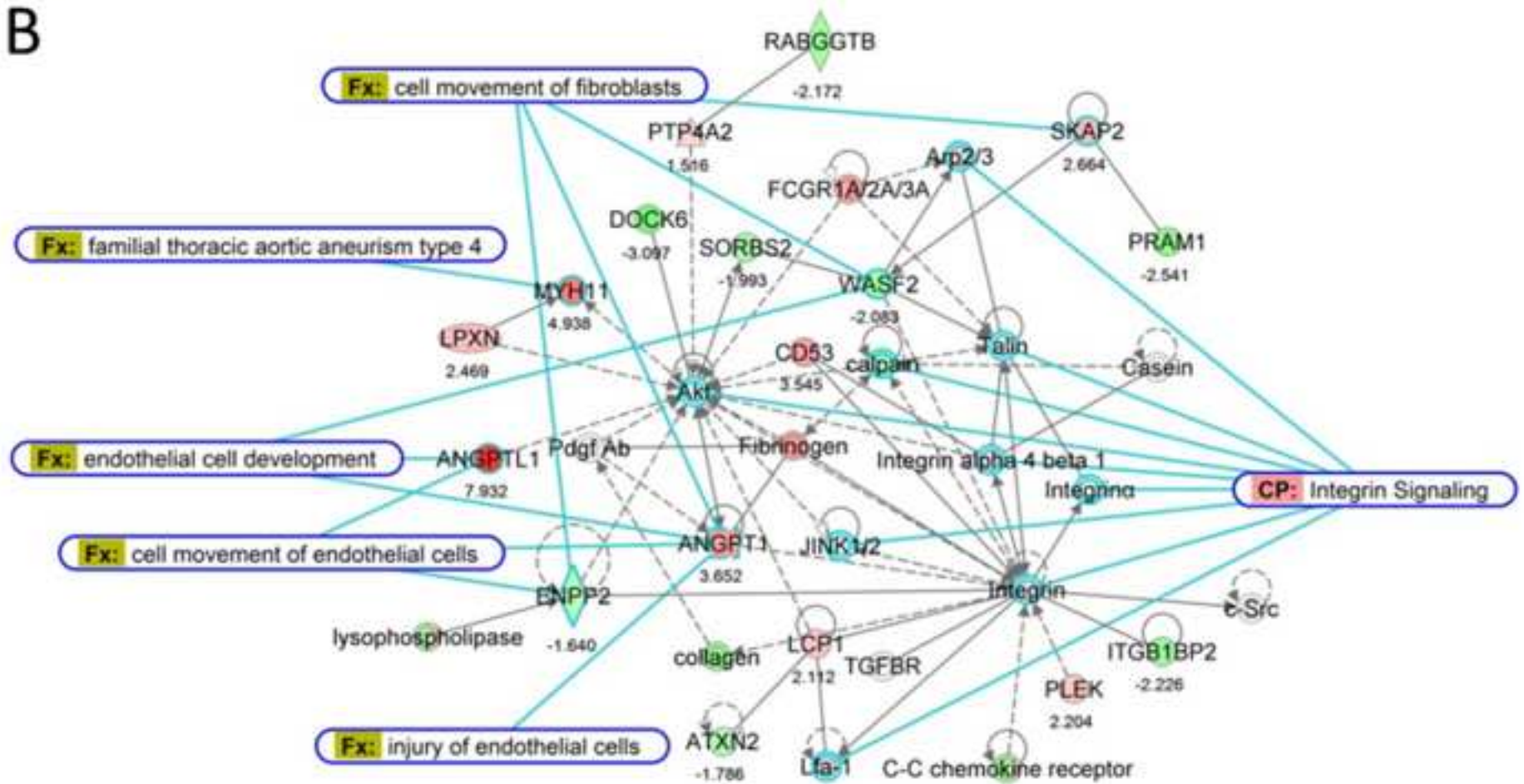
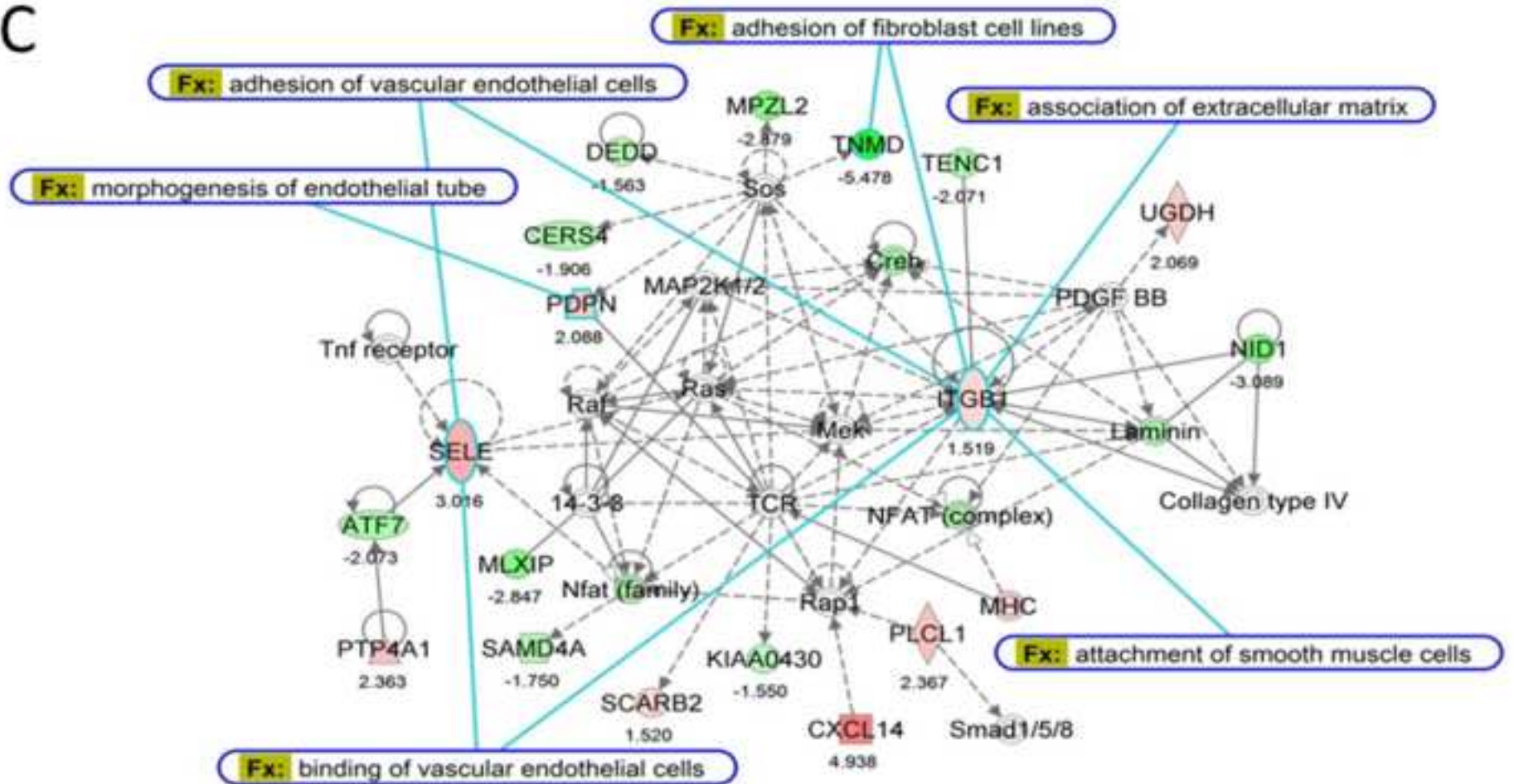


Figure 2C  
[Click here to download high resolution image](#)

Network 23

C



**Supplemental file 1**

[Click here to download Optional e-only supplementary files: Supplemental File 1 Gene List.docx](#)

**Supplemental file2**

[Click here to download Optional e-only supplementary files: Supplemental File 2 Q-PCR validation.docx](#)

**Supplemental file3**

[Click here to download Optional e-only supplementary files: Supplementary File 3 ToppFunn.docx](#)



**Supplemental file5**

[Click here to download Optional e-only supplementary files: Supplementary File 5 IPA.docx](#)

**Supplemental file 4**

[Click here to download Optional e-only supplementary files: Supplementary File 4 DAVID.docx](#)

## Manuscript Highlights

“Gene network and canonical pathway analysis in canine myxomatous mitral valve disease: a microarray study”

1. Most complete coverage of the mitral valve transcriptome, several orders of magnitude better than then only other report from 2006.
2. Application of several gene analysis platforms giving greater levels of data analysis and gene categorisation relevant to disease pathogenesis.
3. Evidence of contribution of endothelial-to-mesenchymal transition to disease pathogenesis; one of the more novel theories of disease to emerge in recent years.
4. Identification of ~600 genes significantly implicated in MMVD giving a picture not only of the complexity of the disease, but important information that will allow hypothesis driven research to be applied to understanding this disease.

Note from the Scientific Editor to the Authors,

Using the edited version of the manuscript (which should be downloaded from the Journal's Web site), please address the comments and suggestions from both reviewers. In addition, please correct the following:

1. Use P in italics

**Corrected**

2. Italicize n

**Corrected**

3. Correct the format of your in-text reference citations (i.e. Corcoran et al., 2014)

**Corrected**

4. Correct the format of your reference list (i.e, all authors, no &, no issue number, no pp. unless it's a book, etc)

**Corrected**

5. Use "L" for liter

**Corrected**

6. In your figures, for titles, axes, etc, please use capital letter only for the first letter of the first word.

**Corrected**

7. For figures 2 and 4, please provide the tables separated in the word document.

**Understand what you mean, but not sure how to go about it correctly; have done as Fig. graphic & Fig. 2 text. What we would want is both together in the MS.**

8. For figure 3, your pathway may be hard to read. Make sure that you provide the highest definition possible for those, and please submit them one pathway/file.

**Re-ordered as Fig.3 a,b,c as separate files and with best resolution we have.**

With kind regards,

Eric Blomme, DVM, PhD, Dipl. ACVP  
Scientific Editor, The Veterinary Journal

Reviewer #1:

This is a well-written and interesting study of gene expression analysis in mitral valve tissue from dogs with myxomatous mitral valve disease. The aim of this study was to make an improved analysis on gene expression compared to previous studies in canine MMVD, in order to identify novel signaling pathways that might contribute to MMVD.

The study has a number of limitations, and most of them are addressed in the limitations. The major limitation is that finding of a difference between MMVD dogs and control dogs may be due to breed differences because the MMVD dogs only include tissue from Cavalier King Charles Spaniels. Previous studies have shown breed differences, for example with regards to platelet aggregation response and circulating biomarkers (Olsen et al 2001; Moesgaard et al 2007).

**This is a valid comment and we will make sure it is mentioned in the limitations. The intention here had been to limit variability by concentrating on this one breed. In fact we have evidence that the cellular changes seen in CKCSs are the same as for other affected dogs, so might argue the CKCS is a good**

**model of the disease in dogs and the propensity for this breed to be affected is primarily a function of the time to onset and not the pathogenesis (although that need one very large study to confirm).**

In addition, it is limitation that histopathology is not performed to confirm the diagnosis. I think this is important also to address these two limitations.

**Histopathological confirmation was undertaken. Have included a short comment in Materials and methods**

In general, more references in relation to the gene analysis would be appropriate.

**We would prefer to leave as is Chen and Huang refer to DAVID and TOPPFUN and were the developers, and IPA is a commercial product for which there is no specific development reference.**

Line 95: Whitney grade for control dogs are missing

**Grade is zero; have amended**

Line 98: How was MMVD clinically confirmed?

**All cases had been clinically confirmed by ourselves using echocardiography; have commented in MS.**

Line 99: Information of therapy is missing

**All dogs had been in the terminal stages of the disease and would have been on standard treatment for congestive heart failure. There might have been subtle differences in types of medication and dosage, but there is no way we could control for those variables; have commented in MS**

Line 142: Was model control for the AVOVA performed

**Parktek 6.6 software only runs ANOVA and so we used that to compare 4 vs 4. We are not too sure what is meant by “model control” in this context.**

Line 165: Description of the statistic in more detail is missing

**See above; since these programmes and platform as have their own in-built statistical methodologies, which we presume have been p[roperly modelled we are not sure of the benefit of providing that detail in the MS. For qRT-PCR Mann-Whitney U test was used with Graphpad prism .**

Reviewer #2: Gene Network and Canonical Pathway Analysis in Canine Myxomatous Mitral Valve Disease: A Microarray Study

In this manuscript the authors used microarray data and several analysis tools to better understand the mechanism and affected genes/pathways in Cavalier King Charles Spaniels with Myxomatous Mitral Valve Disease. These data use the newer and better annotated canine Affymetrix array.

I am accepting this paper but have several comments the authors should address:

General comments:

Capitalize Cavalier King Charles Spaniel throughout the entire manuscript

**This would be incorrect as cavalier and spaniel are not proper nouns, but King and Charles are, so they are capitalised. For the acronym we think capitalisation is the right form hence “CKCS”**

Correct spelling errors throughout the manuscript

**Done**

Abstract

When mentioning the changed gene families please mention the general direction of change (up or down regulated)

**Can we have editorial advice as doing this for each gene might make the abstract look “messy”. We have regrouped the genes as “all down-regulated”, “all up-regulated” or “up and down-regulated”.**

**See amended text and advise if acceptable. An alternative is  $\uparrow\downarrow$  but presume this would not be acceptable.**

Introduction

Include more background about MMVD. What is the incidence of this disease? What are theories about why CKCS are more susceptible to the disease?

**We would normally do so, but are constrained by the word count for the MS. Also much of what is being asked to include would be speculative. Have included one sentence on endothelial damage, VC activation and aberrant remodelling.**

What are the current theories about MMVD formation?

**See previous response**

Please rewrite the aim of the study in the last paragraph as it is unclear (lines 86-91)

**Has been reworded.**

Materials and Methods

How was MMVD diagnosed in the dogs?

**Clinically and confirmed by echocardiography; MS amended**

Correct the discrepancy (or clarify the difference) between  $n = 6$  at line 100 and  $n = 8$  on line 117. How many samples were there?

**Cannot find  $n=8$  in the MS. Numbers are correct.**

What kind of QC parameters were used to accept the microarray data?

**If this means pre-hybridisation it is the RIN number which was  $>7$ , which is in the MS. If it means post-hybridisation we used the PCA plots, which mentioned in new line 128-130.**

Line 145  $\diamond$  specify the cut-off values used

**Now line 134 with values entered for fold-changes etc.**

What was the RNA input for the Q-RT-PCR reaction? Line 161

**Not sure what is being asked here; please clarify. We used the exact same RNA samples for the microarray and the PCR. Have included comment in new line 145.**

Correct the "-" in front of  $\Delta\Delta Ct$  on line 164 or explain what the "-" means.

**No meaning; has been removed.**

Table 2

Clarify what "Gene %" means? Does this refer to the % of the pathway which is changed? Add what general direction the pathways are changed to the table.

**Sentence included to clarify**

Table 3

Include which direction genes were changed (up or down regulated)

**Have included sentence cross-referencing to Figure 3 which shows the fold changes. If that is not sufficient we can include in Table 3, but this this would make it very complicated.**

Figure 4

This figure is very hard to read and interpret; please make it more clear. Suggestions include removing molecules from the networks which do not strengthen the authors point, building a unique heatmap including functional nodes and only necessary/unchanged gene changes, or representing the data in a

different format.

**This has been replaced with Table 4; effectively Figure 4 without the graphic for LDL. Original Table 4 is now Table 5**

Supplemental Figure 4 ◊ change title to "Identity" not Identify

**Done**

Reviewer #3: In this study, the authors used up-to date canine microarray technology to investigate the transcript changes in MMVD of CKCS. They found genes, biological function clusters and pathways related to MMVD. The study was well conducted and the manuscript was well written. The authors only need to correct some punctuations to make the article more accurate and less confusing. eg. in Line58, there should be comma in front of 'less is'; there should be right parenthesis some where in Line110. Please check your MS carefully to correct these issues.

**MS has been checked.**

Editor and Reviewer Comments:

Note from the Scientific Editor to the Authors,

As mentioned below in their comments, both reviewers have recommended your submission to be accepted for publication in The Veterinary Journal. However, for unknown reasons, I am unable to open the manuscript file and to make final edits. Can you please consider the few comments from Reviewer #1 below and save your manuscript in a different version of Word, as this may solve the problem?

In addition, please use the other submission (751: culture of VEC and VIC) that we just send back to you as an example of the edits that will be necessary for you to make before we can accept the manuscript. For example:

*Done*

1. P needs to be capitalized and italicized.
2. "n" needs to be italicized.
3. Do not include ":" after the section titles
4. In your in-text references, use the following format: Corcoran et al., 2014
5. Use "min", "h" and "s"
6. Use Fig., not Figure
7. In your reference list, the volume numbers should not be italicized. And replace all "&" by "and"

*All done*

Reviewer #1:

I find the paper very fine. I have only two minor comments:

I still find it important to address that the findings may be due to breed differences between the control group and the diseased group. I recommend that the sentence in the discussion (line 386-387): "The main limitations of this study were the small sample size, the lack of age-matched controls..." is changed to "The main limitations of this study were the small sample size, the lack of age- and breed-matched controls ..."

*Comment included*

Model control in relation to ANOVA is performed to evaluate if raw data fit the statistical model. Evaluation of variance homogeneity can be performed. Non parametric tests need to be used if data do not fit the model and transformation of the data does not obtain variance homogeneity. I find it important to know if the raw data fit the statistical models, it is not clear to me whether it is performed

*We are unable to determine whether if the raw data fits the statistical model or not due to the small sample size. In PARTEK suite microarray analysis, the data are transformed (log2) such that all values are between 0 and 16. The software then normalises the data irrespective of sample size and analyses using ANOVA regardless of the statistical model (Gaussian). Applying FDR to this bioinformatics platform further corrects for any potential false discovery. This is a routine microarray analysis procedure and widely accepted considering the sample size limitation typical of this type of research. We have consulted with Mick Watson, Director of Edinburgh Genomics (merger of Ark Genomics and TheGenePool) and he is confident the data analysis is robust.*



Ms. No. YTVJL-D-14-00750R2

Gene network and canonical pathway analysis in canine myxomatous mitral valve disease: A microarray study

The Veterinary Journal

Dear Brendan,

Your revised paper has been edited by your Handling Editor, Dr Eric Blomme, and another edited version has been uploaded to the journal website for your approval. Please check this edited manuscript carefully for accuracy and completeness.

I would be grateful if you could address the editorial comments and annotations that Eric has made to your manuscript in the references.

In addition, please attend to the following editorial requests:

- In the Corresponding author's details, please remove "(0)" from the telephone number and change "Brendan Corcoran" to "B.M. Corcoran". Authors' names can be written in full in the main list of authors if desired.

**Done**

- Please change "cavalier king Charles spaniels" to "Cavalier King Charles spaniels".

**Not sure if Cavalier is a proper noun, but done as requested.**

- When writing numbers such as "n = 59", please write "n" in italics.

**Done**

- Please check punctuation and spacing throughout the manuscript.

**Done; according to my own limited grammatical skills!!!**

- Numbers less than 10 that are not linked to a unit should be written in full unless used as the start of a sentence, e.g. change "6 and 10 significantly different biological function clusters" to "six and 10 significantly different biological function clusters".

**Done**

- Please change "Royal (Dick) School of Veterinary Study" to "Royal (Dick) School of Veterinary Studies".

**Done**

- Please write the heading "Conclusions" in bold.

**Done**

- References should be formatted with a hanging indent.

**Done**

- Please write the article titles for Geirsson et al. (2012), Lakatta and Levy (2003), Liu et al. (2003), Mow and Pedersen (1999) and Rabkin et al. (2001) in sentence case.

**Done**

- In the tables, the headings should be written in sentence case, not emboldened and placed on the next line below the headings "Table 1", "Table 2" and "Table 3".

**Done**

- In Table 1, please write sequences in upper case in the format 5'-CCGCATTGAGCTGACAGTAG-3'. Please also change "Probe No." to "Probe number".

**Done**

- In Table 4, please place additional data as footnotes rather than with the heading.

**Done**

- Please check all references carefully for accuracy. Please also check that all references are cited in the text and that all citations are listed in the references.

**Done**

- As recommended by Eric, Fig. 2B should be converted to a Table.

**Done, and other figures re-numbered as appropriate.**

- Please remove the solid borders from around Figs. 3A, B and C.

**Done; now re-labelled Fig 2A, B and C.**

- As noted by Eric, Fig. 4 is missing.

**My mistake; this figure had been removed but the text not amended accordingly.  
The figure does not add to the text.**

Please ensure that your revised paper conforms fully to the requirements of our Guide for Authors.

**IMPORTANT:** Your article has been edited by the Editor handling your paper and the Word filename includes the word 'edited'. You **MUST ONLY** use this version in preparing your revised text. You should click on download submission files link from your author menu on EES and ensure you are making changes to the most up to date version of the manuscript.

You should submit your revision online by logging onto the Elsevier Editorial System for The Veterinary Journal:  
<http://ees.elsevier.com/ytvjl/>

Your username is: \*\*\*\*\*

We look forward to receiving your revised manuscript.

With kind regards,

Adrian

Dr Adrian W. Philbey BVSc(Hon) PhD MANZCVSc(Pathology) MRCVS,

Senior Scientific Editor,  
The Veterinary Journal

On behalf of Dr Andrew Higgins BVetMed MSc PhD FSB MRCVS,

Editor-in-Chief,  
The Veterinary Journal

Editorial Office Web Portal: [www.ees.elsevier.com/ytvjl](http://www.ees.elsevier.com/ytvjl)  
For guidelines on how to submit your revised manuscript please go the following address:  
[http://help.elsevier.com/app/answers/detail/p/7923/a\\_id/91](http://help.elsevier.com/app/answers/detail/p/7923/a_id/91)

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#### EDITOR'S COMMENTS:

Note from the Scientific Editor to the Authors,

Based on your revisions, I have made some final edits to prepare your submission for publication. Please use **ONLY** the edited version of the manuscript (which should be downloaded from the Journal's Web site) to carefully review the text and make sure that I did not alter the meaning of it. I also include a few comments in the reference list for you to address. Make all changes in a font of a different color.

Finally, I could not locate figure 4, and Figure 2B would be better suited as a table.

With kind regards,

Eric Blomme, DVM, PhD, Dipl. ACVP

Scientific Editor,  
The Veterinary Journal



ROYAL (DICK) SCHOOL OF VETERINARY STUDIES  
The University of Edinburgh  
Easter Bush Campus  
Roslin  
Midlothian  
EH25 9RG  
0131 650 6241  
<http://www.vet.ed.ac.uk/>

Dear Editor.

29/7/2014

Please find enclosed manuscript “Gene network and canonical pathway analysis in canine myxomatous mitral valve disease: a microarray study” for consideration for publication in TVJ.

This paper provides a large amount of data on the mitral valve transcriptome that is not currently available to the research community. It markedly improves on the only other study previously reported in 2006, not least because of major advances in transcriptome coverage and bioinformatics platforms.

These data will permit increased numbers of hypothesis driven projects to be generated by the research community interested in canine MMVD, but also provide data of interest to colleagues working on the analogous human disease.

*Brendan Corcoran*

Brendan Corcoran

---

**Professor Brendan Corcoran**  
**Deputy Head of School**