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Citation for published version:

Lu, C, Liu, M, Culshaw, G, French, A & Corcoran, B 2016, 'Comparison of cellular changes in cavalier King Charles spaniel and mixed breed dogs with myxomatous mitral valve disease', *Journal of Veterinary Cardiology*, vol. 18, no. 2, pp. 100-109. <https://doi.org/10.1016/j.jvc.2015.12.003>

Digital Object Identifier (DOI):

[10.1016/j.jvc.2015.12.003](https://doi.org/10.1016/j.jvc.2015.12.003)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Journal of Veterinary Cardiology

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1 Comparison of cellular changes in cavalier King Charles spaniel and mixed breed
2 dogs with myxomatous mitral valve disease

3

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11

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13

14

15 **Abstract**

16 **Objectives:** The aim of this study was to determine if there are differences in cellular
17 changes in cavalier King Charles spaniel (CKCS) myxomatous mitral valves
18 compared to non-CKCS dogs.

19 **Animals:** CKCSs (n=6) and age-matched mixed breed (n=6) with severe MMVD, and
20 normal mixed breed (n=4) dogs.

21 **Methods:** Immunohistochemistry staining and qualitative and quantitative analysis of
22 mitral valves sections, examining for the presence of CD11c and CD45, vimentin,
23 alpha smooth muscle actin (α -SMA) and myosin heavy chain 10 (Smemb),
24 vonWillebrand factor (vWF) and CD31 and Ki-67.

25 **Results:** Vimentin positive cell numbers were increased in the MMVD dogs and
26 distributed throughout the valve with greatest density close to the endothelium. There
27 were no significant differences in cell marker expression for the two diseased groups,
28 but cell numbers were significantly increased compared to controls for α -SMA
29 (CKCS only) and SMemb (CKCS and mixed breed) ($P<0.05$). α -SMA+ cells were
30 primarily located at the valve edge, with Smemb+ cells similarly located, but also
31 present throughout the valve stroma. A small number of cells close to the valve edge
32 co-expressed α -SMA and SMemb. Endothelial vWF expression was identified in all
33 valves, with evidence of disrupted endothelium in the diseased, but was also found in
34 diseased valve stroma. There was no staining for CD11c, CD45 or CD31 in any valve.
35 Ki-67+ cells formed linear clusters at the leaflet tip and were sparsely distributed
36 throughout both myxomatous valve groups.

37 **Conclusions:** The cellular changes notes with advanced stage MMVD are appear
38 similar for CKCS when compared to mixed breed dogs.

39

40 **Key words:** Canine, Cellularity, Endothelial, Immunohistochemistry, Interstitial.

41 **Abbreviations:**

42	CKCS	cavalier King Charles spaniel
43	MMVD	myxomatous mitral valve disease
44	5-HT	5-hydroxytryptamine (serotonin)
45	α -SMA	alpha smooth muscle actin
46	VIC	valve interstitial cell
47	MYH-10	myosin heavy chain 10 (SMemb)
48	Non-muscle embryonic myosin	SMemb (MYH-10)
49	VEC	valve endothelial cell
50	PBS	phosphate-buffered saline
51	vWF	von Willebrand Factor
52	DAPI	4',6-diamidino-2-phenylindole
53	ANOVA	one-way analysis of variance

54

55 **Introduction:**

56 The cavalier King Charles spaniel (CKCS) breed is recognised to be particularly pre-
57 disposed to the development of myxomatous mitral valve disease (MMVD) in terms
58 of age of onset, progression and severity¹⁻³. MMVD is highly heritable in the CKCS
59 breed and it is reasonable to suppose heritability also has a role in disease appearance
60 and progression^{4,5}. There are CKCS-specific traits such as differences in platelet
61 numbers and platelet aggregation tendency and higher serum magnesium and 5-HT
62 (serotonin) levels compared to other dogs that have been examined for correlation
63 with development of MMVD, with varying results⁶⁻¹². Furthermore, the CKCS is
64 more predisposed to chronic fibrosing pancreatitis and syringomyelia than other

65 breeds and with MMVD this might reflect a breed-specific global connective tissue
66 abnormality¹³⁻¹⁵. While there is no clear causal link established between these findings
67 in the CKCS and MMVD it still points towards breed-specific phenotypic
68 characteristics that might have bearing on disease appearance.

69

70 Cellular changes that occur with MMVD are well described. There is an increase in α -
71 smooth muscle actin (α -SMA) positive valve interstitial cells (activated
72 myofibroblasts, aVICs) which locate mainly towards the valve distal free-edge (valve
73 tip) and appears to be related to disease severity¹⁶⁻¹⁹. It is activation of the VICs that is
74 believed to drive the extra-cellular matrix damage seen in MMVD through a
75 combination of increased catabolic activity and aberrant repair and replacement. In
76 normal valve stroma the majority of cells are quiescent vimentin-positive VICs
77 (qVIC), with a few desmin-positive cells^{16, 17}. In overtly myxomatous areas of
78 affected valves some cells also appear to be activated myofibroblasts or with
79 increased differentiation capacity based on their expression of myosin heavy chain-10
80 (MYH-10; non-muscle embryonic myosin, SMemb), with appearance of cells in
81 clusters close to the endothelium and deeper in the valve stroma^{20, 21}. There is no
82 evidence that there is active cell proliferation and while some cells exhibit a pro-
83 apoptotic status they do not undergo programmed cell death^{16, 22}. However, there are
84 regional changes in cell numbers; reduced in overtly myxoid stroma and increased in
85 sub-endothelial zones²¹. A small increase in mast cell numbers is also noted, but there
86 is no increase in macrophages numbers and no inflammatory cells are reported^{16, 17}.
87 Changes in endothelial cell morphology, coupled with endothelial cell (VEC) loss are
88 also reported^{16, 23, 24}. Changes include increased expression of endothelin receptors,
89 evidence of VEC activation, apoptosis, basement membrane loss and damage with

90 cell detachment ^{25,26}. The interaction between the VECs and VICs in the context of the
91 pathogenesis of MMVD is not understood, but the characteristic accumulation of
92 activated myofibroblasts close to the endothelium and in particular areas of damaged
93 endothelium suggest some sort of interaction does occur ²⁶.

94

95 Of the studies to date, all have presented data from a mix of pedigree and cross-bred
96 dogs. Considering the susceptibility of the CKCS to MMVD, the aim of this study
97 was to determine if there are differences in valve cellular changes in the CKCS
98 compared to non-CKCS dogs examining the resident valve interstitial and endothelial
99 cells, to confirm the cell changes noted in previous studies and also examine again for
100 the possible presence of inflammatory cells and macrophages.

101

102 **Animals, Materials and Methods:**

103 *Tissue samples*

104 Anterior mitral valve leaflets were obtained from six elderly CKCS (12-14 yr; 2
105 neutered females & 4 neutered males; 8-12 Kg) and six mixed breed dogs (8-12 yr; 3
106 neutered females & 3 neutered males; 10-18Kg) with MMVD. Control samples were
107 obtained from four young adult mixed breed dogs (1-2 yr; 2 intact males & 2 neutered
108 females; 12-15kg) with no evidence of disease. All samples were collected with full
109 owner consent and the study conformed to national (UK) and institutional ethical
110 guidelines for the use of animals in research. Valves were removed within minutes of
111 death, gently washed with phosphate-buffered saline (PBS), immersed in 4%
112 paraformaldehyde and stored at 4° C for 36 hr. Samples were then rinsed in PBS and
113 stored in 70% ethanol at 4° C until required. Valves were pathologically graded
114 Whitney Grade 3 or 4 by at least two of the authors independently at the time of

115 collection and then a grade agreed (C-CL, BMC, M-ML & GJC)²⁷. Representative
116 samples of similar gross pathological appearance were cut from each valve, paraffin-
117 embedded and 4 consecutive 5 µm sections were collected onto coated slide. Whole
118 valve length and distal transverse sections were collected for longitudinal and
119 horizontal evaluation. Sections were de-waxed in xylene and re-hydrated through
120 serial ethanols and distilled water.

121

122 *Immunohistochemistry*

123 Immunohistochemical staining used a combination of a standard peroxidase method
124 (qualitative) with NovaRed^c as chromagen (~~ABC Elite Kit, Vector~~), and indirect
125 immunofluorescence (qualitative and quantitative) using fluorescent dyes conjugated
126 to IgG^d (~~AlexaFluor488 and 568, Invitrogen~~). Double staining was carried out for α-
127 SMA and SMemb using indirect immunofluorescence to determine if there was co-
128 localisation. ~~For double staining~~ Images were collected using the two different
129 fluorescence channels and then merged. Sections were examined using fluorescence
130 and light microscopy^c (~~Leica DMRB~~) for the presence of the universal mesenchymal
131 cell marker vimentin, the inflammatory cell markers CD11c (macrophage, monocytes)
132 and CD45 (hematopoietic cells, T-cell lymphocytes), the valve endothelial cell (VEC)
133 markers vWf and CD31, the activated myofibroblasts (aVIC) markers α-smooth
134 muscle actin (α-SMA), myosin heavy chain-10 (SMemb) and the cell proliferation
135 marker Ki67. The details for each antibody are shown in Table 1. For antigen retrieval
136 sections were microwaved either in citrate buffer (0.01 M, pH 6.0) or Tris-EDTA
137 (10mM Tris base, 1 mM EDTA solution, pH 8.0).

138

139 For the peroxidase technique sections were incubated with 1% hydrogen peroxide
140 (H₂O₂ in phosphate-buffered saline (PBS pH 7.4) for 10 min and blocked with 10%
141 goat serum (diluted in 0.5% Tween 20)^f (~~Vector Laboratories Inc.~~) for 30 min at
142 room temperature. Sections were then incubated for 60 min at room temperature with
143 primary antibodies, rinsed in 0.5% Tween20 in PBS and incubated for 30 min at room
144 temperature with biotinylated secondary antibodies (goat anti-mouse or anti-rabbit
145 IgG^f; ~~Vector Laboratories Inc.~~). Slides were counterstained with haematoxylin,
146 dehydrated through graded ethanols and xylene and mounted in a xylene-based
147 medium^g (~~DePex; Gurr-BDH Chemicals Ltd~~). For each dog a minimum of four
148 sections were examined for each antibody.

149

150 For immunofluorescence, the basic protocol was the same for the peroxidase
151 technique, without addition of H₂O₂, and the secondary antibody was applied for 60
152 min at room temperature in a dark humid chamber. Slides were washed in PBS (3
153 times for 5min), mounted and nuclear counterstained with DAPI mount (Vectashield).
154 Positive controls included canine spleen (CD11c and CD45), lymph node (CD11c and
155 CD45) and duodenum (Ki67, CD31, vWF, vimentin α -SMA and Smemb), and for
156 negative controls the primary antibody was omitted. Images were taken using a light
157 microscope with fluorescent capability^e (~~Leica-DMRB~~).

158

159 *Image capture and analysis*

160 For quantitative assessment (ImageJ cell counter plug-in^h; ~~National Institutes of~~
161 ~~Health~~) nine randomly selected (x400 magnification) non-overlapping images were
162 digitally captured, beginning close to the valve edge (tip of leaflet) and moving back
163 to the junction with the atrial myocardium ~~where the valve base (zone adjacent to the~~

164 ~~annulus~~) covering the same region of interest for each valve, and the cells were then
165 counted manually. Qualitative descriptive assessment only was carried out on the
166 NovaRed stained sections (longitudinal and horizontal) and qualitative and
167 quantitative assessment on the fluorescent labelled sections (longitudinal only).

168

169 *Statistical analysis*

170 Data were expressed as mean \pm standard deviation of the mean. Since the sample size
171 was small log transformation was undertaken ~~to achieve normality~~, followed by the
172 D'Agostino-Pearson normality test, and inferential statistical analysis applied using
173 one-way analysis of variance (ANOVA) testing with a p value of <0.05 . Where ~~one-~~
174 ~~way analysis of variance~~-(ANOVA)-detected significant difference, inter-group
175 differences were compared using the post-hoc Tukey Simultaneous test.

176

177 **Results:**

178 Gross and histological changes in myxomatous mitral valves from CKCS and non-
179 CKCS were not appreciably different, and not dissimilar to previous reports ^{21,27}.

180

181 *Cellular changes in myxomatous mitral valve disease*

182 Similar changes in the distribution of immunoreactive cells were noted for
183 longitudinal and horizontal sections. In the normal mitral valves, most cells were
184 vimentin positive and evenly distributed, and presumed to be quiescent VICs. In the
185 diseased valves, vimentin positive cells were also evenly distributed, but the highest
186 density was adjacent to the endothelial layer (Fig. 1). No cell staining was observed in
187 any valve for the inflammatory cell markers CD11c or CD45, but positive staining
188 was found in control lymph node. In normal mitral valves very few α -SMA or

189 SMemb⁺ cells were seen and these were typically located adjacent to the
190 endothelium. In diseased valves α -SMA⁺ cells were generally found lying beneath the
191 endothelium, clustering mostly at the distal free-edge with a few cells present in the
192 valve stroma. SMemb positive cells were also found clustering surrounding the
193 myxomatous areas and close to the distal free-edge, but there was positive staining
194 throughout the leaflet depth. There were no clear differences comparing CKCS and
195 the mixed breed dogs (Fig. 1). vWF⁺ endothelial cells were found along the whole
196 leaflet length, but with areas of discontinuity in the diseased dogs. A consistent
197 finding in both diseased groups (5/6 CKCS; 6/6 mixed breed) was clusters of vWF⁺
198 cells in the myxomatous sites away from the valve edge (Fig 2). No CD31⁺ cells were
199 seen in any test or control sample. We have been unsuccessful in identifying CD31
200 positive cells in any canine paraformaldehyde fixed tissues, but have found this
201 antibody to be effective in canine valve endothelial cell cultures.

202

203 Ki-67⁺ cells formed linear clusters typically at the leaflet tip, but also were distributed
204 sparsely throughout the myxomatous valves of both groups (Fig 2). Staining appeared
205 to be a combination of cytoplasmic and nuclear. This sparse distribution was
206 somewhat similar to that seen for α -SMA and SMemb⁺ cells, but cell counting was
207 not feasible. In affected valves, flattened elongated cells at the valve surface
208 (~~presumed to be VECs~~) also showed positive staining for Ki-67.

209

210 *Immunofluorescence; Qualitative and Quantitative Analysis*

211 The total numbers of cells positive for α -SMA was significantly increased in the
212 CKCS group compared to normal (Table 2) ($p<0.05$). SMemb⁺ cells were
213 significantly increased in the two diseased groups compared to normal ($p<0.05$).

214 Vimentin+ cells made up the largest number in all valves, and cell numbers in both
215 diseased valves approached twice that seen in normal valves (Table 2; Fig. 3). While
216 the pattern of staining for α -SMA and SMemb at the valve edge was similar in both
217 affected groups, only a few of those cells co-expressed both antigens (Fig. 3 & Fig. 4).
218 In overtly myxomatous areas, the overall pattern of cellular staining was similar in the
219 CKCS and mixed breed dogs.

220

221 **Discussion:**

222 The cavalier King Charles spaniel (~~CKCS~~) breed predisposition to MMVD is well
223 recognised and heritability has a role in disease appearance and progression^{4,5}. There
224 are some CKCS-specific traits identified and while their association with MMVD can
225 be unclear, it is reasonable to suggest that disease phenotype and pathology might be
226 different to that seen in other dogs⁷⁻¹¹. By assessing for a range of markers for
227 inflammation, proliferation and mesenchymal cell phenotypic differentiation evidence
228 was found that cell changes in the CKCS valve ~~are fundamentally the same~~ appear to
229 be similar to that seen in mixed breed dogs.

230

231 There was no evidence of inflammatory cells in any of the samples examined and this
232 is in agreement with the only other report¹⁶. Small numbers of macrophages and mast
233 cells have been previously found in MMVD valves and there is an increased
234 expression of some inflammatory cytokine transcripts in affected valves, but there still
235 is no clear evidence that inflammation has an important role in this disease^{17, 28, 29}.

236

237 Appearance of activated of α -SMA+ valve interstitial cells in canine MMVD is well
238 recognised, particularly in the distal portion of the valve and close to the valve edge,

239 and this was again noted in the present study in all affected dogs^{16, 17}. Increased α -
240 SMA+ cells numbers were seen in the two affected groups and were moderately
241 higher in the CKCS valves, ~~but this could be a chance finding~~. Increased numbers of
242 α -SMA+ cells is dependent on disease severity and the dogs in the current study were
243 aged and had the most severe form of the disease¹⁹. Increased SMemb+ cell numbers
244 have also been previously reported and were found again to be increased in this study,
245 with a marginally higher number in the non-CKCS dogs¹⁶. The distribution of
246 Smemb+ cells in the valve stroma possibly identifies a cell population with
247 differentiation potential, but the presence of Ki-67 expression would suggest
248 proliferation is on-going as well. Lastly, using vimentin as a general mesenchymal
249 cell maker, total cell numbers in the two affected groups were similar and were
250 approximately twice that of the normal valves. Reports to date on changes in cells
251 numbers are either equivocal or conflicting, but this is likely to be based on the
252 counting techniques used. In myxoid stroma cell numbers decline, but overall cell
253 numbers increase and this is dominated by increased cell numbers in the atrialis layer
254^{15,21}. The current study furthermore confirms there is an overall increase in interstitial
255 cell numbers when the entire valve length is examined. However, it should be noted
256 the normal valves were not age-matched and increased cell numbers associated with
257 aging cannot be discounted. The lack of age-matched normal controls is a study
258 limitation, but it is understood that finding normal mitral valves in aged dogs is not
259 readily achievable.

260

261 While vimentin expression is widespread in many cell types, interstitial cells
262 expressing vimentin are generally considered to be a quiescent phenotype form of
263 VIC in the normal mitral valve^{16, 17, 21}. In the diseased valve cells retaining vimentin

264 expression alone, or expressing one or both of the two activation/differentiation
265 markers (α -SMA and SMemb), may represent an expanded population available for
266 phenotypic alteration in response to appropriate triggers. There was an increased
267 expression of the cell proliferation marker Ki-67 in the MMVD valves and this was
268 most noticeable where there was the largest number of α -SMA positive cells close to
269 the distal free-edge. This finding contrasts with the only other study where Ki-67
270 showed minimal expression in diseased valves ¹⁶. Additionally the similar regional
271 expression of Ki-67 with α -SMA+ cells ~~would~~ might suggest proliferation of
272 activated myofibroblasts close to the valve edge is more likely than migration of these
273 cells from deep in the valve stroma, and it would be reasonable to suggest this
274 proliferation is in response to the endothelial changes and damage noted with MMVD
275 ²⁶. Co-localisation staining would confirm if this was the case. Under normal
276 circumstances VECs and VICs interact to prevent VIC activation and ~~VEC~~
277 endothelial-to-mesenchymal transition (EndoMT) (the process by which endothelial
278 cells invade tissue and differentiate into a mesenchymal phenotype) thereby
279 maintaining a quiescent state ³⁰. However, up-regulation of genes associated EndoMT
280 is found in affected canine valves, suggesting changes in cellular homeostasis occurs
281 with disease progression ^{30,31}. EndoMT is likely to be one mechanism that contributes
282 to accumulation of α -SMA positive cells close to the endothelium.

283

284 Identification of cells positive for ~~a~~ α -SMA and Smemb and the distribution of these
285 cells has been previously reported, but the identification of co-localisation is a novel
286 finding^{16,17,21}. This co-localisation of α -SMA and SMemb in a small number of cells
287 close to the valve edge identifies a population that have greater differentiation
288 potential than those expressing α -SMA or SMemb alone, with Smemb having

289 important functions in cell adhesion and cell migration.- Such cells are believed to
290 have greater phenotypic differentiation capability in response to injury³². In the case
291 of MMVD, a similar capacity for phenotypic versatility might exist where endothelial
292 damage has occurred.

293

294 VonWillebrand factor expression demonstrated the presence of the endothelium in all
295 valves and confirmed the loss of endothelial cells along some sections of affected
296 valve edges. Endothelial damage and denuding is now recognised to be a cardinal
297 feature of MMVD, and might be a major triggering event driving the phenotypic
298 changes seen with VICs and proliferation of aVICs close to the valve edge^{2, 23, 24}. The
299 localised expression of vWF in cells within the stroma of the diseased valves was an
300 un-expected finding, suggesting either migration of endothelial cells or mesenchymal
301 transition of stromal VICs (mesenchymal to endothelial transition). The localisation of
302 Smemb+, vWF+ and Ki-67+ cells in the stroma might support the latter conclusion.
303 This finding might be due to extravasation during sample collection, but the number
304 of cells and consistency of the finding make this less likely. Successful staining for
305 CD31 would have been useful to clarify the endothelial identity or not of these cells,
306 as would double staining to identify co-localisation. Lastly, while there appears to be
307 a common pathological end-point when comparing MMVD in CKCSs and mixed
308 breed dogs this does not presume a shared pathogenesis, although that would not be
309 an unreasonable to presume so conclusion.

310

311 The main limitations of this study is the sample size (a recognized problem with
312 studies of this nature) and the technical problem of comparative sampling from
313 normal and diseased valves that have markedly different geometry. The laminated

314 structure of the normal valve compared to the loss of lamination in the disease valves
315 is also a confounding factor. ~~Lastly, while there appears to be a common pathological~~
316 ~~end-point when comparing MMVD in CKCSs and mixed breed dogs this does not~~
317 ~~presume a shared pathogenesis, although it would not be unreasonable to presume so.~~
318

319 In conclusion, this study confirms that there is no evidence for inflammatory cell
320 involvement in canine MMVD, that the cell changes ~~are similar~~ appear similar for
321 CKCS as for other dogs, and that the changes in cell numbers in MMVD are possibly
322 due to cell proliferation. This study suggests MMVD is not a heterogeneous disease,
323 at least in terms of cellular changes and that the CKCS form of the disease differs
324 only in its time of onset and speed of progression. However, the pathogenesis of
325 MMVD is unknown and it is possible different mechanisms could result in the same
326 end-stage findings. ~~However, for now it seems it is the temporal presentation and not~~
327 ~~the end-stage pathology that is likely to be the heritable feature of MMVD in the~~
328 ~~cavalier King Charles spaniel. Furthermore, it~~ From these studies it would be
329 reasonable to presume that studies of any dog with MMVD are applicable to all dogs
330 and breeds.

331

332 **Acknowledgements:**

333 The authors are indebted to the owners who have donated valve tissue from their pets
334 to this study. Without their support this work would not have been possible. We are
335 particularly indebted to the Cavalier King Charles Club of England for assistance in
336 sourcing the CKCS material and their financial support of C-C Lu.

337

338 **Conflict of Interest:**

339 None of the authors of this paper has a financial or personal relationship with other
340 people or organisations that could inappropriately influence or bias the content of the
341 paper.
342

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465 myocardial infarcts express the embryonic form of smooth muscle myosin heavy
466 chain (SMemb), *Cardiovasc Res* 2000; 48:89-100.
467
468
469

470 Table 1. Antibody source and concentration used for IHC in canine mitral valves.
 471

Primary and Secondary Antibodies	Origin	Dilution	Manufacturer	Catalogue No.
α -SMA	Mouse monoclonal	1:400	Sigma	A2547
SMemb	Rabbit polyclonal	1:1000	Abcam	Ab24761
Vimentin	Mouse monoclonal	1:1600	Sigma	V6389
vWF	Rabbit polyclonal	1:1000	Abcam	Ab6994
Ki-67	Rabbit polyclonal	1:200	Abcam	Ab15580
CD11c	Mouse monoclonal	1:100	Abcam	Ab76911
CD45	Mouse monoclonal	1:100	AbD Serotec	MCA2035S
CD31	Rabbit polyclonal	1:100	Abcam	Ab28364
Alexafluor 488	Goat anti-Mouse IgG (H+L)	1:100	Invitrogen	A10667
Alexafluor 568	Goat anti-Rabbt IgG (H+L)	1:100	Invitrogen	A11011
Biotinylated Antibody	Goat anti-Mouse IgG (H+L)	1:1000	Vector	BA-9200
Biotinylated Antibody	Goat anti-Rabbit IgG (H+L)	1:1000	Vector	BA-1000

472

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474

475 Table 2. Comparison of cell numbers (mean +/-SD) positive for α -smooth muscle
 476 actin (α -SMA), embryonic smooth muscle myosin (Smemb) and vimentin in mitral
 477 valves of cavalier King Charles spaniels (CKCS n=6), mixed breed dogs (n=6) with
 478 myxomatous mitral valve disease (MMVD) and normal mixed breed dogs (n=4). Data
 479 log transformed. * Significantly different from control group $P<0.05$.

480

	α -SMA (Mean \pm SD)	Smemb (Mean \pm SD)	Vimentin (Mean \pm SD)
CKCS n=6	33.95 \pm 12.13*	23.13 \pm 5.72*	40.52 \pm 18.41
non-CKCS n=6	25.7 \pm 2.67	29.55 \pm 3.06*	46.95 \pm 17.65
Control n=4	10.75 \pm 2.95	14.53 \pm 3.96	26.75 \pm 7.24

481

482

483 Figure 1. Immunohistochemistry of myxomatous mitral valves from cavalier King
484 Charles spaniels (CKCS; Left Panel A, B & C) and non-CKCS (Right Panel D, E &
485 F). Photomicrographs for immunostaining of vimentin (A&B) alpha-smooth muscle
486 actin (α -SMA; C&D) and myosin heavy chain-10 (SMemb; E&F) in the distal zone.
487 There is a similar aggregation pattern of α -SMA, SMemb, and vimentin in CKCS and
488 non-CKCS with positive cells forming linear clusters along the valve edge most
489 noticeable with α -SMA . Magnification α -SMA, SMemb, Vimentin 20x, bar = 50 μ m.
490

491 Figure 2. Photomicrographs of Von Willebrand Factor (vWF) immunostained
492 myxomatous mitral valves (vWF; right panel A-C) and Ki-67 (left panel D-F). (A)
493 Continuous layer of endothelium showing expression of vWF, compared with (B)
494 interrupted endothelium. (C) Spindle-shaped vWF positive cells clustering in the
495 spongiosa. (D) Multi-layered Ki-67 positive cells aggregating in the myxomatous
496 region at the distal end of mitral valve leaflets. (E) Clusters of Ki-67 positive cells in
497 the spongiosa. (F) Single layer of endothelial cells showing positive staining for the
498 proliferation marker Ki-67. Magnification (A-D) 20x, Bar = 50 μ m, (E) (F) 40x, Bar =
499 25 μ m.

500
501 Figure 3. Fluorescence photomicrograph of cavalier King Charles spaniel
502 myxomatous mitral valve, taken at the level of the valve edge. Immunofluorescence
503 staining for alpha-smooth muscle actin (α -SMA), myosin heavy chain-10 (SMemb),
504 Vimentin, and 4-6-diamidino-2- phenylindole (DAPI, blue) nuclear counterstaining.
505 Magnification 40x, scale = 25 μ m.
506

507 Figure 4. Double fluorescence photomicrographs of cavalier King Charles spaniel
508 myxomatous mitral valve, taken at the level of the valve edge. Alpha-smooth muscle
509 actin (α -SMA; green) and myosin heavy chain-10 (SMemb; red). There is a different
510 staining pattern of α -SMA and SMemb positive cells. The merged photomicrographs
511 show occasional cells co-expressing α -SMA and SMemb (arrows). Magnification
512 40x, scale = 25 μ m.

513

514 Footnotes:

515 c. ABC Elite Kit, Vector Laboratories Inc.

516 d. AlexaFluor488 and 568, Invitrogen

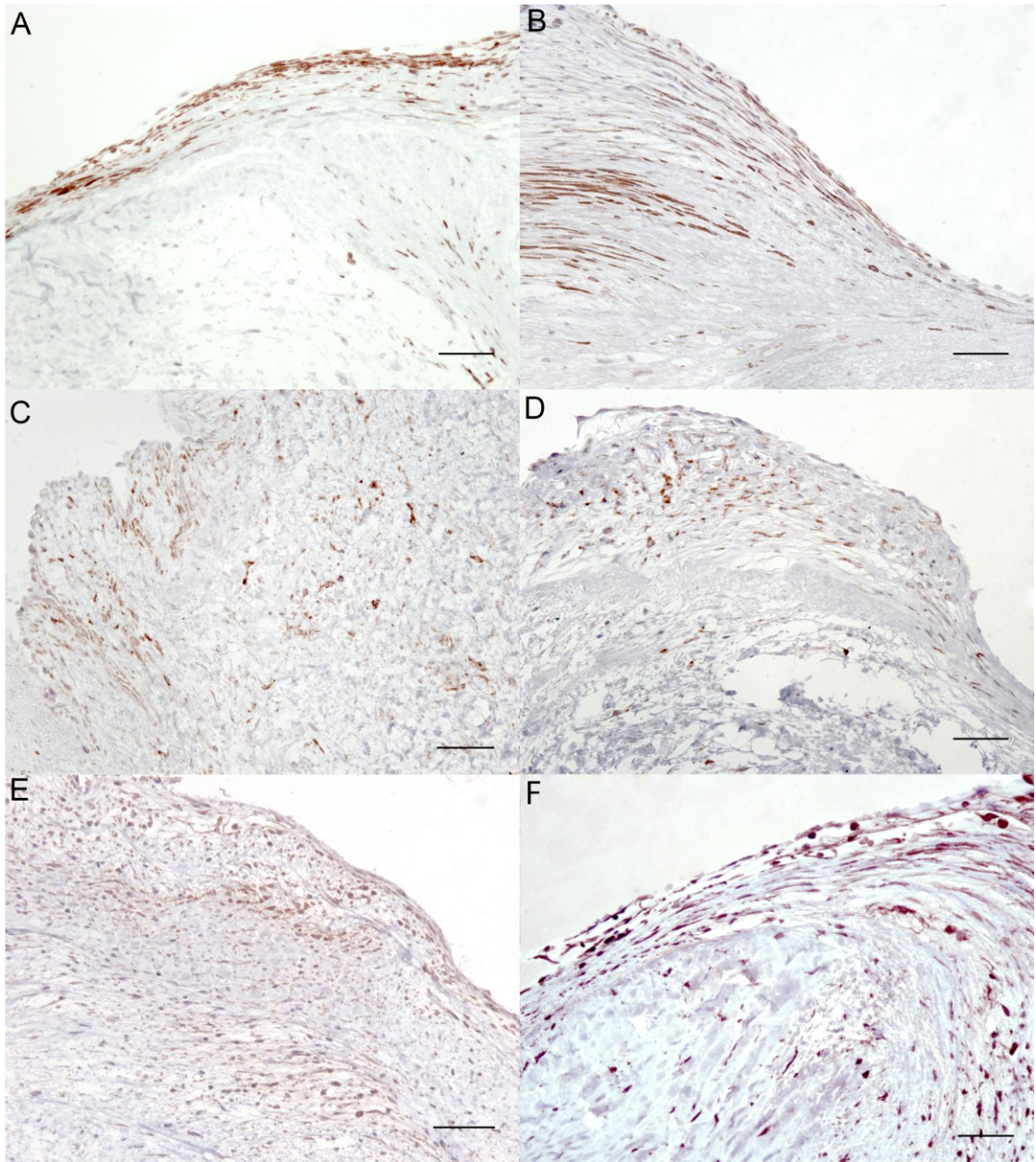
517 e. Leica-DMRB

518 f. Vector Laboratories Inc.

519 g. DePex; Gurr-BDH Chemicals Ltd

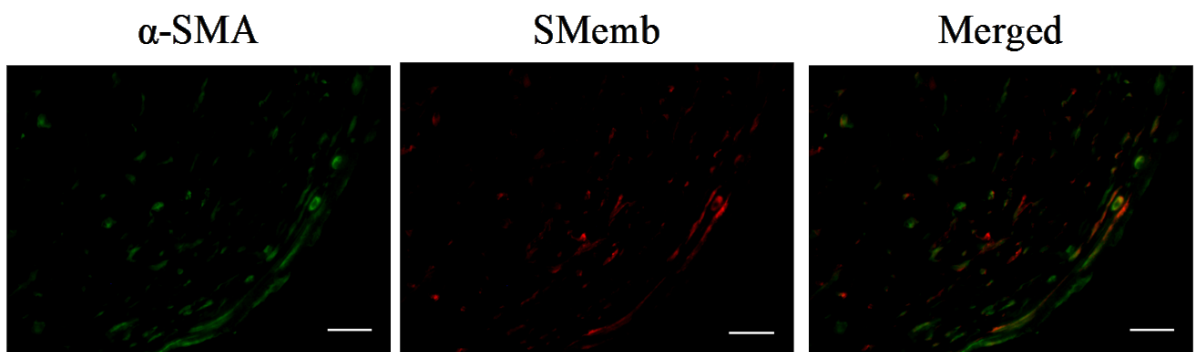
520 h. National Institutes of Health

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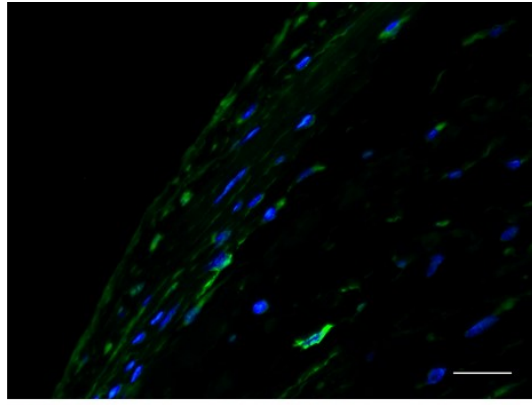
523 Figure 1



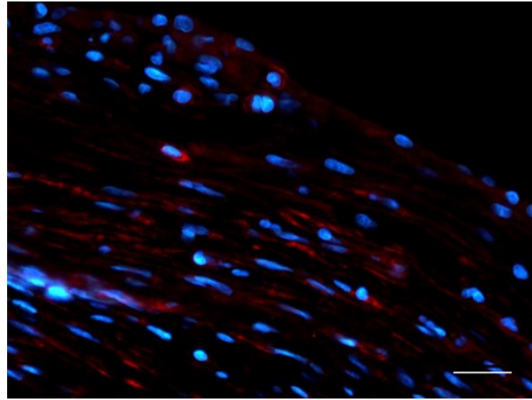
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525 Figure 2

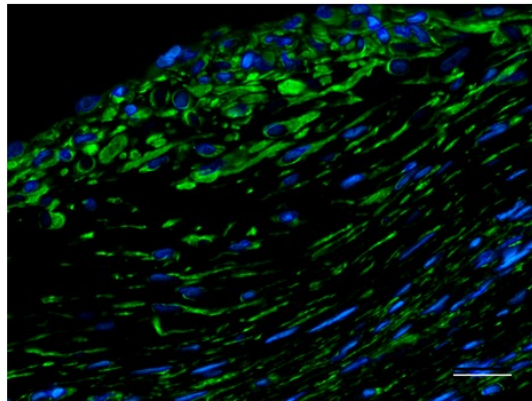
α -SMA



SMemb

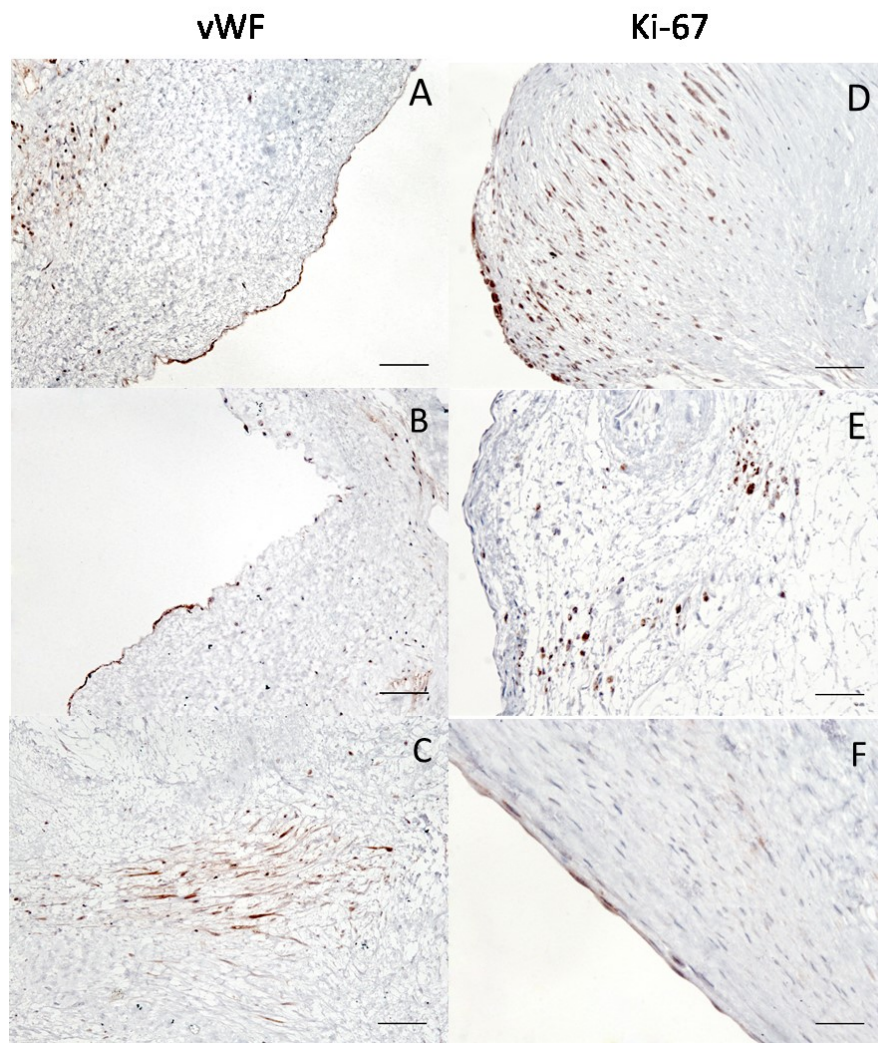


Vimentin



526

527 Figure 3



528

529

Figure 4