



Survivin and Sox9: Potential Stem Cell Markers in Canine Normal, Hyperplastic, and Neoplastic Canine Prostate / Bongiovanni, L;

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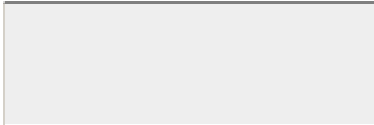
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Survivin and Sox9, potential stem cell markers in canine normal, hyperplastic and neoplastic canine prostate

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Keywords:	Dog < Domestic Mammals < Species, Immunohistochemistry < Technology, prostate carcinoma, Sox9, stem cells, survivin
Abstract:	Canine prostatic carcinoma is a relevant model for human prostatic carcinoma. Survivin is proposed as a cancer biomarker of malignancy in human prostatic cancer. Sox9 is a stem cell marker required for prostate development and expressed in several adult tissues. The aims of the present study were to evaluate the patterns and expression levels of two putative stem cell markers, survivin and Sox9, in canine benign prostatic hyperplasia (BPH) and prostatic carcinoma, in order to investigate their potential as stem cell markers. Immunohistochemistry using specific antibodies was performed on 3 samples of normal prostate gland, 18 samples of canine BPH, and 16 samples of prostatic carcinoma. The basal cell layer of normal and hyperplastic prostatic lobules had nuclear Sox9 immunolabeling, and nuclear and rarely cytoplasmic survivin immunostaining, identifying them as potential stem cell markers. Significantly more frequent ($\geq 10\%$ of nuclei) survivin and Sox9 expression was observed in prostatic carcinoma compared with BPH. The potential co-expression of survivin with Sox9, androgen receptor (AR) and p63 was also investigated in selected BPH and prostatic carcinoma cases using immunofluorescence, and a partial co-localization was observed. Results

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indicate that Sox9 and survivin could be considered as markers of stemness in canine prostate cells. Given its role in proliferation, cells in the basal cell layer with nuclear survivin expression are likely to be transit amplifying cells that maintain some stem cell properties.

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3 **1 Survivin and Sox9, potential stem cell markers in canine normal,**
4 **2 hyperplastic and neoplastic canine prostate**
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24 **Abstract**

25 Canine prostatic carcinoma is a relevant model for human prostatic
26 carcinoma. Survivin is proposed as a cancer biomarker of malignancy in
27 human prostatic cancer. Sox9 is a stem cell marker required for prostate
28 development and expressed in several adult tissues. The aims of the present
29 study were to evaluate the patterns and expression levels of two putative
30 stem cell markers, survivin and Sox9, in canine benign prostatic
31 hyperplasia (BPH) and prostatic carcinoma, in order to investigate their
32 potential as **stem cell markers. Immunohistochemistry using specific**
33 **antibodies was performed on 3 samples of normal prostate gland, 18 samples**
34 **of canine BPH, and 16 samples of prostatic carcinoma. The basal cell layer**
35 **of normal and hyperplastic prostatic lobules had nuclear Sox9**
36 **immunolabeling, and nuclear and rarely cytoplasmic survivin immunostaining,**
37 **identifying them as potential stem cell markers. Significantly more**
38 **frequent ($\geq 10\%$ of nuclei) survivin and Sox9 expression was observed in**
39 **prostatic carcinoma compared with BPH. The potential co-expression of**
40 **survivin with Sox9, androgen receptor (AR) and p63 was also investigated in**
41 **selected BPH and prostatic carcinoma cases using immunofluorescence, and a**
42 **partial co-localization was observed. Results indicate that Sox9 and**
43 **survivin could be considered as markers of stemness in canine prostate**
44 **cells. Given its role in proliferation, cells in the basal cell layer with**
45 nuclear survivin expression are likely to be transit amplifying cells that
46 maintain some stem cell proprieties.

47 Key words: dog; immunohistochemistry; prostate carcinoma; Sox9; stem cells;
48 survivin.

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3 51 Canine prostatic carcinoma is considered a relevant model for studying
4 52 advanced, hormone-refractory prostatic carcinoma in men.^{1,18} Besides man, the
5 53 dog is the only species to spontaneously develop prostate cancer and these
6 54 cancers have certain features in common: age of onset, frequency of
7 55 invasion, sites of metastases, and histological features.¹⁸

11 56 It has been proposed that cancer contains a minor population of cells that
12 57 can self-renew while simultaneously giving rise to tumour cells: cancer
13 58 stem cells (CSCs).³³ Human¹⁵ and canine²⁵ prostatic tumours appear to contain
14 59 a subpopulation of cells with stem cell features. CSCs are highly
15 60 tumorigenic, have a high metastatic potential, and show a relatively high
16 61 resistance to traditional cancer therapies.²⁴ These findings have prompted
17 62 the emergence of a new field of study in cancer treatment involving the
18 63 targeting of CSCs; accordingly this requires the identification of new
19 64 cancer stem cell markers.

23 65 Survivin represents a well-known cancer therapy-resistance factor that is
24 66 overexpressed in several tumour types.² A role for survivin has been
25 67 proposed in tumour initiation and progression, as well as in the
26 68 maintenance of cancer stem cells.³⁰ It has been proposed that survivin be
27 69 used as a biomarker for malignancy in early screening for human prostatic
28 70 cancer.³¹ The overexpression of survivin has been implicated in the
29 71 development of prostatic carcinoma, leading investigators to evaluate the
30 72 efficacy of survivin inhibitors as a possible new therapeutic option.³⁵ High
31 73 expression of survivin has been observed in putative cancer stem cells
32 74 isolated from prostatic adenocarcinoma in a murine model.^{20,21} However, no
33 75 data have been published regarding the expression and significance of
34 76 survivin in canine prostatic tumours.

35 77 Sox9 is a stem cell marker expressed in several adult tissues and is
36 78 required for human prostate development.^{14,32} It contributes to the
37 79 development of human prostatic carcinoma⁷ and is, therefore, considered a

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3 80 potential prognostic marker in human prostatic carcinoma patients.³⁷
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5 81 However, although Sox9 is expressed in a large proportion of prostate
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7 82 cancers, its relevance to prognosis varies depending on the molecular
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9 83 environment: It has recently been demonstrated that loss of Sox9 expression
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11 84 was associated with prostatic carcinoma recurrence in ERG-positive and
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13 85 PTEN-deleted prostate cancers.⁶

14 86 Based on the hypotheses that survivin and Sox9 could represent markers of
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16 87 cancer stem cells and malignancy in canine prostatic tumours, the present
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18 88 study aimed to characterize the immunohistochemical (IHC) patterns and
19
20 89 levels of expression of survivin and Sox9 in canine benign prostatic
21
22 90 hyperplasia (BPH) and prostatic carcinoma.

23
24 91 A further objective was to study the co-localization of survivin with Sox9,
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26 92 p63 and AR in a subset of canine prostatic hyperplastic and neoplastic
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28 93 lesions. p63 has been identified as a prostate basal cell marker required
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30 94 for normal prostatic development^{12,28} and seems to be required for
31
32 95 maintenance of progenitor/stem cells.²⁸ It has also been shown that p63 is
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34 96 discontinuously expressed in the basal cells of both normal canine
35
36 97 prostatic acini and hyperplastic lobules but downregulated in canine
37
38 98 prostatic carcinoma.²⁹

39 99 The androgen/AR axis controls the growth and development of prostate tissue
40
41 100 as well as prostatic carcinoma progression, and AR differentially
42
43 101 influences the characteristics of normal stem cells and prostate cancer
44
45 102 stem cells.⁹ Recently it was shown that AR is expressed in canine BPH and,
46
47 103 to a lesser extent, in Prostatic carcinoma.^{9,29} In the prostate, survivin
48
49 104 expression seems to be regulated by androgen. Survivin
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51 105 expression/overexpression in human prostatic carcinoma has been proposed as
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53 106 one of the molecular mechanisms of progression to androgen independence.
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55 107 The overexpression of survivin appeared to be sufficient to induce
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57 108 androgen-independent growth of androgen-dependent cells.³⁶

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5 110 **Material and methods**6
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8 111 **Histological examination**

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10 112 The present study was carried out using 37 formalin-fixed, paraffin wax-
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12 113 embedded samples of canine prostate tissues from different dogs: 16 BPH, 16
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14 114 primary prostatic carcinoma, and one necropsy sample of normal canine
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16 115 prostate gland retrospectively collected from the University archives, with
17
18 116 unknown fixation time. In addition, fresh material from four additional
19
20 117 dogs, including two normal prostate glands and two BPH cases, was formalin
21
22 118 fixed for a maximum of 24 h. These additional samples were used
23
24 119 specifically to investigate the pattern of Sox9 expression with IHC and
25
26 120 the possible effects of formalin fixation time on the immunostaining.

27 121 Histological diagnosis was performed using haematoxylin and eosin (HE)-
28
29 122 stained slides, according to WHO guidelines.¹⁷ Prostatic carcinoma samples
30
31 123 were further classified based on the histological subtypes²⁷ and the
32
33 124 Gleason-like grading system.²⁶

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35 125 **Immunohistochemistry**

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38 126 Immunohistochemistry (IHC) was performed using specific primary antibodies
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40 127 (Table S1), according to a previously described technique.³ Briefly,
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42 128 deparaffinized and rehydrated sections were incubated with 3% H₂O₂ in
43
44 129 absolute methanol for 45min to inhibit endogenous peroxidase activity and
45
46 130 then rinsed in 0.05M Tris-buffered saline (TBS, pH 7.6) for 5min. Antigen
47
48 131 retrieval was performed by heat treatment in Tris-EDTA buffer, pH9.0 in a
49
50 132 microwave oven for 5min (four cycles). After the last treatment, sections
51
52 133 were left for 20min in the buffer for cooling. To reduce non-specific
53
54 134 binding, slides were then incubated with normal goat serum (code MR*HRP-
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56 135 650, Biospa, Milan, Italy) for 10min at room temperature before overnight
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58 136 incubation with the primary Ab in a humidified chamber at 4°C. After

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3 137 rinsing with TBS, immune complexes were treated at room temperature for
4 138 30min with secondary biotinylated goat anti-mouse or anti-rabbit antibodies
5 139 (1:200 dilution; Vector Laboratories Inc., Burlingame, California, USA) and
6 140 subsequently visualized using an avidin-biotin complex (ABC) method
7 141 (Vectastain ABC Kit, Vector Laboratories) for 30min. Peroxidase activity
8 142 was detected by a 5min application of 0.1% H₂O₂ in 3-3'-diaminobenzidine
9 143 solution (code D5905, Sigma-Aldrich, St. Louis, Mo, USA) followed by
10 144 counterstaining with Mayer's haematoxylin (Merck, Darmstadt, Germany) for
11 145 1min before rinsing, dehydrating and mounting. A negative control was
12 146 performed in all instances by omitting the primary Ab and incubating tissue
13 147 sections with TBS and/or replacing it by an Ab of irrelevant specificity
14 148 (rabbit anti-human von Willebrand factor polyclonal Ab, from DAKO,
15 149 Glostrup, Denmark). Sections of canine tissues known to display expression
16 150 of the investigated molecules, as indicated in Table S1, were used as
17 151 positive controls.

30 152 **Double immunofluorescence**

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32
33 153 Double immunofluorescence was used to qualitatively investigate survivin-
34 154 Sox9, survivin-p63 and survivin-AR nuclear co-expression in 16 selected
35 155 cases (eight BPH and eight prostatic carcinoma) based on our previous
36 156 published study²⁶, using specific antibodies (Table S1). Tissue samples were
37 157 treated as described for the immunohistochemical procedure. A sequential
38 158 protocol was used for double staining. Primary antibodies were applied
39 159 overnight at 4°C. The first secondary Ab, biotinylated goat anti-rabbit
40 160 (for survivin) (1:200 dilution; Vector Laboratories, Burlingame, CA, USA)
41 161 was applied and incubated for 30min at room temperature, and slides were
42 162 then incubated with fluorescein-conjugated avidin (1:100 dilution in 0.1M
43 163 NaHCO₃, 0.15M NaCl buffer, pH 8.2-8.5; Vector Laboratories) for 10min at
44 164 room temperature. An avidin/biotin blocking step was performed by
45 165 incubating slides for 15min with avidin and then biotin (Avidin/Biotin

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3 166 Blocking Kit, code SP-2001, Vector Laboratories) at room temperature. A
4 167 further protein blocking step was performed by incubating slides with
5 168 normal goat serum (Vector Laboratories) for 15min at room temperature,
6 169 before the overnight application of the second primary Ab. The second
7 170 secondary Ab, biotinylated goat anti-rabbit (for Sox9, AR) or anti-mouse
8 171 (for p63) (1:200 dilution; Vector Laboratories) was applied and incubated
9 172 for 30min at room temperature, and slides were then incubated with Texas
10 173 Red-conjugated avidin (Vector Laboratories) diluted 1:100 in a buffer
11 174 composed of 0.1M NaHCO₃ and 0.15M NaCl, pH 8.2-8.5, for 10min at room
12 175 temperature. Nuclei were counterstained with 4',6-diamidino-2-phenylindole
13 176 (DAPI) (Vector Laboratories).

23 177 **Quantification of immunolabelling and statistical analysis**

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25
26 178 Nuclear and cytoplasmic survivin and nuclear Sox9 immunolabelling were
27 179 assessed semiquantitatively by two pathologists (LB and MR) in ten randomly
28 180 selected HPF (40X) and graded as percentage of all neoplastic cells as
29 181 follow: 0, absent; 1 (low), >0 and <10% of cells; 2 (moderate), 10 to <25%
30 182 of cells; 3 (high), 25 to <50% of cells; 4 (very high), >50% of cells.

31 183 Labelling intensity of cytoplasmic survivin was graded as no (0), weak (-
32 184 /+), moderate (+) or strong (++) labelling.

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35 185 Fisher's exact test was used to compare semiquantitatively assessed
36 186 immunoreactivity in BPH and prostatic carcinoma. For this purpose, both
37 187 survivin cytoplasmic score and survivin and Sox9 nuclear score were
38 188 evaluated. The cases were grouped according to the nuclear score as
39 189 follows: <10% positive nuclei (absent and very low number of positive
40 190 nuclei) versus ≥10% positive nuclei (low/moderate and high number of
41 191 positive nuclei). This choice depended on the distribution of nuclear score
42 192 values for each protein and on several statistical analyses performed.
43 193 Analyses were performed using SPSS statistical software, with P <0.05
44 194 considered to be significant.

195

196 Results**197 Histological examination**

198 Histological features are reported in Table S2 (Please, refer to the online
199 version of the manuscript for supplemental material). In half of the
200 prostatic carcinoma cases (8/16), a single histological pattern was seen
201 (four papillary, three solid/undifferentiated and one small acinar/ductal);
202 a mixed pattern was observed in the other 8 cases. Necrosis was observed in
203 12/16 (75%) cases and 2/16 (12.5%) were characterized by abundant fibrous
204 tissue (formerly termed "scirrhous prostatic carcinoma").

205 Immunohistochemistry

206 The IHC results are summarized in Table 1 and S2.

207 Survivin expression in normal gland, BPH and prostatic carcinoma

208 Survivin-positive nuclei were present among the basal/reserve cell layer of
209 normal (Fig.1) and hyperplastic prostatic lobules [0-10% of cells in the
210 11/16 (68,75%) cases with positive labeling] (Fig.2). Even if a single case
211 of normal gland was analyzed, normal prostatic tissue surrounding the BPH
212 lesions was also evaluated, showing the same pattern of expression of the
213 molecule. Most of the BPH cases (12/16, 75%) showed patchy cytoplasmic
214 immunostaining, with a low to moderate (0-25% of neoplastic cells)
215 expression in most of the cases (10/12, 83,3%). In contrast, prostatic
216 carcinomas had more diffuse cytoplasmic expression as well as nuclear
217 expression of survivin. Cytoplasmic staining was observed in all the
218 prostatic carcinoma samples, with half of the cases (8/16) showing low to
219 moderate expression (0-25% of neoplastic cells) and the other half high
220 expression (>25% of neoplastic cells). Positive nuclei were present in all
221 except one case of prostatic carcinoma, with most (9/16, 56%) showing

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3 222 moderate to high expression (10 to >50% of positive nuclei) (Fig.3), present
4 223 mainly among the neoplastic cells with basal cell morphology in the areas
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6 224 with tubular-papillary pattern .
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9 225 **Sox9 expression in normal gland, BPH and prostatic carcinoma**

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11 226 Sox9-positive nuclei were observed in scattered cells among the
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13 227 basal/reserve cell layer of 2 of 3 normal prostatic glands (Fig.4) and 5 of
14
15 228 15 hyperplastic lobules (Fig.5). Of these positive cases, two normal glands
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17 229 and 2 BPH cases were freshly sampled, while the other 3 BPH cases were from
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19 230 the archive and the fixation time was not known, suggesting that prolonged
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21 231 fixation may interfere with Sox9 immunostaining.³⁴ Among prostatic
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23 232 carcinomas, 9/16 cases were positive and most (7/9) showed a moderate to
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25 233 high expression (>25% of neoplastic cells with nuclear
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27 234 immunolabelling) (Fig.6).
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31 236 **Double immunofluorescence**

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33 237 From a qualitative evaluation of the immunofluorescence-stained slides,
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35 238 only a partial co-localization of survivin and p63 (survivin+/Sox9+) was
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37 239 observed. In BPH, survivin-/p63+ nuclei were more numerous (5-10%),
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39 240 survivin+/p63- nuclei were scattered and rare, and survivin+/p63+ nuclei
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41 241 were few (<5% of cells were double-positive) and observed among basal cells
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43 242 (Fig.7). In contrast, in prostatic carcinoma, survivin+/p63- nuclei were
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45 243 more numerous (10-25%), and survivin+/p63+ nuclei were scattered and few
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47 244 (<5% of cells were double-positive). p63 cytoplasmic immunostaining was
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48 also evident (Fig.8).
49

50 246 In BPH cases, a higher number of survivin-/AR+ cells (>50%) was observed
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52 247 compared to survivin+/AR+ cells (5-10%), while no survivin+/AR- cells were
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54 248 observed (Fig.9). Nuclear immunolabelling in prostatic carcinoma was
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56 249 characterized by numerous survivin+/AR+ cells (25-50%) and survivin+/AR-

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3 250 cells were also evident (10-25%) (Fig.10). Several neoplastic cells showed
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5 251 an intense aberrant AR cytoplasmic expression as well as cytoplasmic
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7 252 immunolabelling for survivin.

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9 253 In BPH cases, survivin+/Sox9- cells were more numerous (between 10 and 25%)
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11 254 than survivin+/Sox9+ cells (<5% of double positive cells), while no
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13 255 survivin-/Sox9+ cells were observed (Fig.11). In the prostatic carcinoma
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15 256 cases, a higher number of survivin+/Sox9+ (double-immunostained) cells was
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17 257 observed (Fig.12), with intense nuclear and faint cytoplasmic
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19 258 immunolabelling, and a few (<5%) survivin+/Sox9- and survivin-/Sox9+ cells.

20 259 **Statistical Analysis**

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23 260 Both nuclear survivin and Sox9 expression appeared to be higher ($\geq 10\%$ of
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25 261 neoplastic cells with positively labeled nuclei) in prostatic carcinoma
26
27 262 compared with BPH cases ($p < 0.01$ for both). No significant differences were
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29 263 observed in cytoplasmic survivin expression comparing BPH with prostatic
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31 264 carcinoma cases.

32 265 33 34 35 266 **Discussion**

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37
38 267 This study is the first to characterize the IHC expression of survivin and
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40 268 Sox9 in normal, hyperplastic and neoplastic canine prostate. The main aims
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42 269 were to verify if Sox9 and survivin are expressed by canine prostatic
43
44 270 epithelial cells and if they could represent prostatic stem cell markers.

45
46 271 The nuclear immunostaining observed in the basal cell layer of normal
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48 272 prostate gland is consistent with Sox9 as a stem cell marker in canine
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50 273 prostate, similar to what has been reported in the human literature.⁷ In
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52 274 contrast to our predictions, the first round of testing with Sox9
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54 275 immunohistochemistry, using cases selected from the University's archives,
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56 276 showed no positive cells in the normal glands, and only a few positive

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3 277 samples among the BPH cases. A second round of testing, using freshly
4 278 sampled prostatic tissue fixed in formalin for 24 hours, revealed positive
5 279 staining in all four cases, and it was more intense and evident than that
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7 280 observed in samples with longer fixation time. This suggests that prolonged
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9 281 formalin fixation time decreased Sox9 antigen detection, indicating that
10 282 this antigen is more vulnerable to degradation than the other investigated
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12 283 molecules (such as survivin, p63, AR).³⁴
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16 284 The role of survivin in stem cells¹⁶ might indicate that the positive cells
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18 285 found in the basal cell layer of normal glands are stem cells. However,
19 286 since survivin also has a role in proliferation, it is also possible that
20 287 these cells represent partially differentiated, proliferating, transit
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22 288 amplifying cells that maintain some stem cell properties.
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26 289 The prostate basal cell marker p63 had already been shown to be involved in
27 290 epithelial development by regulating stem cell/transit amplifying cells,
28 291 their differentiation, and cell death.^{8,22} Results obtained from the
29 292 immunofluorescence study showed that in BPH survivin and p63 are expressed
30 293 in different cells, with only partial, minimal co-localization. Our
31 294 hypothesis is that they mark transit amplifying cells in these lesions, an
32 295 undifferentiated population of cells in transition between stem cells and
33 296 differentiated cells, with intermediate features. This would support the
34 297 previously reported theory that at least two biologically distinct
35 298 populations of basal cells exist in the canine prostate gland¹⁹ and that
36 299 these cells can be involved in the development/maintenance of BPH.
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46 300 Other recent studies characterizing the expression of stem cell markers
47 301 have shown that co-expression of CD44 and CD133 detects stem cells in
48 302 canine prostatic cancer cell lines.^{23,24} Further studies should be done in
49 303 order to investigate whether survivin and Sox9 are co-expressed with these
50 304 markers.
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3 305 In human prostatic tissue, survivin expression is regulated by androgen
4 306 stimulation.³⁶ In the canine prostatic tissue studied here, the presence of
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6 307 survivin+/AR- cells in prostatic carcinoma, together with the increased
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8 308 expression of survivin in prostatic carcinoma compared to BPH, would
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10 309 suggest a role for survivin in the progression to androgen independence in
11
12 310 the dog as well. The survivin+/AR- cells could represent a subpopulation of
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14 311 androgen-independent tumour cells. These may be important in the early
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16 312 stages of development of prostate cancer and, if present in human prostatic
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18 313 carcinoma, may be the cause of the failure of androgen ablation therapy
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20 314 that occurs in most human advanced cases.¹⁰ Our findings would suggest the
21
22 315 presence, in canine prostatic carcinoma, of a subpopulation of neoplastic
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24 316 cells with low levels of AR and high levels of survivin and some features
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26 317 of stem cells (prostatic stem cells or transit amplifying cells). This
27
28 318 supports the possibility that this tumour derives from basal/stem cell-like
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30 319 epithelial cells expressing low levels of AR.^{11,18}

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32 320 The origin of CSCs within solid tumours has not yet been clarified and
33
34 321 would appear to vary based on tumour type: tumour cells could originate
35
36 322 from normal tissue stem cells or from partially differentiated transit
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38 323 amplifying cells that undergo oncogenic transformation to produce CSCs.¹³
39
40 324 Further studies should be done to better characterize this subpopulation
41
42 325 and its features of stemness, and to investigate the possible regulation of
43
44 326 the AR-activated pathways on survivin expression in canine prostatic tissue
45
46 327 and prostatic neoplasms.
47

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334

335 **References**

- 336 1. Argyle DJ. Prostate cancer in dogs and men: a unique opportunity to
337 study the disease. *Vet J.* 2009;180(2):137-8.
- 338 2. Athanasoula KCh, Gogas H, Polonifi K, Vaiopoulos AG, Polyzos A,
339 Mantzourani M. Survivin beyond physiology: orchestration of multistep
340 carcinogenesis and therapeutic potentials. *Cancer Lett.*
341 2014;347(2):175-82.
- 342 3. Bongiovanni L, Mazzocchetti F, Malatesta D, et al.
343 Immunohistochemical investigation of cell cycle and apoptosis
344 regulators (survivin, β -catenin, p53, caspase 3) in canine
345 appendicular osteosarcoma. *BMC Vet Res.* 2012; 11;8:78.
- 346 4. Bongiovanni L, Suter MM, Malatesta D, et al. Nuclear survivin
347 expression as a potentially useful tool for the diagnosis of canine
348 cutaneous sebaceous lesions. *Vet Dermatol.* 2012;23(5):394-e73.
- 349 5. Bongiovanni L, Suter MM, Inverso A, et al. Sox9 and CK15 as markers
350 of malignancy in canine cutaneous sebaceous lesions. Abstract in the
351 proceedings of the II Joint European Congress of the ESVP, ECVP, ESTP
352 - Berlin, 27th-30th August 2014.
- 353 6. Burdelski C, Bujupi E, Tsourlakis MC, et al. Loss of SOX9 Expression
354 Is Associated with PSA Recurrence in ERG-Positive and PTEN Deleted
355 Prostate Cancers. *PLoS One.* 2015;10(6):e0128525.
- 356 7. Cai C, Wang H, He HH, et al. ERG induces androgen receptor-mediated
357 regulation of SOX9 in prostate cancer. *J Clin Invest.*
358 2013;123(3):1109-22.

- 1
2
3 359 8. Candi E, Rufini A, Terrinoni A et al. Differential roles of p63
4 360 isoforms in epidermal development: selective genetic complementation
5 361 in p63 null mice. *Cell Death Differ.* 2006; 13: 1037-1047.
6
7
8
9 362 9. Di Zazzo E, Galasso G, Giovannelli P, et al. Prostate cancer stem
10 363 cells: the role of androgen and estrogen receptors. *Oncotarget.* 2016;
11 364 7(1):193-208.
12
13
14
15 365 10. Feldman BJ, Feldman D. The development of androgen-independent
16 366 prostate cancer. *Nat Rev Cancer.* 2001; 1(1):34-45.
17
18
19
20 367 11. Gallardo F, Mogas T, Baró T, et al. Expression of androgen, oestrogen
21 368 alpha and beta, and progesterone receptors in the canine prostate:
22 369 differences between normal, inflamed, hyperplastic and neoplastic
23 370 glands. *J Comp Pathol.* 2007;136(1):1-8.
24
25
26
27
28 371 12. Signoretti S, Waltregny D, Dilks J, et al. p63 is a prostate basal
29 372 cell marker and is required for prostate development. *Am J Pathol.*
30 373 2000;157:1769-1775.
31
32
33
34 374 13. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation.
35 375 *Cell.* 2011;144(5):646-674.
36
37
38
39 376 14. Huang Z, Hurley PJ, Simons BW, et al. Sox9 is required for prostate
40 377 development and prostate cancer initiation. *Oncotarget.*
41 378 2012;3(6):651-63.
42
43
44
45 379 15. Huang CK, Luo J, Lee SO, Chang C. Concise review: androgen receptor
46 380 differential roles in stem/progenitor cells including prostate,
47 381 embryonic, stromal, and hematopoietic lineages. *Stem Cells.*
48 382 2014;32(9):2299-308.
49
50
51
52 383 16. Kapinas K, Kim H, Mandeville M, et al. microRNA-mediated survivin
53 384 control of pluripotency. *J Cell Physiol.* 2015;230(1):63-70.
54
55

- 1
2
3 385 17. Kennedy PC, Cullen JM, Edwards JF, et al. World Health Organisation.
4 386 Histological Classification of Tumors of the Genital System of
5 387 Domestic Animals. Armed Forces Institute of Pathology, Washington DC,
6 388 1998.
7
8
9
10 389 18. Lai CL, L'Eplattenier H, van den Ham R, et al. Androgen receptor CAG
11 390 repeat polymorphisms in canine prostate cancer. *J Vet Intern Med.*
12 391 2008;22(6):1380-4.
13
14
15
16 392 19. Leav I, Schelling KH, Adams JY, Merk FB, Alroy J. Role of canine
17 393 basal cells in postnatal prostatic development, induction of
18 394 hyperplasia, and sex hormone-stimulated growth; and the ductal origin
19 395 of carcinoma. *The Prostate.* 2001;48:210-224.
20
21
22
23
24 396 20. Liao CP, Adisetiyo H, Liang M, Roy-Burman P. Cancer stem cells and
25 397 microenvironment in prostate cancer progression. *Horm Cancer.* 2010;
26 398 1(6):297-305.
27
28
29
30 399 21. Liao CP, Adisetiyo H, Liang M, Roy-Burman P. Cancer-associated
31 400 fibroblasts enhance the gland-forming capability of prostate cancer
32 401 stem cells. *Cancer Res.* 2010;70(18):7294-303.
33
34
35
36 402 22. Little NA, Jochemsen AG. p63. *Int J Biochem Cell Biol.* 2002;34(1):6-
37 403 9.
38
39
40
41 404 23. Liu W, Moulay M, Willenbrock S, et al. Comparative characterization
42 405 of stem cell marker expression, metabolic activity and resistance to
43 406 doxorubicin in adherent and spheroid cells derived from the canine
44 407 prostate adenocarcinoma cell line CT1258. *Anticancer Res.*
45 408 2015;35(4):1917-27.
46
47
48
49
50
51 409 24. Lobo NA, Shimono Y, Qian D, Clarke MF. The biology of cancer stem
52 410 cells. *Annu Rev Cell Dev Biol.* 2007;23:675-99.
53
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56
57
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2
3 411 25. Moulay M, Liu W, Willenbrock S, et al. Evaluation of stem cell marker
4
5 412 gene expression in canine prostate carcinoma- and prostatecyst-
6
7 413 derived cell lines. *Anticancer Res.* 2013;33(12):5421-31.
8
9 414 26. Palmieri C., Grieco V. Proposal of Gleason-like grading system of
10
11 415 canine prostate carcinoma in veterinary pathology. *Res Vet Sci.* 2015.
12
13 416 103:11-5.
14
15 417 27. Palmieri C., Lean FZ, Akter SH, et al. A retrospective analysis of
16
17 418 111 canine prostatic samples: histopathological findings and
18
19 419 classification. *Res Vet Sci.* 2014. 97(3):568-73.
20
21 420 28. Pignon JC, Grisanzio C, Geng Y, Song J, Shivdasani RA, Signoretti S.
22
23 421 p63-expressing cells are the stem cells of developing prostate,
24
25 422 bladder, and colorectal epithelia. *Proc Natl Acad Sci U S A.*
26
27 423 2013;110(20):8105-10.
28
29 424 29. Romanucci M, Frattone L, Ciccarelli A, et al. Immunohistochemical
30
31 425 expression of heat shock proteins, p63 and androgen receptor in
32
33 426 benign prostatic hyperplasia and prostatic carcinoma in the dog. *Vet*
34
35 427 *Comp Oncol.* 2016;14(4):337-349.
36
37 428 30. Ryan BM, O'Donovan N, Duffy MJ. Survivin: a new target for anti-
38
39 429 cancer therapy. *Cancer Treat Rev.* 2009;35(7):553-62.
40
41 430 31. Shariat SF, Lotan Y, Saboorian H, et al. Survivin expression is
42
43 431 associated with features of biologically aggressive prostate
44
45 432 carcinoma. *Cancer.* 2004;100:751-757.
46
47 433 32. Thomsen MK, Francis JC, Swain A. The role of Sox9 in prostate
48
49 434 development. *Differentiation.* 2008;76(6):728-35.
50
51 435 33. Visvader JE, Lindeman GJ. Cancer stem cells: current status and
52
53 436 evolving complexities. *Cell Stem Cell.* 2012;10(6):717-28.
54
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2
3 437 34. Webster JD, Miller MA, Dusold D, Ramos-Vara J. Effects of prolonged
4
5 438 formalin fixation on diagnostic immunohistochemistry in domestic
6
7 439 animals. *J Histochem Cytochem.* 2009. 57(5):753-61.
8
9 440 35. Wiechno PJ, Sadowska M, Kalinowski T, Michalski W, Demkow T. Does
10
11 441 pharmacological castration as adjuvant therapy for prostate cancer
12
13 442 after radiotherapy affect anxiety and depression levels, cognitive
14
15 443 functions and quality of life? *Psychooncology.* 2013;22(2):346-51.
16
17 444 36. Zhang M, Latham DE, Delaney MA, Chakravarti A. Survivin mediates
18
19 445 resistance to antiandrogen therapy in prostate cancer. *Oncogene.*
20
21 446 2005;24(15):2474-82.
22
23 447 37. Zhong WD, Qin GQ, Dai QS, et al. SOXs in human prostate cancer:
24
25 448 implication as progression and prognosis factors. *BMC Cancer.*
26
27 449 2012;12:248.
28
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33 452 **Figure Legends**

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36 453 Figures 1-3. Immunohistochemistry for survivin. Figure 1. Normal prostate
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38 454 gland, dog 1. Scattered positive nuclei among the basal cell layer (arrows)
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40 455 and weak, patchy cytoplasmic immunostaining are present. Figure 2. Benign
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42 456 prostatic hyperplasia (BPH), dog 7. Weak cytoplasmic immunostaining and
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44 457 scattered positive nuclei (arrows), some of which are in the basal cell
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46 458 layer (arrowhead) of hyperplastic prostatic lobules. Figure 3. Prostatic
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48 459 carcinoma, dog 35. Numerous positive nuclei and weak, patchy cytoplasmic
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50 460 immunostaining among the neoplastic cells of a solid prostatic carcinoma,
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52 461 with intensely positive mitotic figures (arrow). Figures 4-6.
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54 462 Immunohistochemistry for Sox9. Figure 4. Normal prostate gland, dog 2.
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56 463 Positive nuclei are evident in the basal cell layer (arrows). Figure 5.
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58 464 BPH, dog 15. Several positive nuclei are shown, mainly present in the basal

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3 465 cell layer(arrows). Figure 6. Prostatic carcinoma, dog 22. Numerous
4 466 positive nuclei were observed among the neoplastic cells of a papillary
5 467 prostatic carcinoma.

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9 468 Figures 7-8: Double immunofluorescence for survivin and p63; nuclei
10 469 counterstained with 4',6-diamidino-2-phenylindole (DAPI). Figure 7: BPH,
11 470 dog 10. Only a partial co-localization of survivin and p63 was observed,
12 471 showing a higher number of survivin-/p63+ nuclei in basal cells and few
13 472 survivin+/p63+ cells. Figure 8.: prostatic carcinoma, dog 19. More frequent
14 473 survivin+/p63- and only few survivin+/p63+ nuclei were observed with
15 474 infrequent p63 cytoplasmic immunostaining. Figures 9-10: Double
16 475 immunofluorescence for survivin and AR; nuclei counterstained with DAPI.
17 476 Figure 9:: BPH, dog 6. Higher number of nuclear survivin-/AR+ cells
18 477 compared to survivin+/AR+ cells was observed in BPH cases, without any
19 478 nuclear survivin+/AR- cells. Most of the cells show cytoplasmic survivin
20 479 expression. Figure 10: prostatic carcinoma, dog 22. Rare cells had nuclear
21 480 labelling for both survivin and AR (arrow), but survivin+/AR- nuclear
22 481 labelling was also evident. Figures 11-12: Double immunofluorescence for
23 482 survivin and Sox9; nuclei counterstained with DAPI. Figure 11: BPH, dog 18.
24 483 Most of the cells have survivin+/Sox9- cytoplasmic labeling (green arrow).
25 484 Figure 12: prostatic carcinoma, dog 32. Numerous neoplastic cells in the
26 485 prostatic carcinoma cases evaluated showed survivin+/Sox9+ double
27 486 immunostaining (arrows), with intense nuclear and faint cytoplasmic
28 487 survivin immunolabelling.

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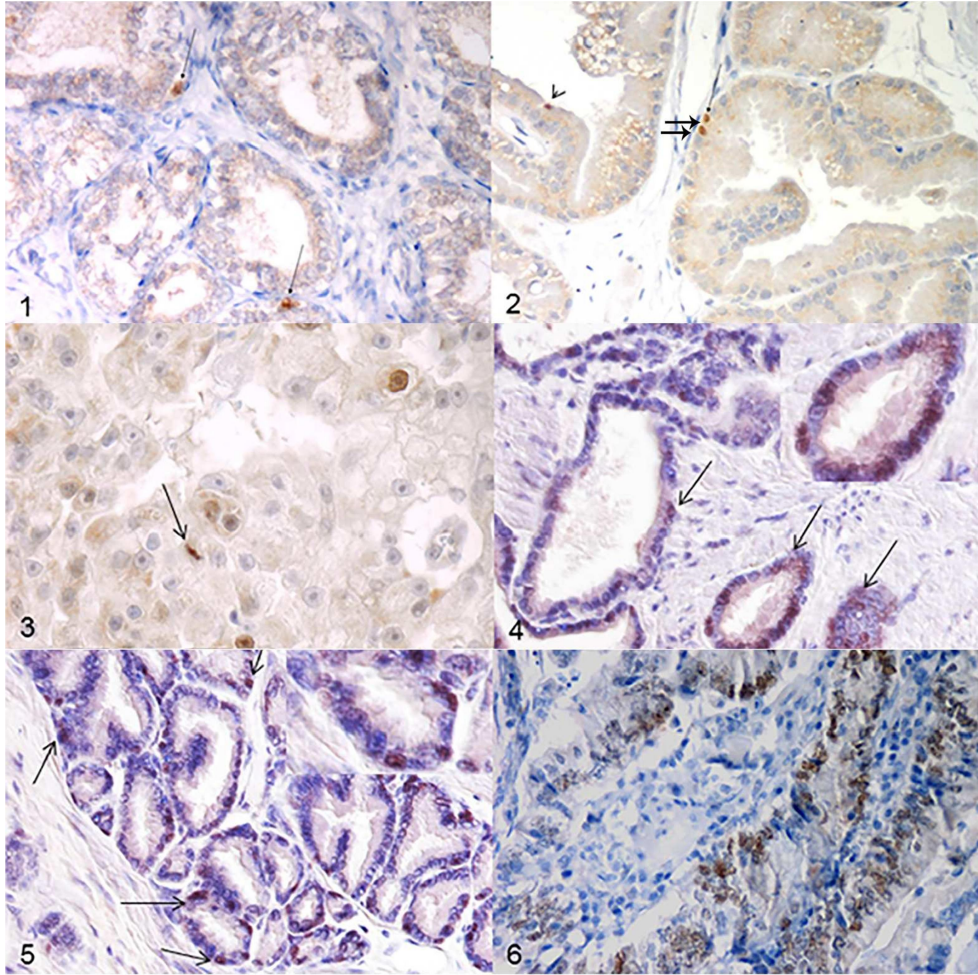
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Table 1. Expression of survivin and Sox9 in normal prostate, benign prostatic hyperplasia, and prostatic carcinoma of dogs. The data show immunohistochemistry grades.

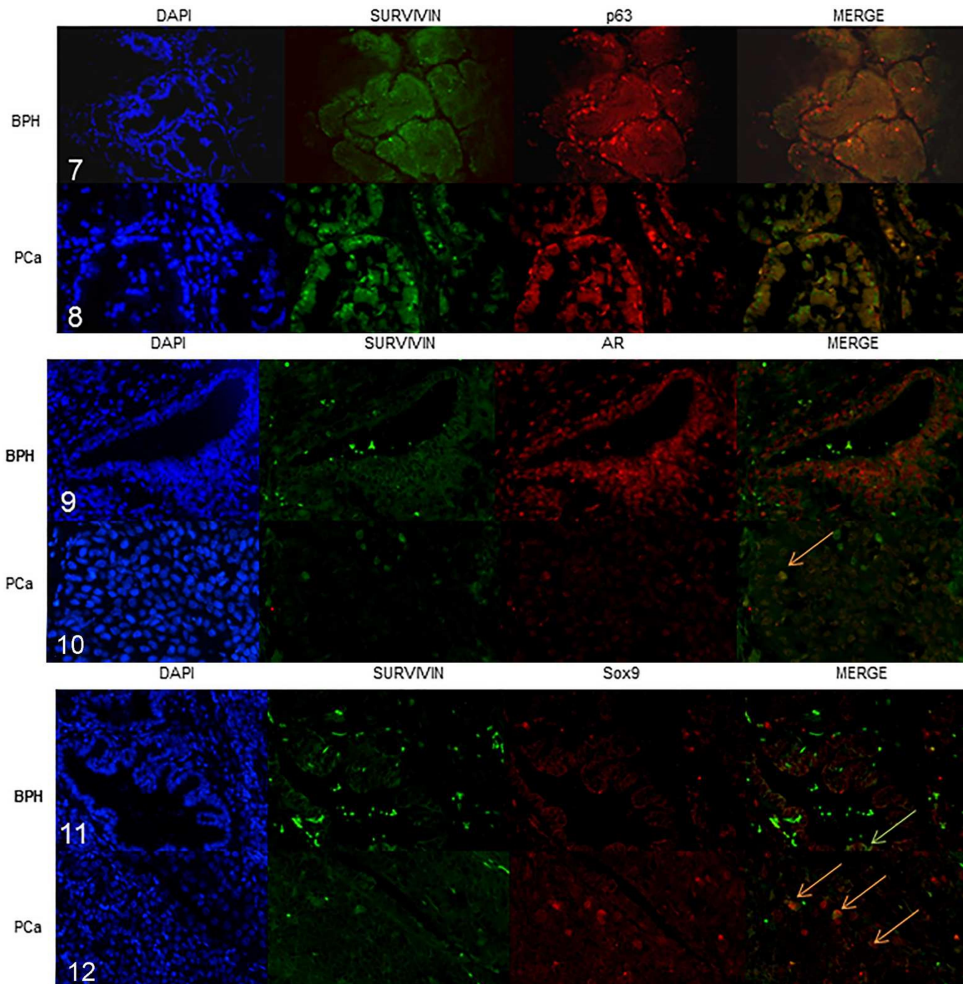
Case N°	Nuclear survivin	Cytoplasmic survivin	Sox9
Normal gland			
1	1	0	0
2*	-	-	1
3*	-	-	1
BPH			
4	1	1, +	0
5	0	1, ++	0
6	1	1, -/+	0
7	1	1, +	0
8	0	1, ++	0
9	1	1, -/+	0
10	1	2, ++	0
11	1	2, ++	0
12	0	1, -/+ to +	1
13	1	4, ++	0
14	1	0	3
15	1	3, ++	0
16	0	0	0
17	1	0	0
18	1	1, +	1
19	0	0	0
20*	-	-	1
21*	-	-	1
PCa			
22	1	1, -/+	0
23	2	4, ++	4
24	1	4, +/++	4
25	1	2, ++	0
26	1	1, -/+	-
27	2	1, +/++	4
28	4	4, ++	4
29	1	4, ++	-
30	4	4, +/++	4
31	1	1, -/+ with small clusters ++	1
32	2	3, ++	0
33	2	Cribiform: 3, +, Rest: 4, +++	2
34	2	3, +	2
35	3	1, +/++	1
36	0	1, -/+	0
37	3	1, -/+	0

Grades for immunolabelling, as percentage of all neoplastic cells: 0, absent; 1, >0 and <10% of cells; 2, 10 to <25% of cells; 3, 25 to <50% of cells; 4, >50% of cells. BPH: benign prostatic hyperplasia. PCa: prostatic carcinoma. *: maximum 24 h of formalin fixation time.

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180x180mm (300 x 300 DPI)



180x181mm (300 x 300 DPI)

Supplemental Table S1 - Methods used for immunohistochemistry.

MOLECULE	ANTIBODY TYPE	SOURCE	WORKING DILUTION	POSITIVE CONTROL
Sox9	Rabbit PAb	Santa Cruz Biotechnology	1:700	Canine normal skin ⁵
Survivin	Rabbit PAb	NOVUS Biologicals	0.7 µg/ml	Canine sebaceous carcinoma ⁴
p63	Mouse MAb	DAKO	1:400	Canine prostatic hyperplasia ²⁶
AR	Rabbit PAb	Santa Cruz Biotechnology	1:500	Canine prostatic hyperplasia ²⁶

PAb: polyclonal antibody; MAb: monoclonal antibody.

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2 **Supplementary table S2.** The table shows tumour type, prostatic carcinoma histo-
3 patterns, Gleason/like score, presence of necrosis and immunohistochemistry
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5 grades.
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Case N°	Histotype	Histo-pattern	Gleason/like score	Necrosis	Nuclear survivin	Cytoplasmic survivin	Sox9
1	Normal gland				1	0	0
2	Normal gland*				-	-	1
3	Normal gland*				-	-	1
4	BPH				1	1, +	0
5	BPH				0	1, ++	0
6	BPH				1	1, -/+	0
7	BPH				1	1, +	0
8	BPH				0	1, ++	0
9	BPH				1	1, -/+	0
10	BPH				1	2, ++	0
11	BPH				1	2, ++	0
12	BPH	Cystic with prostatitis and PIN areas			0	1, -/+ to +	1
13	BPH	With prostatitis			1	4, ++	0
14	BPH	With prostatitis			1	0	3
15	BPH				1	3, ++	0
16	BPH				0	0	0
17	BPH	With prostatitis			1	0	0
18	BPH	With prostatitis			1	1, +	1
19	BPH	With prostatitis			0	0	0
20	BPH*				-	-	1
21	BPH*				-	-	1
22	PCa	Papillary	8	No	1	1, -/+	0
23	PCa	Small acinar/ductal, solid, signet ring	10	Yes	2	4, ++	4
24	PCa	Papillary, solid	9	Yes	1	4, +/++	4

25	PCa	Papillary	8	Yes	1	2, ++	0
26	PCa	Small acinar/ductal, solid	9	No	1	1, -/+	-
27	PCa	Papillary, small acinar/ductal, solid with abundant fibrous stroma	9	Yes	2	1, +/+++	4
28	PCa	Solid	10	Yes	4	4, ++	4
29	PCa	Cribiform, solid	10	Yes	1	4, ++	-
30	PCa	Solid with squamous metaplasia and abundant fibrous stroma	8	Yes	4	4, +/+++	4
31	PCa	Papillary with cystic structures	9	No	1	1, -/+ with small clusters ++	1
32	PCa	Solid, cribriform, papillary	9	Yes	2	3, ++	0
33	PCa	Cribiform, signet-ring, papillary	10	Yes	2	Cribiform: 3, +, Rest: 4, +++	2
34	PCa	Cribiform, papillary	10	Yes	2	3, +	2
35	PCa	Solid	10	Yes	3	1, +/+++	1
36	PCa	Papillary	8	No	0	1, -/+	0
37	PCa	Small acinar /ductal	7	Yes	3	1, -/+	0

Grades for immunolabelling, as percentage of all neoplastic cells: 0, absent; 1, >0 and <10% of cells; 2, 10 to <25% of cells; 3, 25 to <50% of cells; 4, >50% of

cells. BHP: benign prostatic hyperplasia. BHP: benign prostatic hyperplasia.

PCa: prostatic carcinoma. *: maximum 24 h of formalin fixation time.

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60**Supplemental Table S1 - Methods used for immunohistochemistry.**

MOLECULE	ANTIBODY TYPE	SOURCE	WORKING DILUTION	POSITIVE CONTROL
Sox9	Rabbit PAb	Santa Cruz Biotechnology	1:700	Canine normal skin ⁵
Survivin	Rabbit PAb	NOVUS Biologicals	0.7 µg/ml	Canine sabaceous carcinoma ⁴
p63	Mouse MAb	DAKO	1:400	Canine prostatic hyperplasia ²⁶
AR	Rabbit PAb	Santa Cruz Biotechnology	1:500	Canine prostatic hyperplasia ²⁶

PAb: polyclonal antibody; MAb: monoclonal antibody.

Supplementary Table S2. The table shows tumour type, prostatic carcinoma histo-patterns, Gleason/like score, presence of necrosis and immunohistochemistry grades.

Case N°	Histotype	Histo-pattern	Gleason/like score	Necrosis	Nuclear survivin	Cytoplasmic survivin	Sox9
1	Normal gland				1	0	0
2	Normal gland*				-	-	1
3	Normal gland*				-	-	1
4	BPH				1	1, +	0
5	BPH				0	1, ++	0
6	BPH				1	1, -/+	0
7	BPH				1	1, +	0
8	BPH				0	1, ++	0
9	BPH				1	1, -/+	0
10	BPH				1	2, ++	0
11	BPH				1	2, ++	0
12	BPH	Cystic with prostatitis and PIN areas			0	1, -/+ to +	1
13	BPH	With prostatitis			1	4, ++	0
14	BPH	With prostatitis			1	0	3
15	BPH				1	3, ++	0
16	BPH				0	0	0
17	BPH	With prostatitis			1	0	0
18	BPH	With prostatitis			1	1, +	1
19	BPH	With prostatitis			0	0	0
20	BPH*				-	-	1
21	BPH*				-	-	1
22	PCa	Papillary	8	No	1	1, -/+	0
23	PCa	Small acinar/ductal, solid, signet ring	10	Yes	2	4, ++	4
24	PCa	Papillary, solid	9	Yes	1	4, +/++	4
25	PCa	Papillary	8	Yes	1	2, ++	0
26	PCa	Small acinar/ductal, solid	9	No	1	1, -/+	-
27	PCa	Papillary, small acinar/ductal, solid with abundant fibrous stroma	9	Yes	2	1, +/++	4
28	PCa	Solid	10	Yes	4	4, ++	4
29	PCa	Cribiform, solid	10	Yes	1	4, ++	-
30	PCa	Solid with squamous metaplasia and abundant fibrous stroma	8	Yes	4	4, +/++	4
31	PCa	Papillary with cystic structures	9	No	1	1, -/+ with small clusters ++	1
32	PCa	Solid, cribriform, papillary	9	Yes	2	3, ++	0
33	PCa	Cribiform, signet-ring, papillary	10	Yes	2	Cribiform: 3, +, Rest: 4, +++	2
34	PCa	Cribiform, papillary	10	Yes	2	3, +	2
35	PCa	Solid	10	Yes	3	1, +/++	1
36	PCa	Papillary	8	No	0	1, -/+	0
37	PCa	Small acinar /ductal	7	Yes	3	1, -/+	0

Grades for immunolabelling, as percentage of all neoplastic cells: 0, absent; 1, >0 and <10% of cells; 2, 10 to <25% of cells; 3, 25 to <50% of cells; 4, >50% of cells. BPH: benign prostatic hyperplasia. BHP: benign prostatic hyperplasia. PCa: prostatic carcinoma. *: maximum 24 h of formalin fixation time.