Co-localization of PTEN and E-cadherin in canine mammary hyperplasias, benign and malignant mammary tumours

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26 **Summary**

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28 Fifty- four canine mammary lesions (15 hyperplasias, 7 adenomas and 32 carcinomas) were

29 submitted to immunohistochemical analysis for the evaluation of PTEN and E-cadherin co-

30 expression. Subjects bearing mammary carcinomas were also submitted to a 2-year follow-up

31 study to compare immunohistochemical results with overall survival All the hyperplastic

32 samples stained positive for both markers, 100% of adenomas were positive for PTEN and

33 86% for E-cadherin, and 69% and 34% of carcinomas were positive for PTEN and E-

34 cadherin, respectively. Statistical analysis showed a positive correlation between these two

35 proteins both considering all ($p < 0.01$) or malignant tumours ($p < 0.05$). The female dogs

36 bearing tumours positively-stained for both markers had a longer overall survival ($p < 0.05$)

37 and absence of lymphatics invasion ($p < 0.05$). Simultaneous double immunofluorescence

38 confirmed the co-localization of the two proteins in neoplastic cells. Results reported in this

39 study confirm the tumor suppressor effect of these two molecules.

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51 **Introduction**

52   Phosphatase and tensin homolog deleted on chromosome 26 (*PTEN*) in canine species

53 is a tumor suppressor gene that negatively regulates neoplastic growth, survival and

54 invasiveness (Jiang et Liu, 2009). PTEN mutations are widely reported in literature and

55 commonly associated with several human malignancies, such as brain, breast and prostate

56 cancer (Tsutsui et al., 2005; Yashimoto et al., 2007; Endersby and Baker, 2008). In veterinary

57 oncology, PTEN expression has been investigated in canine melanoma (Koenig *et al*., 2002),

58 osteosarcoma (Levine *et al*., 2002), hemangiosarcoma (Dickerson *et al*., 2005) and mammary

59 tumours (Ressel *et al*., 2009). As well as in human medicine, data reported in veterinary

60 oncology suggest that PTEN mutation and loss are associated with tumor development and

61 growth.

62   E-cadherin is a member of the cadherin family involved in regulating intercellular

63 adhesion in epithelial tissues (Takeichi, 1991). Alterations and/or loss of E-cadherin

64 expression is associated with tumor development and increase of metastatic potential in

65 humans (Hirohashi, 1998). Abnormal E-cadherin expression has been detected in several

66 human carcinomas, such as digestive tract (Debruyne *et al*., 1999), urogenital (Giroldi *et al*.,

67 2000), lung (Bremnes *et al*., 2002) and cervical (Li *et al*., 2011) carcinomas. In canine

68 oncology, E-cadherin expression has been investigated in several cancers and particularly in

69 mammary tumours (Brunetti *et al*., 2003. Gama and Schmitt 2012; Yoshida et al., 2014).

70 These reports confirmed E-cadherin membranous immunolocalization as the normal

71 expression (Sarli *et al*., 2004), while cytoplasmic and nuclear location are linked to a

72 downregulation of its tumor suppressor role (Chetty and Serra, 2008).

73   Few reports have suggested the potential interaction between PTEN protein and E-

74 cadherin in the regulation of the morphogenesis and the growth of healthy (Fournier *et al*.,

75 2009) and neoplastic (Li *et al*., 2007) mammary cells. Data arising from these studies

76 proposed that E-cadherin is necessary for PTEN expression, promoting its accumulation

77 preventing its proteasome degradation (Li *et al*., 2007). However, other reports asserted that

78 PTEN is necessary for E-cadherin expression and cell-to-cell adhesion, and not vice versa

79 (Kotelevets *et al*. 2001; Kotelevets *et al*., 2005). In melanoma, it has been demonstrated that

80 PTEN inhibits the PI3K/AKT/mTOR pathway, thereby preventing the switch from E- to N-

81 cadherin, a cadherin subtype associated with tumor progression (Hao *et al*., 2012). A recent

82 review proposed a circular mechanism in which PTEN enhances E-cadherin expression and

83 E-cadherin restores PTEN protein levels thereby reducing tumor proliferation activity (Qiao

84 *et al*., 2008).

85 In order to shed lights on the potential relation between PTEN protein and E-cadherin

86 in canine mammary dysplastic and neoplastic tissues the aim of our study is to explore the co-

87 expression of the two proteins and the possible correlation between their expression patterns

88 and the biological behavior of the tumours.

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90 **Materials and Methods**

91 *Samples*

92 Thirty-nine female dogs (mean age = 9.7 years ± 1.7 years; range = 4-14 years)

93 submitted to mastectomy at the Department of Veterinary Sciences of the University of Pisa

94 were included in this study. Surgical samples obtained by unilateral mastectomy were fixed in

95 10% neutral buffered formalin, routinely processed, and tissue sections stained with

96 haematoxylin and eosin. All nodules from excised mammary glands were examined and

97 lesions were classified accordingly to the World Health Organization Histological

98 Classification of the Mammary tumours of the dog and the cat (Misdorp *et al*., 1999) and

99 tumours displaying multiple features were classified according to the most malignant

100 histologic differentiation. The modified Elston and Ellis histologic grading of non-

101 inflammatory canine mammary carcinomas (Peña *et al.*, 2013) was used to assess the

102 histological grade of the tumors. Furthermore, mitotic index and lymphatic vessel invasion

103 data of the malignant mammary tumours were also recorded. Mitotic index was performend

104 counting mitotic figures in 10 high magnification power fields.

105 In order to can compare immunohistochemical results with the overall survival data,

106 subjects bearing mammary carcinomas (n=32) were submitted to a 2-year post-surgery

107 follow-up examination. Clinical exams and tumour staging were performed 6, 12, 18, and 24

108 months after surgery. The presence of distant organ metastases and the recurrence of primary

109 tumours were investigated by clinical and radiographic examinations. Dogs that died during

110 this period were subjected to necropsy examination to confirm tumor-related death.

111

112 *Immunohistochemistry*

113 For immunohistochemistry (IHC) analysis, 4-µm-thick tissue sections were cut and

114 mounted on Superfrost Plus slides (Thermo Scientific, Menzel GmbH & Co., KG,

115 Braunshweig, Germany) and dried overnight at 37 °C. Sections were dewaxed in xylene,

116 passed through a graded series of alcohols, and rehydrated in deionized water. Antigens were

117 retrieved with a citrate buffer pH 6.0 in a microwave oven with a cycle of 4 minutes at 350

118 watts followed by a cycle of 15 minutes at 650 watts and then cooled at room temperature for

119 20 minutes. Endogenous peroxidases were blocked with Dako Real Peroxidase-Blocking

120 Solution (Dako, Glostrup, Denmark) for 10 minutes, than three washes with 0.05% Tween-

121 Tris-buffered saline solution (TBST) at pH 7.6 were performed. Sections were incubated for

122 10 minutes with the Ultra-V-Block solution (prediluted, Thermo, Fremont, CA, USA) to

123 reduce nonspecific background. After three washes in TBST, sections were incubated for 1

124 hour at room temperature with the primary antibodies: anti-PTEN (mouse monoclonal, clone

125 A2B1, diluted 1:50, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and anti-E-cadherin

126   (rabbit polyclonal, diluted 1:300, Abcam, Cambridge, UK). At the end of the incubation

127   period 3 washes with TBST were performed and then sections were incubated with a

128   biotinylated anti-polyvalent secondary antibody (goat, prediluted, Thermo, Fremont, CA,

129   USA). After three washes with TBST, a straptavidin-peroxidase solution (prediluted, Thermo,

130   Fremont, CA, USA) was placed on the slides, followed by three washes in TBST.

131   Diaminobenzidine (Impact DAB, Vector Labs, Inc., Burlingame, CA, USA) was used with an

132   incubation of 10 minutes to develop the peroxidase reaction, and then a wash with deionized

133   water was performed. After a short-term counter-stain in hematoxylin, sections were

134   dehydrated through a graded series of alcohols, placed in xylene and mounted. Negative

135   controls were performed omitting the primary antibodies and replacing with non-immune

136   rabbit serum or replacing the primary monoclonal antibodies with a murine subclass matched

137   unrelated antibodies. As PTEN positive controls, canine renal glomeruli were used in each

138   experiment and vascular endothelium was used as an internal positive control in each slide as

139   previously described (Koenig *et al.*, 2002). As E-cadherin positive controls, canine skin and

140   liver samples were used. Fifteen hyperplastic mammary gland samples were selected from the

141   archive of the Laboratory of Animal Pathology of the Department of Veterinary Sciences and

142   submitted to IHC as further positive controls.

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144   *Quantification of Immunolabeling*

145   PTEN IHC staining was considered positive by the presence of distinct brown

146   cytoplasmic or both nuclear and cytoplasmic staining. A modified semiquantitative scoring

147   system (range 0–7; positivity $\geq$ 3) was used, as previously described in human (Seow *et al.*,

148   2010) and feline (Maniscalco *et al.*, 2012) mammary tumors. For E-cadherin IHC evaluation,

149   only samples presenting more than 75% positive cells with a membranous preserved pattern

150 was considered positive, while nuclear or cytoplasmic staining were not considered, as

151 previously reported (Sarli *et al.*, 2004).

152

153 *Double indirect immunofluorescence*

154   Dual immunofluorescent (IF) staining for PTEN and E-CAD was performed on

155 sections from malignant tumours selected on the basis of IHC results and designed as

156 PTEN+/E-cadherin+ or PTEN-/E-cadherin-. To investigate the potential co-localization of the

157 proteins, four micron formalin fixed paraffin embedded sections, incubated with anti-E-CAD

158 rabbit polyclonal AB and anti-rabbit Ab conjugated with DyLight549 (AbDSerotec – Dil.

159 1:200), which resulted in red fluorescence; and with mouse monoclonal anti-PTEN and anti-

160 mouse Ab conjugated with DyLight488 (AbDSerotec – Dil 1:200) which resulted in green

161 fluorescence. Blue fluorescent DAPI nuclear counterstaining was also performed and slides

162 were analyzsed using epi-fluorescent microscopy with appropriate filters for each stain.

163 Composite three channel images were obtained using ImageJ® software.

164

165 *Statistical Analysis*

166   Statistical analysis was performed using the statistical package SPSS Advanced

167 Statistics 13.0 (SPSS Inc., Chicago, IL, USA). A chi-square test was used to investigate the

168 significance of the relationship between PTEN protein and E-cadherin expression and

169 between the two markers and individual tumors variables. Statistical significance was based

170 on a 5% (0.05) significance level. Overall survival analysis was performed using the Kaplan-

171 Meyer method, and the Tarone-Ware test was used to investigate the relationship between

172 PTEN and E-cadherin expression and overall survival.

173

174 **Results**

175 *General -findings*

176   Seven of the 39 (18%) canine mammary tumors were diagnosed as adenomas and 32

177 (82%) as carcinomas. Of the 32 carcinomas, 12 (37.5%) were of complex type and 20

178 (62.5%) of simple type. Of these latter, 8 (40%) were tubulopapillary, 10 (50%) solid and 2

179 (10%) anaplastic carcinomas. Twenty-four of the 32 (75%) carcinomas did not invade

180 lymphatic vessels at the time of the diagnosis, the remaining 8 (25%) presented lymphatic

181 invasion in the vessel around the tumor. The modified Elston and Ellis system allowed the

182 histological grading of the tumors: 13 of 32 (40.6%) were well differentiated carcinomas

183 (WDCs), 10 (31.3%) were moderately differentiated carcinomas (MDCs), and 9 (28.1%) were

184 poorly differentiated carcinomas (PDCs). Carcinomas mean mitotic index was 11.6 ± 9.2

185 mitosis/HPF (median = 8.5; range = 1-42). The mitotic rate according to histological grade

186 was 5.4 ± 2.9 for WDCs, 8.8 ± 3.5 for MDCs and 23.6 ± 12.2 for PDCs, with significant

187 differences among WDCs and MDCs and PDCs ($P < 0.000$). Of the 32 subjects bearing

188 mammary carcinoma, 11 (34.4%) died for the progression of the neoplastic disease before the

189 end of the follow-up period, whilst 21 (65.6%) were still alive.

190

191 *Immunohistochemistry*

192   IHC analysis revealed that in all the mammary hyperplasias the epithelial cells were

193 PTEN-positive and revealed a strong membranous E-cadherin staining. All the seven

194 adenomas (100%) and 22 of 32 carcinomas (68.8%) were PTEN-positive, while 6 of the 7

195 adenomas (85.7%) and 11 of 32 carcinomas (34.4%) has a membranous E-cadherin

196 expression. The chi-square test showed a significant difference in the E-cadherin expression

197 between benign and malignant tumours (p=0.013). The relationship between PTEN and E-

198 cadherin expression and clinicopathologic factors is shown in Table 1. Statistical analysis

199 revealed that PTEN loss was related with a simple histotype (p=0.003), presence of lymphatic

vessels invasion (p=0.028) and a shorter overall survival time (p=0.004), while alteration of

E-cadherin expression was observed in simple malignant tumours (p=0.027). Thirteen of the

sixteen tumors with a mitotic index above the median had an altered E-cadherin expression,

but the difference was no statistically significant. All the six E-Cadherin- tumour with

lymphatic invasion had a similar expression in the lymphatic embolies, while of the two E-

Cadherin+ ones one had a similar expression and the other a dowregulation of the expression.

All the eight tumour with lymphatic expression had a similar PTEN expression in embolies

(three PTEN+ and five PTEN-). Considering all the tumors, 17 of 39 samples were

simultaneously positive for PTEN and E-cadherin expression, 12 for PTEN only and 10 were

negative for both (Table 2). No E-cadherin positive and PTEN positive samples were

detected. When both benign and malignant tumours were considered the chi-square test

showed a significant positive correlation between PTEN and E-cadherin expression (p =

0.001). Regarding malignant mammary tumors, of the 32 samples 11 were positive for both

the two markers , 11 were positive for PTEN only and 10 were negative both for PTEN and

E-cadherin expression. As showed in Table 3, there was a significant correlation between

PTEN protein and preserved E-cadherin expression even considering only malignant

mammary tissues (p = 0.006).

The tumour population was divided into three groups according to PTEN and E-

cadherin immunohistochemical results: PTEN and E-cadherin positive (PTEN+/E-cad+),

PTEN positive and E-cadherin negative (PTEN+/E-cad-) and PTEN and E-cadherin negative

(PTEN-/E-cad-). Statistical analysis (Table 3) revealed a significant correlation between

PTEN-/E-cad- group and simple carcinoma type (p = 0.009) and with a poor prognosis

(p=0.017). PTEN-/E-cad- carcinomas had a higher mitotic index when compared with

PTEN+/E-cad+ tumours (p=0.05). When PTEN+/E-cadherin+ and 2 PTEN-/E-cadherin-

tumours were submitted to PTEN/E-cadherin double immunofluorescence stain, the co-

225 localization of the two proteins within the PTEN+/E-cadherin+ tumours was demonstrated

226 (Fig. 1).

227 Tarone-Ware test performed on overall survival data showed that PTEN-/E-cad- group

228 had a shorter survival period if compared to PTEN+/E-cad+ and PTEN+/E-cad- groups, but

229 these differences were not statistically significant. If –PTEN+/E-cad+ and PTEN+/E-cad-

230 groups were unified, a significant difference between the survival of this unified group and

231 the survival of the PTEN-/E-cad- group was observed, as illustrated by the Kaplan Meier plot

232 (Fig. 2). The death for tumour related causes was also correlated to the histological grade: two

233 of the 13 subjctes bearing a WDCs and two of the 10 subjects bearing a MDCs died during

234 the study period, while of the nine subjects bearing a PDCs 7 died during the follow-up

235 (p<0.000)

236

237 **Discussion**

238 The results of the study about PTEN protein expression in this study were similar to

239 those previously reported in human (Li *et al*., 1997; Bose *et al*., 2002) and veterinary (Kanae

240 *et al*., 2006; Qiu *et al*., 2008; Ressel *et al*., 2009) literature. PTEN protein expression was

241 positively correlated with clinico-pathological parameters commonly associated with a

242 favorable prognosis, such as a complex histotype, the absence of lymphatics invasion and a

243 longer overall survival, as previously reported for canine mammary tumours (Ressel *et al*.,

244 2009). Regarding E-cadherin expression, in our study an altered expression was observed in

245 malignant tumours when compared with benign ones, and in simple carcinomas when

246 compared with complex type. In previous studies, reduced membranous E-cadherin

247 expression was statistically correlated with lymphatic invasion, higher cellular proliferation

248 rate and reduced survival (Gama *et al*., 2008; Nowak *et al*., 2007; Restucci *et al*., 2007;

249 Torres *et al*., 2005). In contrast, other studies did not find an association between loss of

250   preserved expression and proliferation or survival (Brunetti *et al*., 2003; Brunetti *et al*., 2005;

251   De Matos *et al*., 2007; Nowak *et al*., 2008). The discrepancy between these and our data

252   could be due to the reduced size of the sample investigated and the different scoring system,

253   particularly considering that the system used in our study has an higher positive cut-off value,

254   fact that lead to a low percentage of E-cadherin positive tumors.

255   The main aim of this study was to investigate the correlation between PTEN protein an

256   E-cadherin expression. Data emerging from our research showed a strong correlation between

257   PTEN and E-cadherin expression both considering all the canine mammary tumors or only

258   the malignant ones. These results enhanced the hypothesis that PTEN and E-cadherin

259   expression are associated, as previous suggested in human medicine (Fournier *et al*., 2009; Li

260   *et al*., 2007). Simultaneous double immunofluorescence for PTEN and E-cadherin identified a

261   co-localization of the two proteins within the same cell compartment in PTEN+/E-cadherin +

262   group. This finding further support the hypothesis of a protein-protein interaction. However,

263   the precise mechanisms responsible of this interaction have not been clearly unraveled. For

264   Fournier and colleagues (2009), cellular accumulation of PTEN is mediated by E-cadherin,

265   and this up-regoulation leads to the control or the arrest of acinar morphogenesis in mammary

266   epithelial cells. In another article Li et al. (2007) proposed that E-cadherin-mediated cell-to-

267   cell adhesion is necessary to prevent PTEN proteasome degradation and to promote its

268   accumulation in human breast carcinoma cells. Other reports focused on PTEN influence on

269   cellular junctions (Kotelevets *et al*. 2001; Kotelevets *et al*., 2005), suggested that PTEN

270   protein is essential for stabilizing cellular junctions, inhibiting the PI3K/AKT/mTOR pathway

271   and preventing the E- to N-cadherin switch, which is a common event in melanoma and

272   prostate cancer cells (Kotelevets *et al*. 2001). A recent report proposed that PTEN prevents

273   the Twist and Snail-mediated switch from E- to N-cadherin thereby inhibiting

274   PI3K/AKT/mTOR pathway (Hao *et al*., 2012). A recent review, investigating the influence of

275 the PI3K/AKT/mTOR pathway on metastatic processes, suggested a circular mechanism with

276 which PTEN promotes E-cadherin preservation and E-cadherin restores PTEN protein levels

277 limiting tumor metastatic and proliferation activity (Qiao *et al*., 2008).

278   Analyzing our data, there was no sample simultaneously negative for PTEN and

279 positive for E-cadherin IHC expression, either in benign or in malignant mammary tumors.

280 Moreover, all the E-cadherin positive samples (6 adenomas and 11 carcinomas) were also

281 PTEN-positive. This finding may suggest that the loss or the reduction of PTEN expression

282 leads to E-cadherin down-regulation, supporting the hypothesis that PTEN is necessary for E-

283 cadherin preserved expression in canine mammary tumors, and not vice versa, as proposed in

284 previous articles (Kotelevets *et al*. 2001; Kotelevets *et al*., 2005). A part of the tumours

285 investigated (1 adenoma and 11 carcinomas) were PTEN-positive and negative for E-cadherin

286 expression. However, it has been widely reported that PTEN protein can be phosphorylated in

287 different sites (Gericke *et al*., 2006; Torres and Pulido, 2001). The phosphorylation of PTEN

288 has been regarded as contributory to its stabilization (Torres and Pulido, 2001), but more

289 recent papers have associated phosphorylation with malignant changes (Roy et al., 2011;

290 Yang et al., 2013), or with the reduction of its biological effects (Torres *et al*., 2005). The

291 anti-PTEN antibody used in our study recognizes all PTEN forms, included phosphorylated

292 PTEN. Therefore, in some of our PTEN positive tumors PTEN activity could be impaired by

293 its phosphorylation, fact that could lead to the lack of PTEN-mediated E-cadherin

294 preservation. However, phospho-PTEN forms are numerous and not completely

295 characterized, so further investigations are needed to confirm this hypothesis.

296   Dividing the population in 3 groups based on IHC results, PTEN-/E-cad- subjects

297 presented a poorer survival than PTEN+/E-cad+ and PTEN+/E-cad- dogs. This finding

298 confirms tumor suppressive role of these two molecules, role that is enhanced by their

299 simultaneous expression. The PTEN-/E-cad- group was also statistically correlated with

300 simple mammary carcinomas, a type of tumor commonly associated with a worse prognosis

301 than complex type (Misdorp *et al.*, 1999). No statistically significant differences were

302 observed between PTEN+/E-cad+ and PTEN+/E-cad- group when compared to clinic-

303 pathological features. However, mitotic activity was increased in PTEN+/E-cad- (55% of

304 samples had a mitotic index higher than the median value) compared with PTEN+/E-cad+

305 group (only 27% of tumor exceeded the mitotic index median value).

306 The absence of statistically significant differences between PTEN+/E-cad+ and

307 PTEN+/E-cad- group when compared to clinic-pathological features seems to suggest that

308 PTEN may be the key molecule in this interaction, both for its maintenance and for tumor

309 suppressive implications, but further studies are needed to confirm this preliminary finding.

310 The fact that the group expressing only PTEN protein presented clinic-pathological features

311 similar to the group expressing both PTEN and E-cadherin could suggest that E-cadherin

312 could play a PTEN-dependent role in tumor suppression.

313 Statistical analysis on overall survival data showed a strong association between

314 PTEN expression and good prognosis, demonstrating its key-role in limiting tumor

315 aggressiveness. The same strong correlation was not observed for E-cadherin, even if only

316 2/11 E-cadherin-positive subjects died before the follow up period while 9/21 E-cadherin-

317 negative dogs died before the 2 years. The simultaneous absence of PTEN expression and E-

318 cadherin membranous expression was related to poor prognosis (p < 0.05) and to a shorter

319 survival period if compared to those of group positive for both markers or for PTEN only.

320 These last two groups had a similar survival trend, and the distribution between subjects that

321 survived or died was exactly the same. These results suggest once again that PTEN may play

322 a predominant role in tumor suppression. Our data seem to suggest that in canine mammary

323 tumors the main role in the maintenance of PTEN and E-cadherin interaction is played by the

324 PTEN protein, and that preserved E-cadherin expression is PTEN-dependent. These data

325 agree with those proposed by Kotevelets and colleagues (2001 and 2005), in which the

326 importance of PTEN in preserving cell-to-cell adhesion molecules expression was

327 highlighted. However, it is not to exclude that to certain extent, when preserved, E-cadherin

328 could stabilize PTEN protein expression as previous reported, limiting PTEN degradation (Li

329 *et al*., 2007) and thus promoting its accumulation (Fournier *et al*., 2009). This redundant

330 mechanism has been proposed by a recent review focused on PI3K/AKT/mTOR pathway

331 influence on metastatic process (Qiao *et al*., 2008).

332 In conclusion, our study confirmed PTEN tumor suppressive role in canine mammary

333 cancer and E-cadherin association with benign neoplastic parameters. The lack of expression

334 of these two markers was correlated with several malignant clinico-pathological features and

335 with shorter overall survival. PTEN and E-cadherin expression were strongly associated, and

336 these interaction could be considered as an important tumor-suppressor mechanism in canine

337 mammary tumors.

Commento [PA1]:

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340 **Conflict of Interest Statement**

341 The authors declare no conflicts of interest.

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References

Bose, S., Chandran, S., Mirocha, J. M., Bose, N., 2002. The Akt pathway in human breast cancer: a tissue-array-based analysis. Modern Pathology 19, 238–245.

Bremnes, R. M., Veve, R., Hirsch, F. R., Franklin, W. A., 2002. The E-cadherin cell–cell adhesion complex and lung cancer invasion, metastasis, and prognosis. Lung Cancer 36, 115-124.

Brunetti, B., Sarli, G., Preziosi, R., Leprotti, S., Benazzi, C., 2003. E-cadherin expression in canine mammary carcinomas with regional lymph node metastases. Journal of Veterinary Medicine Series A 50, 496–500.

Brunetti, B., Sarli, G., Preziosi, R., Monari, I., Benazzi, C., 2005. E-cadherin and beta-catenin reduction influence invasion but not proliferation and survival in canine malignant mammary tumours. Veterinary Pathology 42, 781–787.

Bruni, P., Boccia, A., Baldassarre, G., Trapasso, F., Santoro, M., Chiappetta, G., Fusco, A., Viglietto, G., 2000. PTEN expression is reduced in a subset of sporadic thyroid carcinomas: evidence that PTEN-growth suppressing activity in thyroid cancer cells is mediated by p27^{kip1}. Oncogene 19, 3146-3155.

Chetty, R., Serra, S., 2008. Nuclear E-cadherin immunoexpression: from biology to potential applications in diagnostic pathology. Advances in Anatomic Pathology 15, 234-240.

370

371 De Matos, A. J., Lopes, C. C., Faustino, A. M., Carvalheira, J. G., Rutteman, G. R., Gärtner,

372 F., 2007. E-cadherin, beta-catenin, invasion and lymph node metastases in canine malignant

373 mammary tumours. Acta Pathologica, Microbiologica, et Immunologica Scandinavica 115,

374 327-334.

375

376 Debruyne, P., Vermeulen S., Mareel, M., 1999. The role of the E-cadherin/catenin complex in

377 gastrointestinal cancer. Acta Gastro-enterologica Belgica 62, 393-402.

378

379 Dickerson, E. B., Thomas, R., Fosmire, S. P., Lamerato-Kozicki, A. R., Bianco, S. R.,

380 Wojcieszyn, J. W., Breen, M., Helfand, S. C., Modiano, J.F., 2005. Mutations of phosphatase

381 and tensin homolog deleted from chromosome 10 in canine hemangiosarcoma. Veterinary

382 Pathology 42, 618–632.

383

384 Endersby, R., Baker, S.J., 2008. PTEN signaling in brain: neuropathology and tumorigenesis.

385 Oncogene 27, 5416-5430.

386

387 Fournier, M. V., Fata, J. E., Martin, K. J., Yaswen, P., Bissell, M. J., 2009. Interaction of E-

388 cadherin and PTEN regulates morphogenesis and growth arrest in human mammary epithelial

389 cells. Cancer Research 69, 4545–4552.

390

391

392 Gama A, Schmitt F., 2012. Cadherin cell adhesion system in canine mammary cancer: a

393 review. Veterinary. Vet Med Int. 2012;2012:357187.

394

395   Gama, A., Paredes, J., Gärtner, F., Alves, A., Schmitt, F., 2008. Expression of E-cadherin, P-

396   cadherin and beta-catenin in canine malignant mammary tumours in relation to

397   clinicopathological parameters, proliferation and survival. Veterinary Journal 177, 45-53.

398

399   Gericke, A., Munson, M., Ross, A. H., 2006. Regulation of the PTEN phosphatase. Gene 374,

400   1-9.

401

402

403   Giroldi, L. A., Shimazui, T., Schalken, J. A., Yamasaki, H., Bringuier, P.P., 2000. Classical

404   cadherins in urological cancers. Morphologie 84, 31-38.

405

406   Hao, L., Ha, J. R., Kuzel, P., Garcia, E., Persad, S., 2012. Cadherin switch from E- to N-

407   cadherin in melanoma progression is regulated by the PI3K/PTEN pathway through Twist and

408   Snail. British Journal of Dermatology 166, 1184-1197.

409

410   Hirohashi, S., 1998. Inactivation of the E-cadherin-mediated cell adhesion system in human

411   cancers. American Journal of Pathology 153, 333-339.

412

413   Jiang, B. H., Liu, L. Z., 2009. PI3K/PTEN signaling in angiogenesis and tumorigenesis.

414   Advances in Cancer Researc 102, 19-65.

415

416   Kanae, Y., Endoh, D., Yokota, H., Taniyama, H., Hayashi, M., 2006. Expression of the PTEN

417   tumor suppressor gene in malignant mammary gland tumors of dogs. American Journal of

418   Veterinary Research 67, 127-133.

419

420 Koenig, A., Bianco, S. R., Fosmire, S., Wojcieszyn, J., Modiano, J. F., 2002. Expression and

421 significance of p53, rb, p21/waf-1, p16/ink-4a, and PTEN tumor suppressors in canine

422 melanoma. Veterinary Pathology 39, 458-472.

423

424 Kotelevets, L., van Hengel, J., Bruyneel, E., Mareel, M., van Roy F., Chastre, E., 2001. The

425 lipid phosphatase activity of PTEN is critical for stabilizing intercellular junctions and

426 reverting invasiveness. Journal of Cell Biology 155, 1129-1135.

427

428 Kotelevets, L., van Hengel, J., Bruyneel, E., Mareel, M., van Roy, F., Chastre, E., 2005.

429 Implication of the MAGI-1b/PTEN signalosome in stabilization of adherens junctions and

430 suppression of invasiveness. FASEB Journal 19, 115-117.

431

432 Levine, R. A., Forest, T., Smith, C., 2002. Tumor suppressor PTEN is mutated in canine

433 osteosarcoma cell lines and tumors. Veterinary Pathology 39, 372-378.

434

435 Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C.,

436 Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M., Tycko, B.,

437 Hibshoosh, H., Wigler, M. H., Parsons, R., 1997. PTEN, a putative protein tyrosine

438 phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275, 1943-

439 1947.

440

441 Li, Y., Tang, Y., Zhou, R., Sun, D., Duan, Y., Wang, N., Chen, Z., Shen, N., 2011. Genetic

442 polymorphism in the 3'-untranslated region of the E-cadherin gene is associated with risk of

443 different cancers. Molecular Carcinogenesis 50, 857-862.

444

445 Li, Z., Wang, L., Zhang, W., Fu, Y., Zhao, H., Hu, Y., Prins, B. P., Zha, X., 2007. Restoring

446 E-cadherin-mediated cell-cell adhesion increases PTEN protein level and stability in human

447 breast carcinoma cells. Biochemical and Biophysical Research Communications 363, 165-

448 170.

449

450 Maniscalco, L., Iussich, S., de Las Mulas, J. M., Millán, Y., Biolatti, B., Sasaki, N.,

451 Nakagawa, T., De Maria, R., 2012. Activation of AKT in feline mammary carcinoma: a new

452 prognostic factor for feline mammary tumours. Veterinary Journal 191, 65-71.

453

454 Mikhail, M., Velazquez, E., Shapiro, R., Berman, R., Pavlick, A., Sorhaindo, L., Spira, J.,

455 Mir, C., Panageas, K. S., Polsky, D., Osman, I., 2005. PTEN expression in melanoma:

456 relationship with patient survival, Bcl-2 expression, and proliferation. Clinical Cancer

457 Research 11, 5153-5157.

458

459 Misdorp, W., Else, R. W., Hellmén, E., Lipscomb, T. P., 1999. Histological Classification of

460 Mammary Tumors of the Dog and Cat. Washington DC: AFIP, WHO.

461

462 Nowak, M., Madej, J. A., Dziegiel, P., 2007. Expression of E-cadherin, beta-catenin and Ki-

463 67 antigen and their reciprocal relationships in mammary adenocarcinomas in bitches. Folia

464 Histochemica et Cytobiologica 45, 233-238.

465

466 Nowak, M., Madej, J. A., Podhorska-Okolow, M., Dziegiel, P., 2008. Expression of

467 extracellular matrix metalloproteinase (MMP-9), E-cadherin and proliferation-associated

468 antigen Ki-67 and their reciprocal correlation in canine mammary adenocarcinomas. In Vivo

469 22, 463-469.

470

471 Peña, L., De Andrés, P. J., Clemente, M., Cuesta, P., Pérez-Alenza, M. D., (2013). Prognostic

472 value of histological grading in noninflammatory canine mammary carcinomas in a

473 prospective study with two-year follow-up: relationship with clinical and histological

474 characteristics. Veterinary Pathology 0, 94-105.

475

476 Qiao, M., Sheng, S., Pardee, A. B., 2008. Metastasis and AKT activation. Cell Cycle 7, 2991-

477 2996.

478

479 Qiu, C., Lin, D., Wang, J., Wang, L. (2008) Expression and significance of PTEN in canine

480 mammary gland tumours. Research in Vetericnary Science 85, 383-388.

481

482 Ressel, L., Millanta, F., Caleri, E., Innocenti, V. M., Poli, A., 2009. Reduced PTEN protein

483 expression and its prognostic implications in canine and feline mammary tumors. Veterinary

484 Pathology 46, 860-868.

485

486 Restucci, B., Maiolino, P., Martano, M., Esposito, G., De Filippis, D., Borzacchiello, G., Lo

487 Muzio, L., 2007. Expression of beta-catenin, E-cadherin and APC in canine mammary

488 tumors. Anticancer Research 27, 3083-3089.

489

490 Roy, D., Dittmer, D. P., 2011. Phosphatase and tensin homolog on chromosome 10 is

491 phosphorylated in primary effusion lymphoma and Kaposi's sarcoma. American Journal of

492 Pathology 179, 2108-2119.

493

494 Sakai, A., Thieblemont, C., Wellmann, A., Jaffe, E. S., Raffeld, M., 1998. PTEN gene

495 alterations in lymphoid neoplasms. Blood 92, 3410-3415.

496

497 Sarli, G., Preziosi, R., De Tolla, L., Brunetti, B., Benazzi, C., 2004. E-cadherin

498 immunoreactivity in canine mammary tumors. Journal of Veterinary Diagnostic Investigation

499 16, 542-547.

500

501 Seow, H. F., Yip, W. K., Loh, H. W., Ithnin, H., Por, P., Rohaizak, M., 2010.

502 Immunohistochemical detection of phospho-Akt, phospho-BAD, HER2 and oestrogen

503 receptors alpha and beta in Malaysian breast cancer patients. Pathology Oncology Research

504 16, 239-248.

505

506 Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., Langford, L.

507 A., Baumgard, M. L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D. H.,

508 Tavtigian, S. V., 1997. Identification of a candidate tumour suppressor gene, MMAC1, at

509 chromosome 10q23.3 that is mutated in multiple advanced cancers. Nature Genetics 15, 356-

510 362.

511

512 Takeichi, M., 1991. Cadherin cell adhesion receptors as a morphogenetic regulator. Science

513 251, 1451-1455.

514

515 Tashiro, H., Blazes, M. S., Wu, R., Cho, K. R., Bose, S., Wang, S. I., Li, J., Parsons, R.,

516 Ellenson, L. H., 1997. Mutations in PTEN are frequent in endometrial carcinoma but rare in

517 other common gynecological malignancies. Cancer Research 57, 3935-3940.

518

Formattato: Nessuna sottolineatura, Colore carattere: Automatico

Formattato: Nessuna sottolineatura, Colore carattere: Automatico

519   Torres, J., Pulido, R., 2001. The tumor suppressor PTEN is phosphorylated by the protein

520   kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated

521   degradation. Journal of Biological Chemistry 276, 993-998.

522

523   Torres, L.N., Matera, J. M., Vasconcellos, C. H., Avanzo, J. L., Hernandez-Blazquez, F. J.,

524   Dagli, M. L., 2005. Expression of connexins 26 and 43 in canine hyperplastic and neoplastic

525   mammary glands. Veterinary Pathology 42, 633-641.

526

527   Tsutsui S, Inoue H, Yasuda K, Suzuki K, Higashi H, Era S, Mori M: Reduced expression of

528   PTEN protein and its prognostic implications in invasive ductal carcinoma of the breast.

529   Oncology 2005, 68:398-404.

530

531   Yang, Z., Yuan, X. G., Chen, J., Luo, S. W., Luo, Z. J., Lu, N. H., 2013. Reduced expression

532   of PTEN and increased PTEN phosphorylation at residue Ser380 in gastric cancer tissues: a

533   novel mechanism of PTEN inactivation. Clinics and Research in Hepatology and

534   Gastroenterology 37, 72-79.

535

536   Yao, Y. J., Ping, X. L., Zhang, H., Chen, F. F., Lee, P. K., Ahsan, H., Chen, C. J., Lee, P. H.,

537   Peacocke, M., Santella, R. M., Tsou, H.C., 1999. PTEN/MMAC1 mutations in hepatocellular

538   carcinomas. Oncogene 18, 3181-3185.

539

540   Yoshida K, Yoshida S, Choisunirachon N, Saito T, Matsumoto K, Saeki K, Mochizuki M,

541   Nishimura R, Sasaki N, Nakagawa T. The relationship between clinicopathological features

542   and expression of epithelial and mesenchymal markers in spontaneous canine mammary

543   gland tumors. J Vet Med Sci. 2014 Oct;76(10):1321-7.

544

545 Yoshimoto, M., Cunha, I. W, Coudry, R. A, Fonseca, F. P., Torres, C.H., Soares, F. A.,

546 Squire, J. A., 2007. FISH analysis of 107 prostate cancers shows that PTEN genomic deletion

547 is associated with poor clinical outcome. British Journal of Cancer 97, 678–685.

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557 **Figure Legends**

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559 **Figure 1.** Dog, E-Cadherin and PTEN expression in mammary carcinomas. A-B) E-Cadherin

560 + and PTEN positive simple mammary carcinoma. Membranous expression of E-Cadherin in

561 neoplastic epithelial cells (A) and cytoplasmic expression of PTEN (B). Formalin fixed

562 sections labeled with antibodies against E-Cadherin and PTEN, haematoxylin counterstained.

563 Bar = 50 mm. C-D) E-Cadherin – and PTEN – simple mammary carcinoma. Weak

564 cytoplasmic expression of E-Cadherin (C) and lack of PTEN expression in neoplastic

565 epithelial cells (D), while some stromal cells were PTEN positive. Formalin fixed sections

566 labeled with antibodies against E-Cadherin and PTEN, haematoxylin counterstained. Bar = 50

567 mm.

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Commento [PA2]: E-cadherin é rabbir polyclonal

569 Figure 2. Dog, PTEN-positive/E-Cadherin-positive mammary carcinoma. Double indirect

570 immunofluorescent stain of PTEN and E-cadherin. (A) PTEN stainoning is evident in the

571 green channel (scale bar=50 microns). (B) E-cadherin positivity is evident in the red channel

572 as intense strong membranous staining (scale bar=50 microns). (C) In the composite image,

573 PTEN staining is evident in the stomal cells and also in the majority of epithelial neoplastic

574 cells and co-localizes with E-cadherin stainining (inset).   E-CAD stain is fain and not

575 continuous through the cell membrane (inset). Indirect immunofluorescence.

576

577 **Figure 32.** Kaplan Mayer estimates of overall survival in PTEN+ and/or E-Cad+ tumors

578 group (green line) -and PTEN-/E-Cad- tumors group (blue line). $P < 0.05$.