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Canine mammary carcinoma with vacuolated cytoplasm: glycogen-rich carcinoma, a histological type distinct from lipid-rich carcinoma.

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25 Abstract

26 Lipid-rich carcinoma is a rare histotype of canine mammary tumors with cytoplasmic
27 vacuolation. In humans, glycogen-rich carcinoma, secretory carcinoma, and myoepithelial
28 neoplasms are among the differential diagnosis for lipid-rich carcinoma. The aims of the study
29 were to investigate the existence of histotypes other than lipid-rich in canine mammary
30 carcinomas with vacuolated cytoplasm using a diagnostic algorithm based on histochemistry,
31 immunohistochemistry and ultrastructure, and to evaluate the molecular phenotype of these
32 neoplasms. Ten mammary carcinomas were collected, histologically reviewed and subjected
33 to histochemistry (PAS, PAS with diastase, Alcian blue, Sudan III (one case) and Congo red
34 (one case)), immunohistochemistry for CK19, CK5/6, CK14, p63, calponin, vimentin, ER, PR
35 and HER2, and transmission electron microscopy (TEM). CKs demonstrated the epithelial
36 origin of all the tumors. Sudan III and TEM confirmed the diagnosis of lipid-rich carcinoma in 8
37 tumors (one amyloid-producing). One tumor was reclassified as a glycogen-rich carcinoma
38 based on PAS reactivity that was diastase-labile, and a second tumor was reclassified as a
39 carcinoma-and-malignant myoepithelioma based on the differentiation markers. Lipid-rich
40 carcinomas were basal-like (5/8), null-type (2/8) and luminal A phenotype (1/8). The
41 glycogen-rich carcinoma was basal-like, while the carcinoma-and-malignant myoepithelioma
42 was luminal A. Vacuolated morphology of neoplastic cells in canine mammary carcinoma can
43 indicate either a neoplasm of epithelial origin with cytoplasmic lipid or glycogen, or vacuolated
44 neoplastic suprabasal myoepithelial cells. Glycogen-rich carcinoma is a novel histological
45 type that should be considered in the differential diagnosis for canine mammary carcinoma
46 with vacuolated cytoplasm.

47 **Keywords:** dog, mammary gland, histochemistry, electron microscopy,
48 immunohistochemistry, lipid-rich carcinoma, glycogen-rich carcinoma.

49

50

51 Clear cell carcinomas of the breast were originally described as tumors with vacuolated,
52 optically empty, clear cell cytoplasm.¹⁷ Exceptionally rare types and variants of breast
53 carcinoma with vacuolated cytoplasm are classified as secretory, sebaceous, lipid-rich,
54 glycogen-rich, and acinic cell carcinomas and myoepithelial neoplasms.¹¹ In the classification
55 of canine mammary carcinomas, lipid-rich carcinoma is the only histological type with
56 morphological features of large cytoplasmic vacuoles.^{13,33} Lipid-rich carcinoma is extremely
57 rare, usually found in young intact bitches, and has an unfavorable biological behavior with
58 lymphatic invasion and nodal and distant metastasis.^{10,13,33} Similarly, human lipid-rich
59 carcinoma is frequently classified as histological grade III with triple negative phenotype,
60 nodal metastasis at presentation, and a high first-year mortality rate.¹¹ Lipid-rich carcinoma of
61 the human breast is diagnosed when no fewer than 90% of the cells contain abundant,
62 cytoplasmic neutral lipid, either on histochemical or ultrastructural evaluation.¹¹ By contrast, in
63 dogs, a diagnosis of lipid-rich carcinoma should only be made when more than 50% of the
64 neoplastic cells have vacuolated cytoplasm.³³

65 The differential diagnosis for human lipid-rich carcinoma includes breast glycogen-rich,
66 histiocytoid, secretory, signet-ring, myoepithelial and metastatic renal carcinomas, and
67 carcinomas modified by hormonal therapy and chemotherapy.¹¹ Glycogen-rich carcinoma is a
68 rare subtype of human invasive mammary gland carcinoma, in which at least 90% of the
69 neoplastic cells have abundant, clear cytoplasm containing glycogen.¹¹ Several reports
70 suggest that glycogen-rich carcinoma in women is an aggressive type, with a high incidence
71 of axillary lymph node metastasis, high histological grade, and short disease-free survival and
72 overall survival.³⁴

73 In humans, secretory carcinoma is a rare, translocation-associated (ETV6-NTRK3 gene),
74 invasive carcinoma with a solid, microcystic and tubular architecture composed of cells that

75 specifically produce intracellular and extracellular secretory material.^{2,11} Secretory carcinoma
76 has a low-grade clinical course with extremely rare distant metastasis and a favorable
77 prognosis in younger human patients.¹¹ Secretory carcinoma is a rarely described canine
78 mammary carcinoma composed of lobules and tubules of neoplastic cells with clear
79 cytoplasm and prominent vacuoles, together with luminal spaces filled with eosinophilic
80 secretion.⁸

81 The aim of the study was to investigate the existence of histological subtypes other than lipid-
82 rich carcinoma in canine mammary carcinomas with vacuolated cytoplasm, using a diagnostic
83 algorithm based on histochemical staining, immunohistochemical markers of differentiation
84 and ultrastructural characterization. The second aim was to evaluate the molecular phenotype
85 of these neoplasms.^{4,6,5,24,33}

86

87 **Material and methods**

88 Case collection and clinical data

89 Ten formalin-fixed paraffin-embedded (FFPE) canine mammary carcinomas were
90 retrospectively collected (2007-2012) from the database of the Pathology Service of the
91 Department of Veterinary Medical Science of Bologna University, from the Department of
92 Veterinary Sciences of Pisa University, and from the Department of Veterinary Medicine,
93 University of Milan. The selection criterion was a diagnosis of lipid-rich carcinoma.

94 Tumor size data were collected during the trimming of the samples. Lymph node metastases
95 were histologically investigated. Systemic metastases were histologically or cytologically and
96 radiographically documented. Tumor-specific survival was clinically confirmed, and a follow-
97 up period of 2-years was recorded.

98

99 *Histology and histochemistry*

100 Sections 4 μm thick were cut from FFPE tissue and routinely stained with hematoxylin and
101 eosin for the histological review of tumors. The slides were reviewed by two board-certified
102 pathologists (LVM and BB). The tumors were graded according to the canine-adapted
103 Nottingham system, based on the histological features of tubule formation, nuclear
104 pleomorphism and mitotic count.²⁵
105 Histochemical stains including periodic acid–Schiff (PAS), PAS with diastase, and Alcian blue
106 were performed on all the collected neoplasms. Wet tissue was available in 1 case, and a
107 portion of the neoplasm was snap-frozen in isopentane and cooled at $-196\text{ }^{\circ}\text{C}$, cryosectioned
108 at $5\text{-}\mu\text{m}$, and stained with Sudan III.³ In one case, where there was a morphological suspicion
109 of amyloid, $8\text{-}\mu\text{m}$ -thick sections were stained with Congo red.

110

111 *Immunohistochemistry*

112 Seven consecutive sections from each block underwent immunohistochemistry (IHC) using
113 antibodies to estrogen receptor (ER), progesterone receptor (PR), human epidermal growth
114 factor receptor 2 (HER2), cytokeratin 5/6 (CK5/6), cytokeratin 14 (CK14), cytokeratin 19
115 (CK19), p63, calponin and vimentin (Supplemental Table S1).
116 Sections were dewaxed and rehydrated. Endogenous peroxidase was blocked by immersion
117 in 0.3% H_2O_2 in methanol for 30 min. Sections were then rinsed in Tris buffer and antigen
118 retrieval was performed by incubation in citrate buffer (pH 6.0 except for sections labelled for
119 CK5/6, which were incubated with EDTA, pH 8.0) and heated for two 5 min periods in a
120 microwave oven at 750 W, followed by cooling at room temperature for 20 min.

121 All antibodies were incubated with the tissue sections overnight at 4°C. Binding sites were
122 revealed by secondary biotinylated antibodies (dilution 1:200) and amplified using a
123 commercial avidin-biotin peroxidase kit (VECTASTAIN ABC Kits, Peterborough, UK). The
124 chromogen DAB (3,3'-diaminobenzidine; 0.05% for 3 minutes at room temperature) was used
125 (ACH500-IFU, ScyTek Laboratories). Slides were counterstained with Harris' hematoxylin.
126 The primary antibody was omitted in the negative control. As positive controls to assess the
127 cross reactivity with canine tissues and the specificity of the immunohistochemical
128 procedure, sections of normal canine mammary gland (for anti-ER and -PR, -CK5/6, -CK14, -
129 CK19, -p63, -calponin and -vimentin), normal canine uterus (for anti-ER and -PR antibodies)
130 and canine skin (for anti-CK5/6, -CK14, -CK19, -p63 antibodies) were used following the
131 same protocols. A human mammary ductal carcinoma (kindly provided by P. Viacava,
132 Department of Oncology, University of Pisa, Italy) known to react with HER2 antibody and a
133 scored 3+³² canine mammary carcinoma were used as positive controls for HER2. HER2
134 was evaluated according to the current ASCO/CAP guidelines (score from 0 to 3+ where only
135 3+ are considered positive).³² The immunohistochemical panel was evaluated following the
136 recommended guidelines for canine mammary tumors.²⁶ The tumors were classified into five
137 molecular subtypes according to the previous literature.^{4,6,5,24,33} Immunohistochemical
138 expression of luminal and basal cytokeratins in the different subtypes was interpreted based
139 on the criteria for human breast tumor investigation.¹

140

141 Transmission electron microscopy

142 All the tumors were processed for TEM.¹⁴ Formalin-fixed, paraffin-embedded (FFPE) tissues
143 were available for review. From all FFPEs, 5 mm³ areas of interest were selected by
144 comparison with related hematoxylin and eosin-stained sections and dewaxed in xylene,

145 washed in a graded series of ethanol (100%, 95%, 70%), rehydrated rapidly in distilled water
146 and then rinsed in 0.15 M cacodylate buffer overnight. Rehydrated tissue samples were
147 postfixed in 1% OsO₄ in cacodylate buffer, dehydrated in graded ethanol, and embedded in
148 Araldite. Ultrathin sections, stained with uranyl acetate and lead citrate, were examined with a
149 Philips TEM CM100 Transmission Electron Microscope.

150
151 *Histochemical, immunohistochemical and ultrastructural algorithm.*

152 Based on the human breast cancer literature on the differential diagnosis of carcinoma with
153 clear vacuoles,¹¹ we created a diagnostic algorithm to investigate the chemical components
154 and differentiation lineage of canine carcinoma with clear vacuoles (Table 1 and
155 Supplemental Figure S1). The histological entities that we investigated were lipid-rich
156 carcinoma, glycogen-rich carcinoma, secretory carcinoma, and carcinoma-and-malignant
157 myoepithelioma.

158 The diagnostic scheme was based on:

- 159 • histological features: presence of optically empty vacuoles with distinct or indistinct
160 edges and/or diffuse diaphanous (translucent) cytoplasm. Vacuoles were
161 morphologically characterized and semi-quantitatively scored as the percentage of
162 neoplastic cells affected (from 50% to 100%).
- 163 • histochemical stains: PAS, PAS with diastase, Alcian blue and Sudan III (the latter only
164 on frozen samples).
- 165 • immunohistochemistry: p63, calponin, cytokeratin 14, cytokeratin 5/6 and cytokeratin
166 19.
- 167 • ultrastructural features: presence of intercellular junctions, lipid vacuoles and glycogen.

168

169

170 **Results**

171

172 Revised diagnosis based on the proposed algorithm

173 Based on the proposed algorithm, by integration of morphological, histochemical,
174 immunohistochemical and ultrastructural data, the 10 lipid-rich carcinomas were reclassified
175 as 8 lipid-rich carcinomas (one of which was amyloid-producing), 1 glycogen-rich carcinoma
176 and 1 carcinoma-and-malignant myoepithelioma.

177

178 *Lipid-rich carcinoma*

179 The 8 cases of lipid-rich carcinoma included 7 females (3 entire and 4 spayed), and 1 male
180 with a concurrent testicular interstitial cell tumor. The mean age at the time of diagnosis was 7
181 years. The breeds represented were mixed breed (2), German Shepherd, Rottweiler, English
182 Setter, Maltese, Bolognese and Bulldog (1 for each breed). The mean tumor size was 3.8 cm,
183 and the affected mammary gland were the IV and V. A clinical 2-year-follow up was available
184 in 4 cases, with tumor specific survival times of 3, 17, 18 and 24 months. Systemic
185 metastases were histologically confirmed in one case (n.6, Supplemental Table S2) and
186 radiographically and cytologically in one other case (n.7, Supplemental Table S2).

187 Lipid-rich carcinomas were composed of polygonal cells with a moderate amount of
188 cytoplasm containing single or multiple, optically empty vacuoles with distinct edges (Figure
189 1). The percentage of neoplastic cells with vacuoles in their cytoplasm ranged from 60 to
190 100%. Mitotic count ranged from 3 to 24 in 10 high-power fields (2.37 mm²).

191 On the one formalin-fixed frozen sample available, neoplastic cells stained positively with
192 Sudan III (Figure 2). Clear vacuoles were negative by PAS and Alcian blue stains in all cases.

193 The electron-lucent vacuoles were ultrastructurally identified as lipid vacuoles, confirming the
194 diagnosis of lipid-rich carcinoma (Figure 3).

195 In one carcinoma, in addition to the lipid content, abundant extracellular eosinophilic material
196 was detectable (Figure 4). The eosinophilic material was PAS-positive and diastase-resistant,
197 and Alcian blue and Congo red positive (Figure 5), and therefore was interpreted as amyloid.
198 The ultrastructural features of both the intracytoplasmic and extracellular material (Figure 6)
199 were of haphazardly arranged, 8-10 nm diameter, non-branching fibrils, consistent with
200 amyloid (Figure 7). The neoplasm was classified as amyloid-producing lipid-rich carcinoma.

201 Three lipid-rich carcinomas were grade III, and 5 were grade II. Lymphovascular invasion was
202 observed in 2 cases. One case (n.6 Supplemental Table S2) had lymph node metastases and
203 systemic metastasis to the liver, lung and spleen. Another case (n.7 Supplemental Table S2)
204 had metastases to liver and lung.

205 In all the examined cases, the epithelial origin of the neoplasms was confirmed by the
206 presence of intercellular junctions by TEM and immunohistochemical expression of
207 cytokeratins. Vimentin was negative in all but one case that was also characterized by
208 concurrent immunolabeling for CK14.

209 The immunophenotype of lipid-rich carcinoma was triple negative basal-like in 5 of 8 primary
210 mammary tumors, based on positive labeling of basal cytokeratins (CK14, CK5/6) (Figure 8).
211 All the neoplasms were negative for ER, but one lipid-rich carcinoma showed expression of
212 PR and was therefore classified as luminal A. None of the examined neoplasms showed
213 overexpression of HER2. Two neoplasms were classified as null type, displaying negativity to
214 all antibodies in the panel. CK19 was expressed in the cytoplasm of neoplastic cells in 6 of 8
215 lipid-rich carcinomas (Figure 9). Co-expression of luminal and basal cytokeratin was present
216 in 3 of 5 basal-like carcinomas, defining a mixed subtype. There was no immunolabeling of

217 neoplastic epithelial cells for myoepithelial markers (p63, calponin) . p63 and calponin labeling
218 were diffusely lost in all 8 carcinomas, confirming stromal invasion. The immunophenotype in
219 one lymph node metastasis was concordant with the primary site (basal-like), while the other
220 lymph node metastasis was discordant with the primary tumor (null-type and basal-like,
221 respectively). Overall results are summarized in Supplemental Table S2.

222

223 *Glycogen-rich carcinoma*

224 The single case of glycogen-rich carcinoma was an 11-year-old, intact female Boxer dog with
225 a 1.5 cm mass in the fifth mammary gland. No follow up information was available. The
226 glycogen-rich carcinoma was composed of lobules, tubules and cords of polygonal cells with
227 sharply distinct borders, moderate to abundant amounts of clear, diaphanous cytoplasm
228 (Figure 10) or with optically empty vacuoles and an intermediate N/C ratio. There were 25
229 mitoses in 10 high-power fields (2.37 mm²). The percentage of neoplastic cells with diffusely
230 vacuolated, diaphanous cytoplasm was 60%. The neoplasm was graded as III.

231 The cytoplasm of neoplastic cells was diffusely and strongly positive for PAS (Figure 11) and
232 diastase-labile (consistent with glycogen) (Figure 12). Ultrastructural analysis confirmed the
233 presence of abundant, granular, electron-dense cytoplasmic material (glycogen) in the
234 neoplastic cells, supporting the diagnosis of glycogen-rich carcinoma. However, glycogen was
235 only visible in a few areas (Figure 13, Figure 14) due to partial extraction during routine
236 processing of FFPE specimens for TEM study.

237 A draining lymph node was massively invaded by the glycogen-rich carcinoma with the same
238 morphological and histochemical features seen in the primary tumor. The glycogen-rich
239 carcinoma was immunolabeled for CK19 and CK5/6 (Figure 15), scattered expression of
240 CK14, and no labeling for ER, PR or HER2. It had a basal-like mixed phenotype in the

241 primary tumor and lymph node metastasis. No immunoexpression of p63 or calponin was
242 present.

243

244

245 *Carcinoma-and-malignant myoepithelioma*

246 The single case of carcinoma-and-malignant myoepithelioma was a 10 year-old, female,
247 mixed breed. Carcinoma-andmalignant myoepithelioma was composed of tubules, papillae
248 and solid sheets of polygonal cells. The biphasic nature of the tumour was subsequently
249 defined by immunohistochemistry. The solid component was characterized by optically empty
250 cytoplasmic vacuoles with blurred edges (Figure 16) and an intermediate nucleus to
251 cytoplasm ratio. There were 17 mitoses in 10 high-power fields (2.37 mm²). The neoplasm
252 was grade III. Transmission electron microscopy demonstrated rudimentary intercellular
253 desmosome-like junctions between cells featuring an elongated nucleus (Figure 17). Basal
254 lamina and remnant cytoplasmic myofibrils were not clearly recognizable due to retrieval from
255 FFPE specimens.

256 The immunophenotype of the carcinoma-and-malignant myoepithelioma revealed the
257 biphasic nature of the neoplasm. The tubular and papillary components were CK19-positive.
258 The solid sheets of vacuolated cells expressed CK14, CK5/6, p63 (Figure 18) and calponin.
259 There was expression of PR and no staining for ER and HER2, compatible with a luminal A
260 immunophenotype.

261

262 **Discussion**

263 According to the current classification system for dogs, lipid-rich carcinoma is the only
264 mammary carcinoma histotype that can be diagnosed in the presence of cytoplasmic
265 vacuolation of neoplastic cells.³³ By contrast, in humans, breast cancers with clear cells
266 include several tumor types: lipid-rich carcinoma, glycogen-rich carcinoma, apocrine
267 carcinoma, secretory carcinoma, and myoepithelial neoplasms.¹¹ A diagnostic algorithm was
268 applied to investigate other histotypes of mammary carcinoma with vacuolated cytoplasm in
269 dogs. In the present study, 8 lipid-rich carcinomas (one of which was amyloid-producing), 1
270 glycogen-rich carcinoma, and 1 carcinoma-and-malignant myoepithelioma were identified,
271 confirming that canine mammary carcinomas with vacuolated cytoplasm can include different
272 tumour entities other than lipid-rich carcinoma, as in the human counterpart.

273 The first tumor parameter to be evaluated in the diagnostic algorithm is morphological. In fact,
274 based on the data obtained and on the reviewed classification, the morphological
275 characteristics of the clear cytoplasm can be useful for guiding the diagnostic procedure.
276 Lipid-rich tumors always have cytoplasm with optically empty vacuoles that have distinct
277 margins. On the contrary, the solid myoepithelial component of carcinoma-and malignant
278 myoepithelioma has optically empty cytoplasmic vacuoles with blurred, poorly defined
279 margins. Finally, glycogen-rich carcinomas have diffusely clear cytoplasm with a central
280 nucleus and prominent cell margins (colloquially referred to as “fried eggs
281 appearance”).^{16,17,28,23,18,12} Histochemistry and immunohistochemistry are supportive in
282 characterizing the different histological subtypes, and ultrastructural analysis can confirm the
283 diagnosis.

284 Demonstration of the lipid content can be achieved with Sudan III stain on frozen sections. In
285 the absence of frozen samples, ultrastructural analysis can be performed on FFPE material.⁹
286 Based on our results, the diagnosis of lipid-rich carcinoma should require the presence of

287 vacuoles in at least 50% of neoplastic cells as well as the demonstration of lipid content within
288 the vacuoles, as defined by the current classification.³³

289 Localized amyloid deposition has been reported in canine mammary carcinoma²⁹ and
290 amyloid-producing lipid-rich carcinoma is a very rarely described variant of lipid-rich
291 carcinoma in which the amyloid and the neoplastic cells are β -casein positive.³⁰ Amyloid is
292 ultrastructurally defined by the presence of aggregates of non-branching fibrils with a
293 diameter of 8–10 nm.²¹ The present case of amyloid-producing lipid-rich carcinoma was
294 Congo red-positive and had the canonical ultrastructural features reported in the literature.²¹

295 According to the literature, the epithelial cells of lipid-rich carcinoma can express both luminal
296 and basal cytokeratins²⁷ and usually do not express estrogen and progesterone receptors.¹⁰

297 Lipid-rich carcinomas in our study expressed luminal cytokeratin (CK19), basal cytokeratins
298 (CK5 / 6 and CK14), no hormone receptors (ER, PR; with one exception), and no HER2, in
299 line with the canine literature.^{10,27} In humans^{20,11} and dogs¹⁰, lipid-rich carcinoma has a triple
300 negative immunophenotype with loss of hormone receptors, and its behavior is typically
301 aggressive with nodal or distant metastasis. In the present study, the lipid-rich neoplasms
302 were triple negative in 7 of 8 cases (5 basal-like and 2 null-type). Three of five basal-like
303 carcinomas co-expressed the luminal CK19, defining a mixed subtype. In human breast
304 cancer, expression of CK19 is suggestive of maintained glandular differentiation;¹ it is almost
305 always expressed by carcinomas regardless of the phenotype^{15,31} and is a useful marker in
306 the detection of micrometastasis in lymph node core biopsies.³¹ A mixed luminal/basal subtype
307 characterized by co-expression of luminal and basal cytokeratins has been described in human
308 breast cancer with a biological behavior more similar to the basal-like phenotype.¹ In only one
309 case was there expression of PR, and the neoplasm classified as luminal A. Both concordant

310 and discordant immunophenotypes were observed in the metastatic sites compared to the
311 primary tumors, as previously described in the dog.^{4,5}

312 Secretory carcinoma is an additional histological type that has been reported in dogs in one
313 previous publication.⁸ In the current canine classification system, the term secretory
314 carcinoma is used as a synonym of lipid-rich carcinoma.³³ By contrast, in humans, secretory
315 carcinoma is considered a separate entity characterized by secretory material within
316 neoplastic tubules and translocation of ETV6-NTRK3 gene. As our study confirms the
317 presence of several analogies between canine and human carcinomas, we believe that the
318 use of the term secretory as a synonym for lipid-rich carcinoma should be discouraged, and
319 that it should be restricted to those neoplasms showing both intracellular and extracellular
320 material, and the translocation should be investigated.

321 Glycogen-rich clear cell carcinoma is a rare breast carcinoma accounting for 0.9-3% of
322 human breast neoplasms.^{23,12} The typical cellular feature of human glycogen-rich clear cell
323 carcinoma of the breast is the “fried eggs appearance” with clear cytoplasm and small, dark,
324 punctate central nuclei.^{16,17,28,23,18,12} The diagnosis in humans is confirmed by PAS-positive,
325 diastase-labile, intracytoplasmic glycogen granules in more than 90% of neoplastic
326 cells^{16,17,28,23,12} Ultrastructural analysis confirms the presence of tight junctions and abundant
327 non-membrane bound glycogen in the neoplastic cells.¹⁷ In the present canine case,
328 histological features were similar to the human cases, but glycogen was present in only 60%
329 of neoplastic cells. A canine adapted cut-off value should be considered, similar to lipid-rich
330 carcinoma in dog.³³ Moreover, the glycogen component was maintained in the lymph node
331 metastasis. In this case, a basal-like phenotype was confirmed, suggesting a poor
332 prognosis.¹⁹ Prognosis of glycogen-rich carcinoma of the human breast is also generally poor
333 and is typically associated with a high-grade, advanced-stage, triple negative hormone

334 receptor status, lymph node metastasis and high mortality rate.^{17,22,34} Glycogen-rich
335 mammary carcinoma has also been reported in cats.⁷

336 Canine carcinoma-and-malignant myoepithelioma is a biphasic neoplasm characterized by
337 the presence of malignant luminal epithelial and malignant myoepithelial cells.³³ The
338 myoepithelial cells may have different morphology^{5,33} ranging from spindle to stellate and
339 polygonal. The presence of cytoplasmic vacuoles can occur in myoepithelial neoplasms,
340 therefore further immunohistochemical characterization in mammary carcinoma with clear
341 vacuoles should be considered to investigate a myoepithelial origin of the neoplasm.³³

342 In conclusion, the proposed algorithm can be useful in distinguishing different histological
343 entities among mammary carcinomas with vacuolated cell morphology, which may be
344 associated with different biologic behavior. According to the results of this study, vacuolated
345 morphology in canine mammary carcinoma can indicate either an epithelial phenotype with
346 lipid or glycogen content or a myoepithelial phenotype. As expected, the lipid-rich carcinoma
347 was the most prevalent. Glycogen-rich carcinoma is a newly described, rare histological
348 subtype of canine mammary carcinoma, characterized by clear cytoplasm, diastase-sensitive
349 PAS positivity, and a triple negative phenotype. Lipid-rich carcinoma and glycogen-rich
350 carcinoma should be considered as a potential differential diagnosis for mammary carcinoma
351 with vacuolated cytoplasm in dogs.

352

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357

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450

451 **Figure legends**

452 **Figures 1-3.** Lipid-rich carcinoma, mammary gland, dog. Figure 1: Nodules of polygonal
453 neoplastic cells, with a moderate amount of cytoplasm containing distinct, optically empty
454 vacuoles. Hematoxylin and eosin (HE). Figure 2: Red-staining (Sudan III-positive) material in
455 the cytoplasmic vacuoles of the neoplastic epithelial cells. Cryosection, Sudan III stain. Figure
456 3: Ultrastructure of electron-lucent vacuoles consistent with lipid-containing vacuoles.
457 Transmission electron microscopy (TEM).

458 **Figures 4-7.** Amyloid-producing lipid-rich carcinoma, mammary gland, dog. HE. Figure 4:
459 Abundant intracytoplasmic and extracellular compact eosinophilic material. Figure 5: The
460 eosinophilic secretory material is Congo red-positive, interpreted as amyloid. Congo red.
461 Figures 6-7: Intracytoplasmic and extracellular electron-dense material (Fig. 6) is characterized
462 by non-branching 8- to 10-nm-diameter fibrils (Fig. 7) consistent with amyloid. TEM.

463 **Figure 8.** Lipid-rich carcinoma, lymph node, dog. The subcapsular sinus contains nodules of
464 neoplastic cells with scattered intense cytoplasmic immunolabeling for cytokeratin 14. **Figure**
465 **9.** Lipid-rich carcinoma, mammary gland, dog. Nodules of neoplastic cells have diffuse and
466 intensely positive luminal immunolabeling for cytokeratin 19.

467

468 **Figure 10-15.** Glycogen-rich carcinoma, mammary gland, dog. Figure 10: Nodules and cords
469 of neoplastic cells with sharply distinct borders and a moderate amount of clear, diaphanous
470 cytoplasm, and central punctate nuclei (fried eggs appearance). Hematoxylin and eosin (HE).
471 Figure 11: Neoplastic cells with intense, diffuse, granular PAS-positive cytoplasm with polarized
472 effect. Figure 12: After treatment with diastase, PAS reactivity is negative (consistent with
473 glycogen). Figure 13: The neoplastic cells have an increased amount of granular electron-dense

474 cytoplasmic material. Transmission electron microscopy (TEM). Figure 14. Glycogen granules
475 within the cytoplasm of neoplastic cells. TEM. Figure 15: There is moderate and diffuse
476 membranous and cytoplasmic immunolabeling for cytokeratins 5/6.

477 **Figure 16-18.** Carcinoma-and-malignant epithelioma, mammary gland, dog. Figure 16: Solid
478 pattern of neoplastic malignant myoepithelial cells characterized by optically empty cytoplasmic
479 vacuoles. HE. **Figure 17:** Rudimentary desmosome-like junctions (arrows) are visible between
480 two neoplastic cells. TEM. **Figure 18:** Diffuse and intense nuclear immunolabeling for p63.

481

482 **Figure S1:** Graphic scheme of the diagnostic algorithm.

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