VETERINARSKI ARHIV 92 (4), 421-442, 2022 DOI: 10.24099/vet. arhiv.1336

Canine circumanal gland tumors and epithelial – mesenchymal transition: an immunohistochemical study

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ŠOŠTARIĆ-ZUCKERMANN, I.-C., M. HOHŠTETER, B. ARTUKOVIĆ, M. HUZAK, K. SEVERIN, Ž. GRABAREVIĆ, A. GUDAN KURILJ: Canine circumanal gland tumors and epithelial – mesenchymal transition: an immunohistochemical study. Vet. arhiv 92, 421-442, 2022.

ABSTRACT

Circumanal gland (CG) tumors are common neoplasms of older dogs. Of these, only circumanal gland carcinomas show clear metastatic potential, and even so metastases seem to be uncommon. Epithelial-mesenchymal transition (EMT) is a process that facilitates the initial steps of metastasis, and to date it has not been systematically investigated in CG tumors. Insight into the occurrence of this process would be a valuable asset in understanding the biology of these tumors. To test the occurrence of EMT we used three immunohistochemical markers that alter their expression in this process, namely – E-cadherin, N-cadherin, and matrix metalloproteinase-9 (MMP-9). Additionally, we used the Ki-67 marker of proliferation. The samples used consisted of 15 adenomas, 11 epitheliomas, 21 well-differentiated carcinomas, seven poorly differentiated carcinomas, and ten samples of normal CG. The results of N-cadherin were negative for all samples. E-cadherin was highly expressed in all groups, but was slightly lower in semi-malignant and malignant tumors; MMP-9 marking was generally very low, but significantly higher in semi-malignant or malignant tumors when compared to benign or non-neoplastic CG. The index of proliferation (Ki-67) was significantly higher for semi-malignant or malignant CG tumors when compared to benign CG tumors or normal CG. These results show that with an increase in the histologic malignancy of these tumors there is a slight drop in E-cadherin, a slight rise in MMP-9, and a significant increase in Ki-67. Therefore, these results suggest the possibility that EMT occurs within the malignant or even semi-malignant forms of CG tumors, but probably as a rare and late event. Further studies are needed to prove or disprove these statements.

Key words: canine circumanal (perianal, hepatoid) gland tumors; epithelial – mesenchymal transition; E-cadherin; matrix metalloproteinase-9; Ki-67; immunohistochemistry

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ISSN 0372-5480
Printed in Croatia

Introduction

Circumanal (also known as hepatoid or perianal) glands are modified sebaceous glands located in the canine skin surrounding the anus. Their importance in the health of the dog is primarily associated with the common neoplasms that arise from these glands. Grossly, these tumors arise as nodular or multinodular outgrowths in the perianal region of aged animals (LEE GROSS et al., 2005.). Histologically, these tumors are classified into the benign and more frequent adenomas, the less frequent semi-malignant (or low-grade malignant) epitheliomas, and malignant carcinomas (GOLDSCHMIDT et al., 1998). Although attempts have been made to differentiate these tumors on the basis of cytological findings (SABATTINI et al., 2019), the gold standard for diagnosing these neoplasms remains biopsy sampling and microscopic examination. Microscopy discerns sufficiently between the types mentioned and thus helps with prognosis. Nonetheless, as with many neoplasms, additional immunohistochemical markers that prove the diagnosis or help with prognosis are sometimes used. Such examples involve the use of Ki-67 (PEREIRA et al., 2013, BRODZKI et al., 2014), AgNOR (PREZIOSI et al., 1995), vimentin when calculating fractal dimension (ŠOŠTARIĆ-ZUCKERMANN et al., 2016), cytokeratin (JARDIM et al., 2018), estrogen receptor, progesterone receptor, Akt (KIM et al. 2018) and others. Ki-67 is so far one of the most widely used indicators of cellular proliferation, and one of the markers that can often help establish tumor prognosis (SUN and KAUFMAN, 2018.). This applies to numerous human neoplasms (SCHOLZEN and GERDES, 2000;) and canine neoplasms e.g. soft tissue sarcomas (ETTINGER et al., 2006), mast cell tumors (SCASE et al., 2006), lymphomas (PONCE et al., 2003), mammary gland tumors (PENA et al., 1998; GUDAN KURILJ et al., 2011), and many others.

Not so long ago, the phenomenon of epithelial to mesenchymal transition was identified as an integral part in the development of migratory and metastatic phenotypes in malignant carcinoma cells (KALLURI and WEINBERG, 2009). This process of epithelial to mesenchymal transition can be closely monitored by detection of either the gain or loss of expression of certain proteins. One of the most important proteins involved in this process are cadherins. They are a family of cell surface glycoproteins with a repeated extracellular domain and a cytoplasmic tail, bound to the actin cytoskeleton via catenins. Cadherins are important members of adherens junctions and they mediate calcium dependant cell-cell adhesions. There are distinct types of cadherins (E-, N-, P-cadherin) that show different expressions depending on the cell (tissue) type and stage of development (HULPIAU and VAN ROY, 2009). E-cadherin is the main cadherin type expressed in polarized epithelial cells. Malignant epithelial cells commonly downregulate E-cadherin, which has been detected in numerous canine tissues (MATOS et al., 2006; POLTON et al., 2007; SILVESTRI et al., 2020).

In the process of tumorigenesis, this downregulation of E-cadherin is commonly preceded by the upregulation of N-cadherin expression (LOH et al., 2019). Within human tissues, N-cadherin expression is evident in various normal tissues: neurons, endothelial and muscle cells, but also as a subpopulation of early hematopoietic cells (PUCH et al., 2001), and numerous tumor types, e.g., mesotheliomas, chordomas, synovial carcinomas, melanomas, and some mammary tumors (LASKIN and MIETTINEN, 2002; HAN et al., 1999). A few reports of N-cadherin expression in dogs include choroid plexus tumors (REGINATO et al., 2016), meningioma (MANDARA et al., 2015; IDE et al., 2010) and mammary gland tumors (YOSHIDA et al., 2014).

Within the process of EMT, there is a special place for matrix metalloproteinases (also known as matrix metallopeptidases). These zinc-dependent enzymes digest various proteins of the extracellular matrix and basement membranes, and are essential for any tissue remodeling. MMP-9 (together with MMP-2) is a gelatinase that digests collagen type IV, an integral part of the basement membranes (HUANG, 2018; VANDOOREN et al., 2013). It is exactly for that reason that the action of these two matrix metalloproteinases allows the carcinoma cells to breach the basement membrane, escape into the surrounding tissue, and ultimately enter

the lymphatic and blood vessels. Moreover, MMP-9 is directly implicated in tumor angiogenesis (DERYUGINA and QUIGLEY, 2006). Therefore, within many canine tumors there is an increase in the expression levels of these enzymes (; NOWAK et al., 2008, DOCAMPO et al., 2011).

Taking into consideration all of the above, and encouraged by the results of a previous study in which the same subset of cases showed the wide expression of vimentin (ŠOŠTARIĆ-ZUCKERMANN et al., 2016), we decided to test for additional markers of EMT. Therefore, this study aimed to detect whether the process of EMT occurs within any of these neoplasms, and whether it is associated with the proliferative potential of malignant cells. To do this, we tested immunohistochemically the level of expression of four different proteins – the three indicators of EMT mentioned (E-cadherin, N- cadherin, and MMP-9), and one indicator of proliferative activity (Ki-67) within the various types of circumanal gland tumors and normal circumanal glands.

Material and methods

Samples, microscopic examination and classification. The case series used in this study were the same as those used in a previous study (ŠOŠTARIĆ-ZUCKERMANN et al., 2016), with the inclusion of one additional sample of a poorly differentiated circumanal gland carcinoma (this case was included since it was part of the original case series but it was discarded in the previous study due to procedural errors). The samples were classified histologically as described in that publication (ŠOŠTARIĆ-ZUCKERMANN et al., 2016). The number of samples per histopathological diagnosis is shown in Table 1.

Table 1. Samples by diagnosis (samples included in the study)

	Number of samples	Mean age of dogs (in years)	Sex ratio (M/F)	Most common breed
Normal circumanal glands	10	2.37	6/4	Mixed breed (4)
Circumanal gland adenomas	15	8.13	12/3	Mixed breed (4)
Circumanal gland epitheliomas	11	10.91	11/0	Mixed breed (5)
Well-differentiatedcircumanal gland carcinomas	21	11.25	21/0	Poodle (3)
Poorly differentiated circumanal gland carcinomas	7	12.29	5/2	Mixed breed (2). Poodle (2)
Overall number of samples	64	9.44	55/9	Mixed breed (17)

Immunohistochemical analysis. Immunohistochemical analysis was conducted using four different antibodies: Ki-67, matrix metalloproteinase-9 (MMP-9), E- and N cadherin. Details regarding antigen retrieval, specific antibodies, and incubation period are given in Table 2. In short, for all antibodies a 4µm thick section of each chosen paraffin block sample was mounted on coated glass slides (DAKO, K8020), dewaxed, and rehydrated. Antigen retrieval was performed with a corresponding buffer, according to the manufacturer's instructions (see Table 2). Endogenous peroxidase activity was blocked by incubating the sections in Dako REALTM Peroxidase-Blocking solution for 5 minutes. Sections were then incubated with the selected primary antibody (see Table 2 for manufacturer and dilution). This was followed by incubation for 30 minutes with a ready-to-use secondary antibody (Dako REALTM EnVisionTM/Horseradish Peroxidase, Rabbit/Mouse), and with the substrate Dako REALTM Diaminobenzidine + Chromogen for a further 10 minutes. Rinsing was performed with DakoCytomation Wash Buffer between each step. All of the previous steps (from antigen retrieval) were conducted in a DakoAutostainer. Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted with

coverslips. Samples of small intestine, large intestine, haired skin, and brain tissue were used as positive controls for Ki-67, MMP-9, E-cadherin, and N-cadherin, respectively. Negative controls were obtained by substitution of the primary antibody with phosphate-buffered saline.

Table 2. Primary antibodies, dilutions, incubation periods and antigen retrieval solutions

Primary antibody	Manufacturer, code, clone	Dilution	Incubation time	Antigen retrieval solution and incubation time
Ki-67	Dako, M7240, monocolonal mouse, anti-human, MIB-1 clone	1:75	30 min	EDTA buffer (Dako S2367); 20 min
MMP-9	Novocastra, NCL-MMP-9-439, monoclonal, mouse, antihuman, clone 15W2	1:60	15 min	EDTA buffer (Dako S2367); 15 min
E-cadherin	Dako, M3612, monoclonal, mouse, antihuman, NHC-38 clone	1:100	30min	Modified citrate buffer (Dako S1700); 20 min
N-Cadherin	Dako, M3613, monoclonal, mouse, antihuman, clone 6G11	1:50	30 min	Modified citrate buffer (Dako S1700); 2x20 min

Scoring of IHC sections. Ki-67. The Ki-67 proliferation index was assessed by counting 100 circumanal gland cells (either tumor or normal, depending on the sample) in ten random and non-overlapping fields of view that contained the tissue of interest. Positively stained nuclei within these cells were counted separately. The sum of positive nuclei was divided by 1000 (the total number of counted cells), thus yielding the percentage of Ki-67 positive cells within the target cell population.

E-cadherin. For E-cadherin, the so-called percentage of immunopositive cells was determined. This represented the ratio of marked cells to all the tumor cells (IDE et al., 2011). This ratio was determined after assessment of ten non-overlapping, random high-power fields.

To address the staining pattern more precisely, the localization (membrane and/or cytoplasm) and intensity of the staining were also determined. The intensity of staining was scored as follows: 0 - no reaction; 1 – weak reaction; 2 – moderate reaction; 3 – strong reaction.

N-cadherin. The percentage of immunopositive cells was determined as for E-cadherin. Due to a very scarce number of positively stained cells, the intensity of staining was not assessed.

Matrix metalloproteinase-9. This stain was assessed by counting positively marked cells within 100 tumor cells per 10 representative and random high-power fields. The positively stained cells were measured as the percentage of positively stained cytoplasms or nuclei of tumor cells, and assigned to four categories: 0 - negative; 1 - less than 10%; 2 - more than 10% and less than 50%; 3 - more than 50% positive staining (as previously described by DE VICENTE et al., 2005). All slides were scored in a blinded and random fashion without the knowledge of the previous histopathological diagnosis. All the scoring for Ki-67 and assessment of immunopositive cells (E-, N- cadherin, and MMP-9) was conducted by two investigators, the final values being the arithmetic mean of the two values. Scoring of intensity or localization of E-cadherin and stained slides was also conducted by two investigators. In these cases, in the case of disagreements between the two scores, a third independent investigator scored the slide/s and the majority decision was thus considered final.

Statistical analysis. All data were analyzed using Statistica version 10. 0 data analyzing software. To address the significance of the differences between the scores of particular IHC markers, 2x2 frequency tables were assembled and the chi-square test was conducted. We used the histological diagnosis as an ordinal variable since we had five different diagnoses from the same tissue that could be easily arranged according to biological behavior. The diagnoses were therefore listed in the following order, with increasing malignant behavior: 1. normal CG, 2. CG adenoma, 3. CG epithelioma,

4. well-differentiated CG carcinoma, 5. poorly differentiated CG carcinoma. Associations between diagnoses and values of E-cadherin and MMP-9 were tested using Goodman and Kruskal's Gamma statistics and Spearman's rank-order correlation (as shown later in the results), while the association of diagnoses with Ki-67 values (continuous variable) was tested using the Pearson correlation coefficient and Spearman correlation coefficient.

Results

Ki-67. All the samples contained at least some positive signals, but these values varied considerably. The mean values of Ki-67 expression within normal or tumorous circumanal gland cells were: normal CG - 5.7%; CG adenomas - 4.9%; CG epitheliomas - 14.4%; well-differentiated CG carcinomas - 16.4%; poorly differentiated CG carcinomas - 20.8%. These values are depicted in Figure 1. By using the Tukey HSD test, significant differences between these values were shown between normal CG and either well-differentiated or poorly-differentiated carcinomas ($P < 0.01$); between CG adenomas and CG epitheliomas ($P<0.05$); and between CG adenomas and either well-differentiated or poorly-differentiated carcinomas ($P<0.01$). Figures 2 (Ki-67 staining of one normal circumanal gland) and 3 (Ki-67 staining of one poorly differentiated CG carcinoma) illustrate this level of difference in the staining of this marker. The value of the Pearson correlation coefficient between the histological tumor subtype and Ki-67 proliferation index was 0.531, while the Spearman correlation coefficient was 0.677. Most of the positive staining was observed within reserved or basaloid cells (the normally proliferating pool of cells in these glands), but also within the polyhedral hepatoid cells in the case of well and poorly differentiated CG carcinomas.

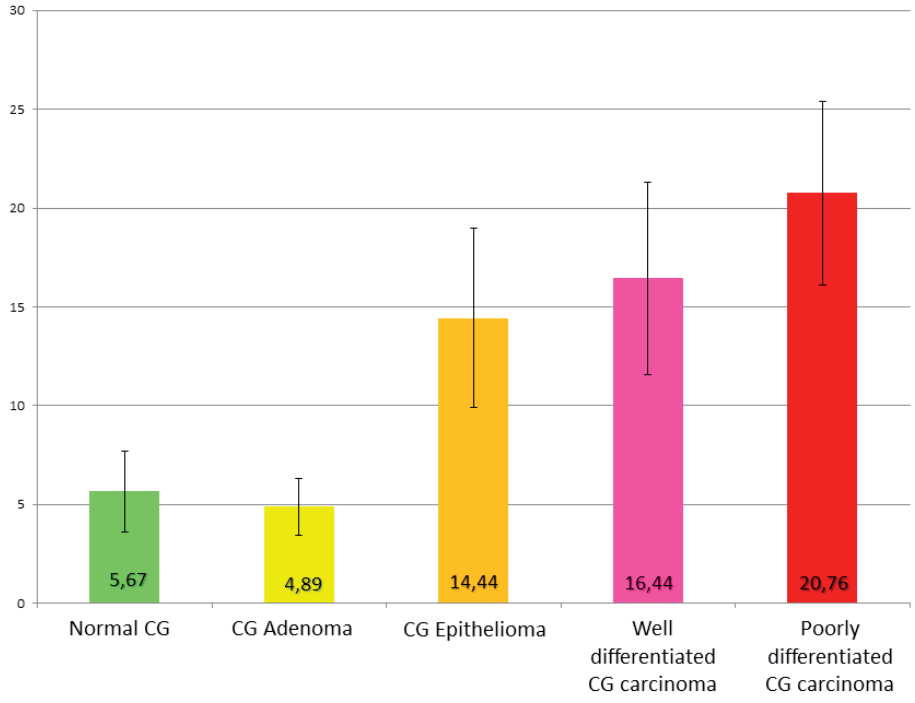

Fig. 1. Ki-67 values per different circumanal gland tumors. Bar chart illustrating the values of Ki-67 per different circumanal gland tumors and normal CG glands, values are given on the "Y" axis as percentages. On the "X" axis normal and different subtypes of circumanal gland tumors are aligned. Note the increase in the Ki-67 values as less favorable diagnoses are reached. The whiskers indicate the values of double standard error (95% confidence intervals).

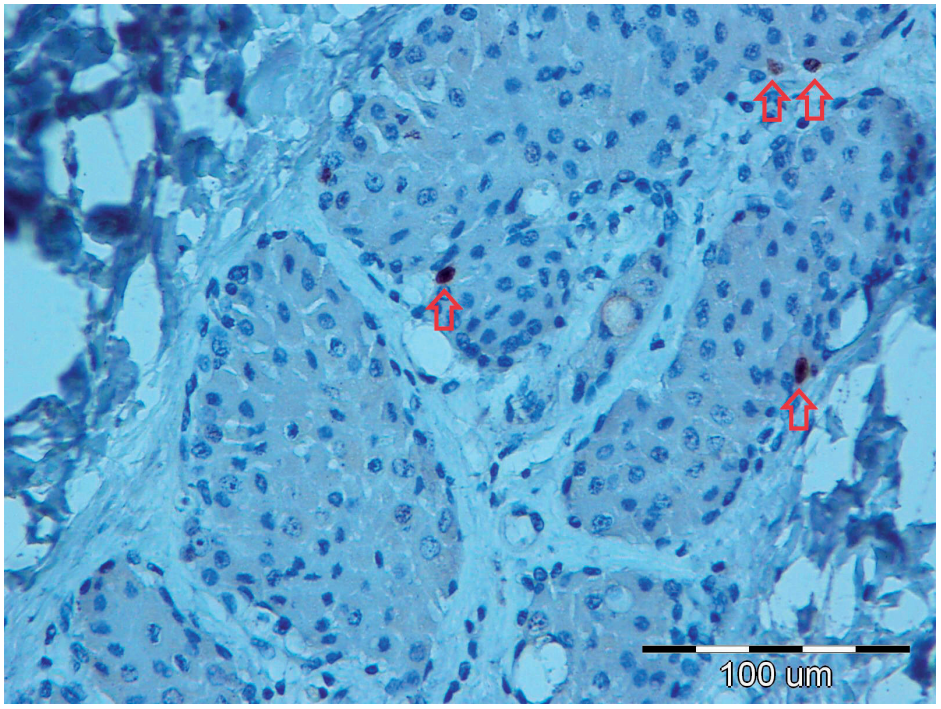

Fig. 2. Micrograph of IHC Ki-67 staining, normal circumanal gland. The red arrows indicate the only four positively stained nuclei in this field of view. Note that these are all reserve (basaloid) cells located at the periphery of the CG lobule.

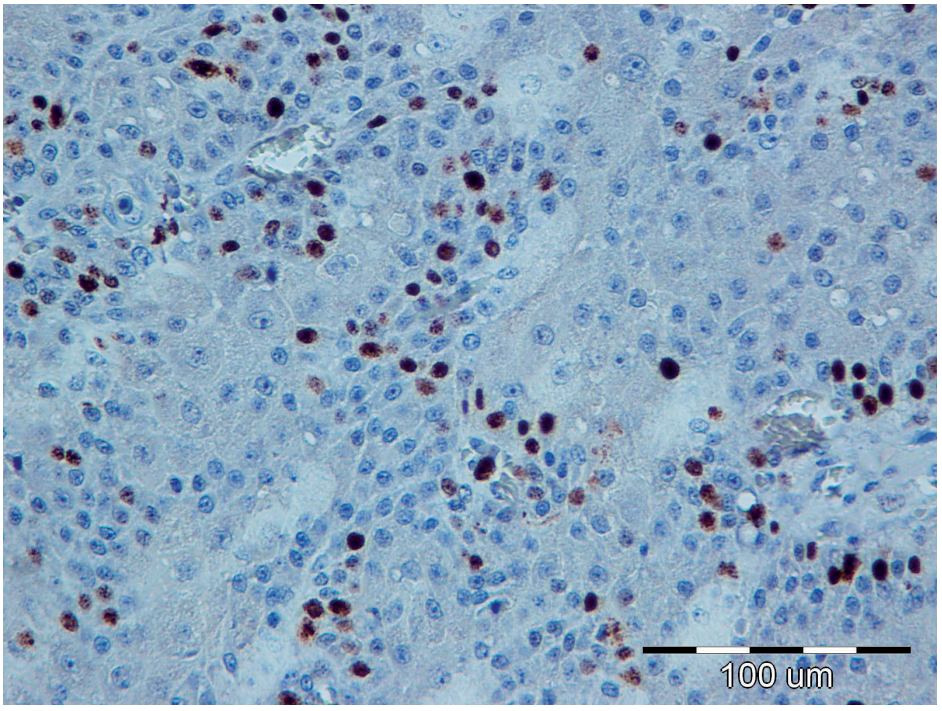

Fig. 3. Micrograph of IHC Ki-67 staining, of a poorly differentiated circumanal gland tumor. Note that roughly 20% of tumor cell nuclei are positively marked. Positive signals are observed mostly in reserve (basaloid) cells, but also in polyhedral hepatoid cells.

E-cadherin. All the samples included in the study showed a positive reaction to E-cadherin. Examples of this staining are given in Figures 4 and 5. The percentage of immunopositive cells was generally very high, in most cases above 90%.

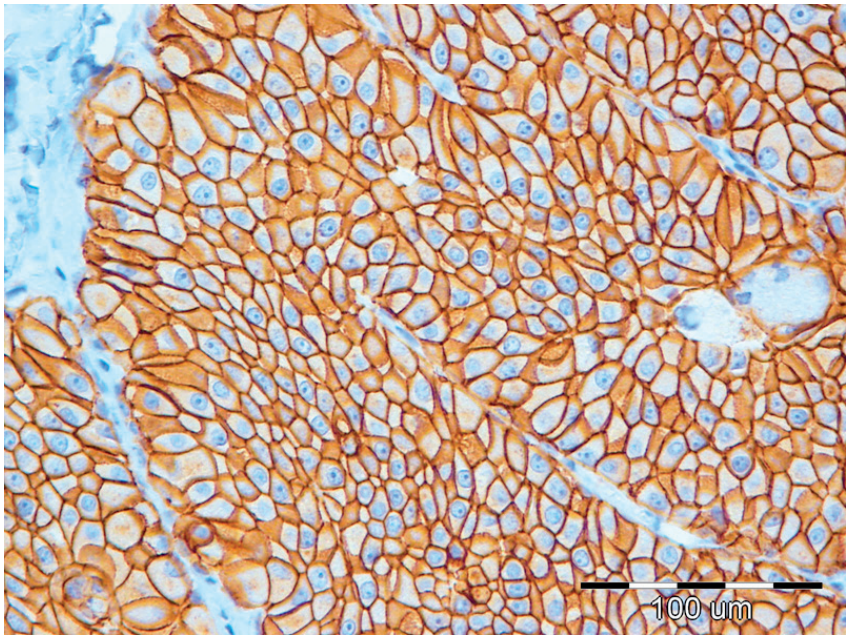

Fig. 4. E-cadherin IHC stain, circumanal gland adenoma, magnification 400X. Note intense membranous and mild to moderate cytoplasmic staining. Both reserve (basaloid) and polyhedral (hepatoid) cells have reacted equally.

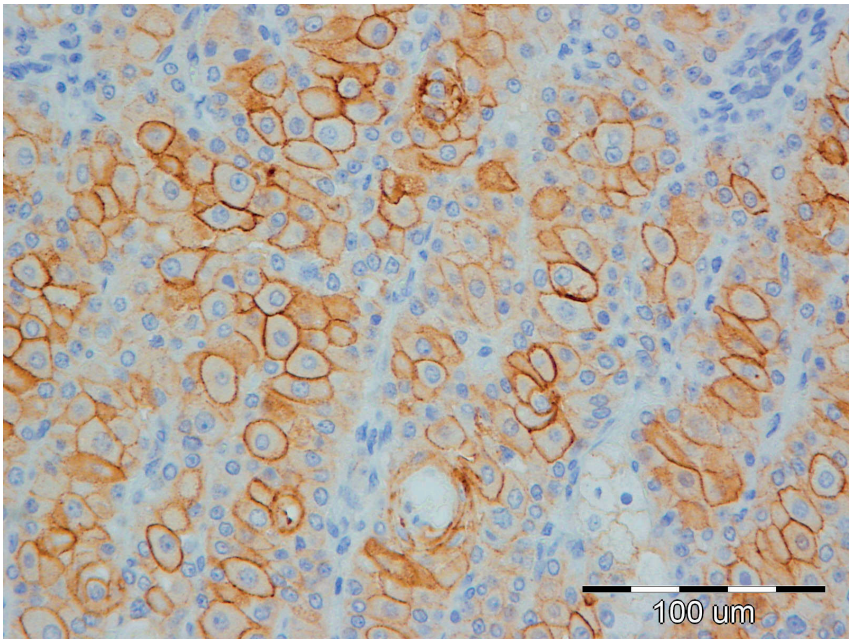

Fig. 5. E-cadherin IHC stain, poorly differentiated circumanal gland carcinoma, magnification 400X. Note the quite variable staining between tumor cells. Most of the reserve (basaloid) cells have weakly stained membranes and/or cytoplasms, or there is no reaction at all. Most of the polyhedral (hepatoid) cells have a positive reaction, similar to but somewhat weaker than in the case of CG adenoma (compare with figure 4).

Small differences were observed between different CG tumor subtypes, however, the HSD Tukey test did not reveal any significant differences. The intensity of the cytoplasmic or nuclear staining was variable but without significant difference within the different tumor subtypes. However, it was noted that both values (the percentage of immunopositive cells and the intensity of staining) were lowest for the poorly differentiated CG carcinomas. Table 3 gives detailed values of E-cadherin, Ki-67 and MMP-9 for each case, and Table 4 reveals the detailed percentages of immunopositive cells and intensity of staining per different tumor subtypes. Figure 4 shows one highly positive and intense staining in the case of one adenoma, while Figure 5 shows the less positive and less intense reaction in one sample of poorly differentiated circumanal gland carcinoma. To emphasize the difference between entirely positive and not entirely positive tumors, we grouped the results of the E-cadherin percentage of immunopositive cells into two categories: samples with 90% or less immunopositive cells, and samples with more than 90% positive cells. Additionally, due to the relatively low number of samples within individual investigated groups, the five groups studied were merged into two new groups according to the two following models:

1. First group: normal circumanal glands + CG adenomas + CG epitheliomas; Second group: well and poorly differentiated circumanal gland carcinomas

2. First group: normal circumanal glands + CG adenomas; Second group: CG epitheliomas + well and poorly differentiated circumanal gland carcinomas

Table 3. Overview of all cases included in the study, histopathological diagnoses, Ki-67, E-cadherin membrane and cytoplasm staining intensity with overall percentage of positive cells and the degree of MMP-9 staining are given. M-male, F-female, E-cadh – E-cadherin, MMP-9 – Matrix metalloproteinase 9

No.	Breed	Sex	Age (in years)	Histopathological diagnosis	Ki-67 (%)	E-cadh membrane intensity (0-3)	E-cadh cytoplasm intensity (0-3)	E-cadh %	MMP-9 degree of staining* (0-3)
1	Samoyed	M	0.2	Normal gland	10.2	3	2	100	0
2	Newfoundland dog	M	4	Normal gland	1.8	2	2	100	0
3	Dachshund	F	2	Normal gland	6.5	3	1	100	0
4	Mongrel	M	1	Normal gland	4.5	1	1	99.7	0
5	Mongrel	F	5	Normal gland	2.8	3	2	100	0
6	German shepherd dog	M	3	Normal gland	10.9	1	2	100	0
7	Alaskan malamute	M	1	Normal gland	5.2	3	2	100	1
8	Beagle	Ž	0.5	Normal gland	4.9	2	1	99.9	0

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No.	Breed	Sex	Age (in years)	Histopathological diagnosis	Ki-67 (%)	E-cadh membrane intensity (0-3)	E-cadh cytoplasm intensity (0-3)	E-cadh %	MMP-9 degree of staining* (0-3)
9	Mongrel	Ž	4	Normal gland	8.1	3	2	100	0
10	Mongrel	M	3	Normal gland	1.8	2	3	100	0
11	Pyrenean Mountain Dog	M	12	Adenoma	1.6	3	2	100	0
12	German shepherd dog	Ž	6	Adenoma	4.5	3	2	100	0
13	Poodle	M	14	Adenoma	7.5	3	2	100	0
14	Golden retriever	M	6	Adenoma	3.5	1	2	100	0
15	Mongrel	M	8	Adenoma	5.3	2	2	100	1
16	Maltese	M	1	Adenoma	3.9	3	2	100	0
17	Pekingese	Ž	4	Adenoma	1.0	1	1	100	0
18	Mongrel	Ž	10	Adenoma	4.7	2	1	100	0
19	Golden retriever	M	7	Adenoma	2.5	3	2	100	0
20	Golden retriever	M	1	Adenoma	2.8	3	2	100	0
21	Mongrel	M	8	Adenoma	4.7	3	2	100	1
22	Mongrel	M	13	Adenoma	8.2	3	1	100	0
23	Dalmatian	M	10	Adenoma	4.6	3	2	100	0

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No.	Breed	Sex	Age (in years)	Histopathological diagnosis	Ki-67 (%)	E-cadh membrane intensity (0-3)	E-cadh cytoplasm intensity (0-3)	E-cadh %	MMP-9 degree of staining* (0-3)
24	Tibetan terrier	M	10	Adenoma	12.0	3	2	100	1
25	Siberian husky	M	12	Adenoma	6.5	3	2	100	0
26	Samoyed	M	11	Epithelioma	12.1	1	2	85	1
27	Mongrel	M	14	Epithelioma	16.7	2	2	60	2
28	Siberian Husky	M	9	Epithelioma	17.0	2	3	100	1
29	Cocker Spaniel	M	5	Epithelioma	21.1	1	1	70	1
30	Mongrel	M	10	Epithelioma	9.5	2	2	90	0
31	Istrian hound	M	12	Epithelioma	6.1	2	1	90	0
32	Mongrel	M	8	Epithelioma	30.9	3	2	100	1
33	Poodle	M	14	Epithelioma	8.4	2	2	100	0
34	Mongrel	M	15	Epithelioma	9.9	2	2	100	0
35	Mongrel	M	9	Epithelioma	20.0	2	1	100	0
36	Cocker Spaniel	M	13	Epithelioma	7.1	1	1	100	0
37	Alaskan Malamute	M	10	Well-differentiated carcinoma	9.2	2	1	100	1
38	Yorkshire Terrier	M	13	Well-differentiated carcinoma	19.1	2	1	100	0

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No.	Breed	Sex	Age (in years)	Histopathological diagnosis	Ki-67 (%)	E-cadh membrane intensity (0-3)	E-cadh cytoplasm intensity (0-3)	E-cadh %	MMP-9 degree of staining* (0-3)
39	Cocker Spaniel	M	12	Well-differentiated carcinoma	23.7	1	1	40	1
40	Greyhound	M	10	Well-differentiated carcinoma	6.7	2	1	100	0
41	Labrador Retriever	M	15	Well-differentiated carcinoma	7.9	2	1	100	1
42	Miniature Poodle	M	7	Well-differentiated carcinoma	23.4	3	2	100	1
43	Labrador Retriever	M	12	Well-differentiated carcinoma	10.4	2	2	100	1
44	Newfoundland dog	M	9	Well-differentiated carcinoma	10.3	3	2	100	0
45	Akita	M	11	Well-differentiated carcinoma	2.6	3	2	100	1
46	Mongrel	M	11	Well-differentiated carcinoma	14.7	2	1	90	1
47	Newfoundland dog	M	10	Well-differentiated carcinoma	15.0	2	2	100	0
48	Mongrel	M	19	Well-differentiated carcinoma	58.4	2	2	80	0
49	Unknown	M	11	Well-differentiated carcinoma	23.4	2	1	90	0
50	Bichon Havanese	M	9	Well-differentiated carcinoma	9.3	2	1	100	0
51	Unknown	M	-	Well-differentiated carcinoma	15.9	3	1	100	2
52	Poodle	M	14	Well-differentiated carcinoma	17.2	3	2	100	0
53	Alaskan malamute	M	10	Well-differentiated carcinoma	12.7	1	2	100	0

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No.	Breed	Sex	Age (in years)	Histopathological diagnosis	Ki-67 (%)	E-cadh membrane intensity (0-3)	E-cadh cytoplasm intensity (0-3)	E-cadh %	MMP-9 degree of staining* (0-3)
54	Yorkshire Terrier	M	11	Well-differentiated carcinoma	16.8	2	2	100	0
55	Poodle	M	15	Well-differentiated carcinoma	16.1	2	2	90	1
56	Airedale terrier	M	8	Well-differentiated carcinoma	17.3	3	2	100	0
57	Siberian Husky	M	8	Well-differentiated carcinoma	15.2	3	1	100	0
58	Mongrel	M	10	Poorly-differentiated carcinoma	9.4	1	2	80	1
59	Poodle	F	14	Poorly-differentiated carcinoma	19.5	1	1	60	0
60	Brittany Spaniel	F	16	Poorly-differentiated carcinoma	29.4	1	2	100	0
61	Pekingese	M	11	Poorly-differentiated carcinoma	22.6	2	2	90	1
62	Scottish terrier	M	15	Poorly-differentiated carcinoma	19.4	1	1	90	0
63	Pekingese	M	9	Poorly-differentiated carcinoma	24.8	1	1	100	1
64	Mongrel	M	11	Poorly-differentiated carcinoma	20.2	3	2	100	1

* The degree of staining was expressed as previously described by DE VICENTE et al., 2005

Table 4. E cadherin – staining intensity and percentage of immunopositive cells in different histological subtypes of circumanal gland tumors and normal circumanal glands (NCG – normal circumanal gland, CGA – circumanal gland adenoma, CGE – circumanal gland epithelioma, WDCGC – well-differentiatedcircumanal gland carcinoma, PDCGC – poorly differentiated circumanal gland carcinoma)

	Membranous staining intensity		Cytoplasmic staining intensity		Percentage of immunopositive cells (mean)
	Median	Mode	Median	Mode	
NCG	2	3	2	2	99.96
CGA	2	3	1.5	2	100
CGE	2	2	2	2	90.45
WDCGC	2	2	1.5	2	94.76
PDCGC	2	1	1.5	2	88.57

These models of grouping are shown in Tables 5 and 6. From these tables, it is evident that there were no significant differences in the values for E-cadherin immunopositivity when epitheliomas were added to normal CG and adenomas (P=0.0797), but there was a significant difference when epitheliomas are added to well and poorly differentiated circumanal gland carcinomas (P=0.0007). The value of Goodman and Kruskal's Gamma statistics was 0.619, and Spearman's rank-order correlation was 0.377 (P=0.002) when associating the E-cadherin values (either equal or below 90%, or higher than 90%) with the ordinally arranged values of histopathological diagnosis.

Table 5. First model of grouping of E-cadherin scores to make the data amenable for Hi-square test (epitheliomas are added to normal circumanal glands and CG adenomas)

	E-cadherin percentage of immunopositive cells		TOTAL
	≤ 90%	> 90%	
Normal CG, adenomas and epitheliomas	5	31	36
Well and poorly differentiated carcinomas	9	19	28
TOTAL	14	50	64
P value for significant difference between the proposed groups	0.079708		

Table 6. Second model of grouping of E-cadherin scores to make the data amenable for Hi-square test (epitheliomas are added to well and poorly differentiated CG carcinomas)

	E-cadherin percentage of immunopositive cells		TOTAL
	≤ 90%	> 90%	
Normal CG, adenomas and epitheliomas	0	25	25
Well and poorly differentiated carcinomas	14	25	39
TOTAL	14	50	64
P value for significant difference between the proposed groups	0.000701		

N-Cadherin. Simply put, the reactions to this marker were negative. To be more precise, in all the studied groups the percent of immunopositive cells was below 1%. The exact values were as follows: normal CG and CG adenomas – 0.44%, CG epitheliomas 0.84%, well-differentiated CG carcinomas 0.43%, poorly differentiated CG carcinomas 0.24%. The HSD Tukey test did not yield significant differences between these values. Figure 6 shows the only tumor (one CG epithelioma) with a percentage of immunopositive cells above 1% (the exact value was 5.4%).

Matrix metalloproteinase-9. A positive reaction to this marker was evident as the weak staining of the cytoplasms of normal or tumorous cells of circumanal glands. This positive reaction was evident in one of ten samples of normal CG (10%), three of 15 adenomas (20%), five of 11 epitheliomas (45.5%), six of 21 well-differentiated CG carcinomas (28.6%) and four of seven (57.1%) poorly differentiated CG carcinomas. In all the positive cases mentioned, the positivity score was

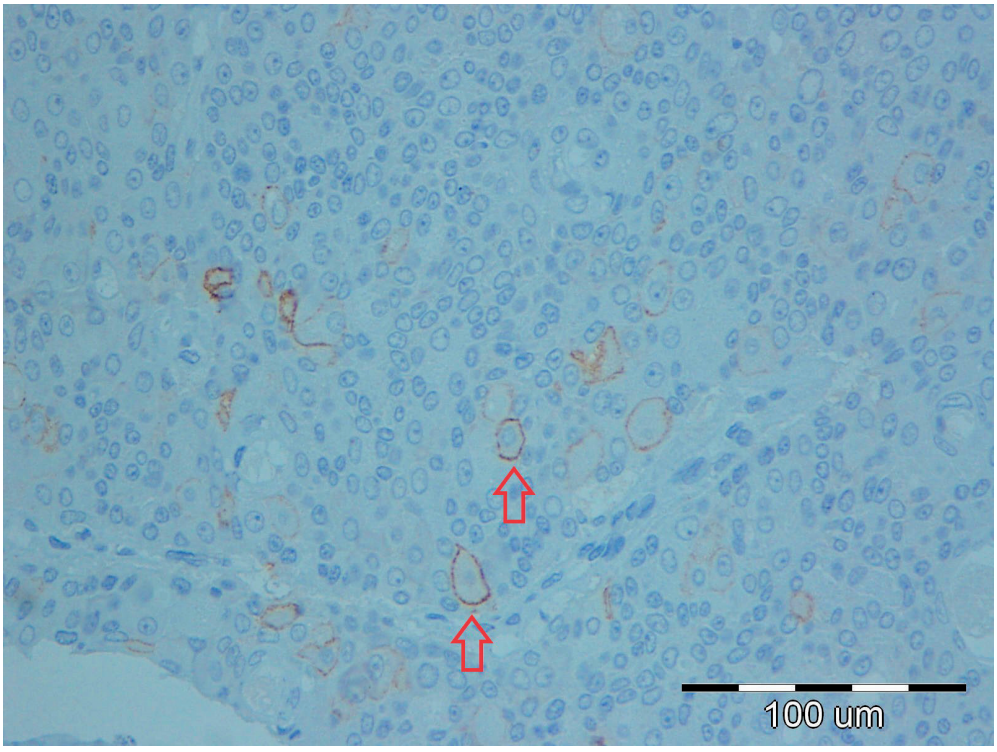

Fig. 6. N-cadherin IHC stain, circumanal gland epithelioma, magnification 400X. The vast majority of cells show no reaction at all. A few cells (two of these are indicated with red arrows) show membranous positivity that ranges from weak to moderate. Most of the marked cells have a morphology consistent with polyhedral (hepatoid) cells.

within category 1 (up to only 10% of positive cells), with the exception of one case of epithelioma and one well-differentiated CG carcinoma (Figure 7), where it reached category 2 (10-50% of cells positive). As previously for E-cadherin, the same two models of groupings were created (Tables 7 and 8). From these tables, it is evident that there were no significant differences in the values for MMP-9 positivity when epitheliomas are added to normal CG and adenomas (P=0.0734), but there was a significant difference when epitheliomas are added to well- and poorly-differentiated circumanal gland carcinomas (P=0.0132). When associating the values of MMP-9 (either none or some - any value from 1 to 3) with the ordinally arranged values of histopathological diagnosis, the value of Goodman and Kruskal's Gamma statistics was 0.457, and of Spearman's rank-order correlation 0.306 (P=0.014).

Table 7. First model of grouping of MMP-9 scores to make the data amenable for Hi-square test (epitheliomas are added to normal circumanal glands and CG adenomas)

	MMP-9 positivityscore		TOTAL
	0	1-3	
Normal CG, adenomas and epitheliomas	27	9	36
Well and poorly differentiated carcinomas	15	13	28
TOTAL	42	22	64
P value for significant difference between the proposed groups	0.0734		

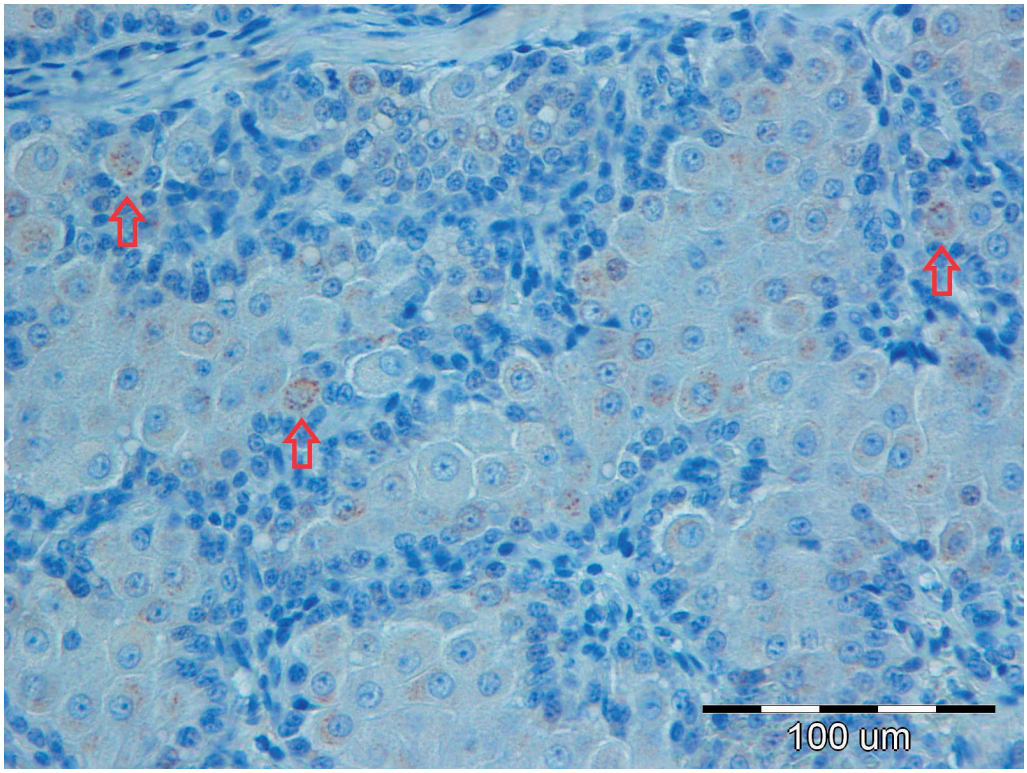

Fig. 7. MMP-9 IHC stain, well-differentiatedcircumanal gland carcinoma, magnification 400X. Individual polyhedral (hepatoid) cells with positive "granular" signals within the cytoplasm (red arrows).

Table 8. Second model of grouping of MMP-9 scores to make the data amenable for Hi-square test (epitheliomas are added to well and poorly differentiated CG carcinomas)

	MMP-9 positivityscore		TOTAL
	0	1-3	
Normal CG and adenomas	21	4	25
Epitheliomas, Well and poorly differentiated carcinomas	21	18	39
TOTAL	42	22	64
P value for significant difference between the proposed groups	0.0132		

Associations within individual IHC markers. To simplify the results, E-cadherin values were classified as previously mentioned, into two groups. Namely, these were groups of tissues with either very high positivity (>90% of immunopositive cells) or high positivity (≤ 90% of immunopositive cells). Similarly, Ki-67 values were classified as either "high" (≥ 12%), or "low" values (< 12%). MMP-9 results were viewed as either positive (any score from 1 to 3, according to DE VICENTE et al., 2005), or negative (score 0 in the previously mentioned scale). Results expressed in this way were amenable for 2x2 frequency tables and calculation of Pearson's chi-square test, to detect significant differences in distribution. The 2x2 frequency tables between Ki-67, E-cadherin, and MMP-9 are given in Tables 9-11. All the frequency tables showed significant differences between the investigated markers ($P < 0.05$).

Table 9. 2x2 frequency table for Ki-67 and E cadherin

High (≤ 90%)		E-cadherin percentage of positive cells		TOTAL
		Very high (> 90%)		
Ki-67	high (≥ 12%)	11	16	27
	low (< 12%)	3	34	37
TOTAL		14	50	64
P value for significant difference between the groups		0.001816		

Table 10. 2x2 frequency table for Ki-67 and MMP-9

0		MMP-9 positivityscore		TOTAL
		1-3		
Ki-67	high (≥ 12%)	13	14	27
	low (< 12%)	29	8	37
TOTAL		42	22	64
P value for significant difference between the groups		0.011915		

Table 11. 2x2 frequency table for E-cadherin and MMP-9

0		MMP-9 positivityscore		TOTAL
		1-3		
E-cadherin percentage of positive cells	High (≤ 90%)	6	8	14
	Very high (> 90%)	36	14	50
TOTAL		42	22	64
P value for significant difference between the groups		0.042433		

Discussion

The goal of this study was to evaluate the expression of several immunohistochemical markers that could point to EMT (E-, N-cadherin, MMP-9), and one marker of cellular proliferation (Ki-67) in a retrospective case series of normal and tumorous circumanal glands.

As our results show, within the investigated groups (normal CG, CG adenoma, CG epitheliomas, and poorly and well-differentiated CG carcinomas) there were subtle differences in the expression of E-cadherin and MMP-9, and no or minimal expression of N-cadherin. On the other hand, the findings of the Ki-67 varied considerably between groups, and demonstrated significant differences between benign diagnoses (normal circumanal glands and adenomas) and semi-malignant or malignant diagnoses (CG epitheliomas, well and poorly differentiated carcinomas).

This is the first study to use E-cadherin, N-cadherin, and MMP-9 markers systematically on a considerable number of samples that originated from tumorous or normal circumanal glands. To our knowledge, the only such reported case of using any of these markers was the usage of E-cadherin in one recent case report of metastatic well-differentiated circumanal gland carcinoma (JARDIM et al., 2018). The central question of this study was whether EMT occurs within these glands at any point in tumor development. Our results should be evaluated and commented on with care. The general positive marking of E-cadherin, at the same time as the quite consistent negative results of N-cadherin and MMP-9, prompt us to conclude that EMT probably does not occurs within any of the groups of normal or tumorous CG. This seemingly irrefutable statement, however, is not entirely true. For example, even though there were no significant differences in E-cadherin expression between different histological diagnoses, there was an observable drop in its level of expression within semi-malignant or malignant diagnoses (epitheliomas, well- and poorly-differentiated CG carcinomas) when compared with benign ones (normal CG, and adenomas). Moreover, this was also statistically significant when the results of E-cadherin expression are modeled, as in Table 5, and both the values of Goodman and Kruskal's Gamma statistics and Spearman's rank-order correlation showed considerable correlation (0.619 and 0.377, respectively) between diagnosis and either very high (above 90%) or "lower" (90% or lower) values of E-cadherin. Visually, this can be also appreciated when comparing Figures 4 and 5. In the same fashion, although generally minimal to low, the expression of MMP-9 was significantly higher in semi-malignant or malignant diagnoses when compared to benign ones (p=0.0132, see Table 7). There was also a somewhat smaller (when compared to E-cadherin) but still detectable correlation between diagnosis and MMP-9 (0.457 for Goodman and Kruskal's Gamma statistics, and 0.306 for Spearman's rank-order correlation). These two facts – the subtle lowering of E-cadherin coupled with a minimal but significant increase in the level of MMP-9 within the semi-malignant and malignant diagnoses, could be a sign that EMT is actually occurring, but at a very low rate, in rare cells, or as an uncommon event. This is consistent with, and could potentially explain the fact that CG carcinomas metastasize infrequently, and only poorly differentiated carcinomas are incriminated as having more metastases (LEE GROSS, 2005).

Nevertheless, cases of well-differentiated circumanal (hepatoid) gland carcinomas with distant metastases have been described (MCCOURT et al., 2018). An additional argument that EMT can occur in circumanal carcinomas is evidenced in a report by JARDIM et al., 2018. This was a case report dealing with a large intrapelvic well-differentiated hepatoid gland carcinoma that had a loss of E-cadherin within undifferentiated tumor cells and clusters of cells that invaded lymphatic vessels (JARDIM et al., 2018). These same cells were at the same time negative for high molecular weight cytokeratin, and positive for vimentin, leading the author to interpret these changes as suggestive of EMT. Other studies also reported vimentin staining within CG tumors, and at least indirectly support the idea that CG carcinomas undergo EMT (VOS et al., 1992; VOS et al., 1993; ŠOŠTARIĆ-ZUCKERMANN et al., 2016; PIEPER et al., 2015).

The clearest finding of this study is the fact that the level of Ki-67 expression varies considerably, and increases as the malignancy of these tumors increases. This is nothing new, and was previously described in two studies (PEREIRA et al., 2013, and BRODZKI et al., 2014). The differences in the values obtained in the level of expression of Ki-67 between these two studies and our study can be attributed to the subjectivity of the human eye and the variability of these values within the investigated samples. In a study by PEREIRA et al., 2013, the computer-assisted measured values of Ki-67 were several folds smaller than the values obtained by manual counting, suggesting the inherent overzealous counting of positively reacting nuclei by the human eye. In our case, this increase in the level of Ki-67 expression toward malignant diagnoses was particularly evident between adenoma and epitheliomas, which could at least in part be because epitheliomas contain more basaloid (reserve) cells, which are even in normal glands the cells that replenish hepatoid cells.

Analysis of the association between Ki-67, E-cadherin and MMP-9 generally shows: (1) with the decrease in Ki-67 values there tends to be a decrease in MMP-9 scores; (2) with the lowering of Ki-67 values the expression of E-cadherin reaches its maximum; and (3) with lower MMP-9 score E-cadherin again reaches its maximum (tables 8-10.). Although the 2x2 tables presented rely on the custom cut-off values of Ki-67 and E-cadherin, these findings are suggestive of a trend showing with the increase in Ki-67 there is also an increase in MMP-9, yet a decrease in E-cadherin. This is in turn suggestive of a scenario in which CG tumors that are more proliferative also start to lose their association with other cells (lowered expression of E-cadherin) and initiate production of MMP-9, which would ultimately facilitate early metastatic steps.

There are clear weaknesses and limitations of the study which have to be addressed. First of all, any histopathological scoring system without the assistance of a computer is clearly biased (as evidenced in the paper by PEREIRA et al., 2013), and there is no universal manual scoring system which is easily comparable to any other scoring system, especially if it is applied for some other marker. We tried to at least partially overcome this issue by having two different pathologists estimate the percentage of positive cells (for all the markers) and treating the mean values as final. The scoring was also conducted in a blinded and random fashion making it minimally biased. Finally, even though computer-assisted measurement of IHC staining is more objective, so far most of the results to which we could compare our results were done in a manual (computer unassisted) manner. Another clear drawback of this study is that it is a retrospective study and that there are no data regarding the clinical course of the disease after tumor extraction. We were aware of that drawback from the beginning of the study, but felt that the retrospective case series at our disposal were a good starting point to study these tumors. Moreover, we wanted to complete and complement the same case series which had already been used in our initial (and already published) study (ŠOŠTARIĆ-ZUCKERMANN et al., 2016). At first, it may be argued that the number of samples in the study is generally low. However, the number of samples used in this study is comparable to other similar studies (PEREIRA et al., 2013., BRODZKI et al., 2014).

In conclusion, semi-malignant and malignant CG tumors show marked and significant differences to benign counterparts or normal circumanal glands in the case of the Ki-67 proliferative marker, and subtle but detectable differences in some markers of EMT used (E-cadherin, MMP-9). For others (N-cadherin) the results were very similar i.e. negative. Such findings leave the possibility that EMT does indeed occur within the malignant (or even semi-malignant) forms of CG tumors, but probably as a rare scenario and likely very late within the clinical course of tumor development. Further prospective and more detailed studies are needed to prove or disprove these statements.

Acknowledgements
This study was supported by a project of the Ministry of Science and Technology, Republic of Croatia: Comparative diagnostic, morphometry, and analysis of human and animal tumors (053–05322642260, principal investigator: prof. dr. sc. Željko Grabarević).

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Received: 8 October 2020
Accepted: 25 November 2020

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SAŽETAK

Tumori cirkumanalnih žlijezdi su česte neoplazije u starijih pasa. Od ovih, karcinomi cirkumanalnih žlijezdi pokazuju metastatski potencijal koji nije previsok s obzirom da metastaze nisu česte. Epitelno-mezenhimska tranzicija (EMT) je proces koji omogućava početne korake u procesu metastaziranja, a budući da do sada nije bio sistematično istraživan u ovih tumora, znanje o prisutnosti ovog procesa zacijelo bi pomoglo u razumijevanju njihove biologije. Kako bi testirali prisutnost EMT-a, koristili smo tri različita imunohistokemijska biljega čija se ekspresija mijenja u sklopu ovog procesa – to su E-kadherin, N-kadherin i matriksalna metaloproteinsaza-9 (MMP-9). Osim toga, koristili smo i Ki-67 biljeg proliferacije. Uzorci korišteni u radu sastojali su se od 15 adenoma, 11 epitelioma, 21 dobro diferenciranog karcinoma, 7 slabo diferenciranih karcinoma te 10 uzoraka normalnih cirkumanalnih žlijezdi. Markiranje N-kadherinom je bilo negativno u svih uzoraka. E-kadherin je bio visoko eksprimiran u svih istraživanih grupa, sa nešto slabijom ekspresijom u semimalignih i malignih tumora. Markiranje sa MMP-9 je općenito bilo vrlo nisko, no značajno više u semimalignih i malignih tumora kad se usporede sa benignim tumorima ili normalnim cirkumanalnim žlijezdama. Ki-67 proliferacijski indeks je bio znakovito viši za semimaligne i maligne tumore cirkumanalnih žlijezdi kada se usporede sa benignim tumorima cirkumanalnih žlijezdi ili normalnim cirkumanalnim žlijezdama. Rezultati ukazuju da sa porastom histološke malignosti ovih tumora dolazi do blagog pada ekspresije E-kadherina, blagog porasta MMP-9 markera te znakovitog porasta u markiranju sa Ki-67. Navedeno upućuju na mogućnost da unutar malignih ili čak semimalignih tumora ovih žlijezdi dolazi do procesa EMT-a. Ipak, ovaj proces je vjerojatno relativno rijedak te nastupa kasno u fazi razvoja tumora. Stoga su potrebna su daljnja istraživanja kako bi se pretpostavke prihvatile ili odbacile.

Ključne riječi: tumori cirkumanalnih žlijezdi pasa; epitelno – mezenhimska tranzicija; E-kadherin; matriksalna metaloproteinaza-9; Ki-67; imunohistokemija