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Original Communication

Phenotypical, cell-adhesion, hormonal receptors and cell-cycle-associated markers: an integrated immunohistochemical study on feline mammary lesions

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ABSTRACT

The need for reliable prognostic markers and promising therapeutic approaches to treat feline mammary carcinomas, has led to an intensive research aiming to accomplish a molecular characterization of feline mammary tumours, that reflects their biological behaviour, similar to what has been previously performed in human breast cancer. The aim of this study is to characterize, by immunohistochemistry, the molecular markers of normal mammary gland, hyperplastic/dysplastic mammary lesions, and benign and malignant feline mammary tumours, applying phenotypical molecular markers (AE1/AE3, vimentin, p63), hormonal receptor status (estrogen receptor (ER), progesterone receptor (PR)) and markers of proliferative activity (Ki-67 index) as well as the classical cadherins P and E's expression. In feline mammary carcinomas there was an overexpression of vimentin and p63, a higher proliferation index and a reduction in estrogen receptor expression, when compared with benign tumours. Carcinomas that were simultaneously P-cadherin-positive, vimentin-positive and estrogen-negative were associated with a higher histological grade. Moreover, P-cadherin and vimentin-positive tumours were also associated with the presence of neoplastic emboli, presenting a threefold likelihood of intravascular dissemination when compared with tumors in which the expression of these markers was absent. P-cadherin, vimentin and ER expression seem to be relevant molecular markers in feline mammary carcinomas associated with a more aggressive behaviour.

KEYWORDS: feline, mammary tumours, hormone receptors, Ki-67, vimentin, p63, cadherins

INTRODUCTION

Mammary tumours are the third most common neoplasm in queens, with more than 90% classified

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as malignant. The prognosis is guarded for most cases, with a mean post-diagnosis survival time of 10-12 months, and death or euthanasia is mainly attributable to local recurrence or metastasis [1, 2]. Several features have been studied as prognostic indicators in feline mammary tumours, including clinical features such as tumour size, lymph node status, and metastases; pathological features, namely histological grade; and molecular markers, such as cell-cycle-associated molecules, estrogen and progesterone receptors (ER and PR), and cell adhesion molecules, among others [3-8]. Currently, lymph node involvement, lymphovascular invasion, and tumour histological grade are the most widely accepted prognostic parameters [9]. Constitutional and functional changes of cell-cell adhesion molecules such as E- and P-cadherins, have been implicated in the progression of human breast cancer and related to a more aggressive carcinomal behaviour and poor prognosis [10-14], and changes in the expression of E- and P-cadherins are associated with feline mammary carcinogenesis [8, 15-18].

The search for prognostic and predictive factors has led to the development of studies directed towards molecular and immunophenotypic markers, in order to classify feline mammary tumours more accurately, and provide additional information in terms of diagnosis, prognosis and therapeutic targets [19-21], similar to what has previously been performed in human breast cancer [22, 23]. The identification of those molecular markers in carcinogenesis requires the knowledge of the pathways involved in the process, not only in laboratory models but also in spontaneous cases, for the benefit of both animals and humans affected by cancers with similar development and clinical behaviour. Feline mammary tumours have been suggested as suitable spontaneous animal models for some sub-types of human breast cancer [19]; thus our aim was to evaluate, by immunohistochemistry, samples of normal mammary glands, hyperplastic/dysplastic, benign and malignant feline mammary tumours, using phenotypical markers (AE1/AE3, vimentin, p63), hormone receptors (ER, PR) and cell-cycle-associated molecules (Ki-67), as well as cell adhesion molecules (namely P- and E-cadherins), in order to contribute to the molecular characterization of feline mammary tumours, enhancing their value as models of human breast cancer.

MATERIALS AND METHODS

Tissue samples

Samples from 75 queens with naturally occurring mammary lesions surgically excised with curative intent and nine normal mammary glands (obtained from queens that were humanely euthanized for reasons unrelated to neoplastic disease) are included in this study. In each case, an informed consent was granted by the owners. All specimens were fixed in 10% neutral buffered formalin and routinely processed. Consecutive histological sections (2 μm) were cut from each paraffin block. One was stained with haematoxylin and eosin (HE) for histological examination, and the others were used for immunohistochemistry (IHC). When available, local and regional lymph nodes were also processed and examined for the presence of metastases, as described in Figueira *et al*., 2014 [18].

The histological classification of the tumours was independently performed by three observers (ACF, PDP and FG), based on the criteria of the World Health Organization (WHO), for the histological classification of the mammary tumours of domestic animals [24].

Carcinomas were graded in accordance with the Nottingham grading system for human breast carcinomas, based on the assessment of three morphological features: tubule formation, nuclear pleomorphism, and mitotic counts, and classified as grade I (well-differentiated), grade II (moderately differentiated) and grade III (poorly differentiated) [25]. Variables with known prognostic value, such as mode of growth (infiltrative or expansive), tumour diameter (<2 cm, 2-3 cm, >3 cm), presence of necrosis, skin ulceration, lymph node metastases, and intravascular neoplastic emboli [2, 26] were also recorded.

Immunoexpression

Immunohistochemistry (IHC) was performed using a polymer-based system (Novolink Max Polymer Detection System, Product No: RE7280-K, Leica Biosystems, Newcastle, UK), according to the manufacturer's instructions. Sections were

dewaxed in xylene, rehydrated through graded alcohols and treated with 10 mM citrate buffer, pH 6.0, for 3 minutes in a pressure cooker. Endogenous peroxidase activity was blocked by treating the sections with 3% hydrogen peroxide in methanol for 10 minutes and rinsing in Tris-buffered saline (TBS, pH 7.6, 0.5 M). Sections were incubated overnight at 4 °C in a humid chamber, with specific mouse monoclonal antibodies against human pan-cytokeratin AE1/AE3 (clone AE1/AE3, Zymed/Invitrogen, Camarillo, CA, USA), vimentin (clone V9, Dako, Gostrup, Denmark), p63 (clone 4A4, Thermo Scientific, Fremont, USA), ER (clone 6F11, Novocastra, Newcastle Upon Tyne, United Kingdom), PR (clone 1A6, Novocastra, Newcastle Upon Tyne, United Kingdom), and Ki-67 (clone MIB-1, DakoCytomation, Denmark). The antibodies were diluted to ratios of 1:300, 1:500, 1:200, 1:40, 1:40 and 1:50, respectively, in TBS with 5% bovine serum albumin (BSA). Immunolabelling was detected with 3,3'-diaminobenzidine tetrahydrochloride (DAB) incubated at room temperature and sections were then counterstained with Mayer's haematoxylin, dehydrated and mounted. For negative controls, the primary antibody was replaced with TBS. Sections of feline normal mammary glands were used as positive controls.

Evaluation of immunolabelling

All samples were evaluated regarding the expression of pan-cytokeratin AE1/AE3 (epithelial cells), vimentin (mesenchymal cells), and p63 (myoepithelial cells) for phenotypical characterization. Positivity was indicated by the presence of distinct, dark brown nuclear (p63), cytoplasmic (vimentin) and/or membranous (AE1/AE3) staining.

The AE1/AE3 immunoexpression was considered reduced when less than 75% of the epithelial cells were stained. Vimentin and p63 were considered overexpressed when the epithelial cells showed cytoplasmic and nuclear staining, respectively. For data analysis, tumours with less than 10% stained cells were considered negative and those with ≥ 10% stained cells were considered positive (adapted from [27-29]).

The assessment of ER and PR was also based on a semi-quantitative analysis, according to the percentage of stained nuclei and the intensity of staining. The percentage of tumour cells with nuclear staining (proportion score (PS)) was graded as 0: < 5%, 1: 5-19%, 2: 20-60%, and 3: > 60%. The staining intensity (intensity score (IS)) was estimated according to the average staining intensity of positive cells and scored as 0 = negative, 1 = light staining, 2 = moderate staining, 3 = strong staining. PS and IS were added to obtain the total hormone-receptor score (TS) (range 0-6). Tumours were considered hormone-receptor-positive when PS was ≥ 1 and TS ≥ 2 [30].

The proliferation activity was determined by assessing the Ki-67 index [31] determined by counting 1000 neoplastic cells, in 10 representative fields, at high magnification (40x objective) and expressing the percentage of positive cells (nuclear staining, regardless of the intensity) [3, 32]. For statistical analysis, tumours were grouped into four quartiles based on the percentage of cells labelled for Ki-67 (<47.46; 47.46-61.1; 61.2-74.8; >74.8), following the approach of Castagnaro *et al*., 1998 [3].

The staining and evaluation method for the expression of P- and E-cadherins was performed as previously described in [18].

Statistical methods

Data was organized into contingency tables and the likelihood ratio chi-square test was used to determine the significance of the relationship between the expression of the catenins and the tumours' clinicopathological parameters as well as the cadherins' expression. Whenever biologically consistent, 2 × 2 tables of contingency were built and Fisher's exact test was performed. All statistical analysis was performed using SAS/STAT, 1989 (SAS Institute Inc., Cary, NC, USA) [33] and, in all instances, $p < 0.05$ was considered to be statistically significant.

RESULTS

Nine normal mammary gland samples, 13 hyperplastic/dysplastic lesions (seven fibrocystic disease cases and six fibroadenomatous changes), 10 benign tumours (seven simple adenomas and three fibroadenomas) and 60 malignant tumours (32 tubulopapillary carcinomas, 16 solid carcinomas,

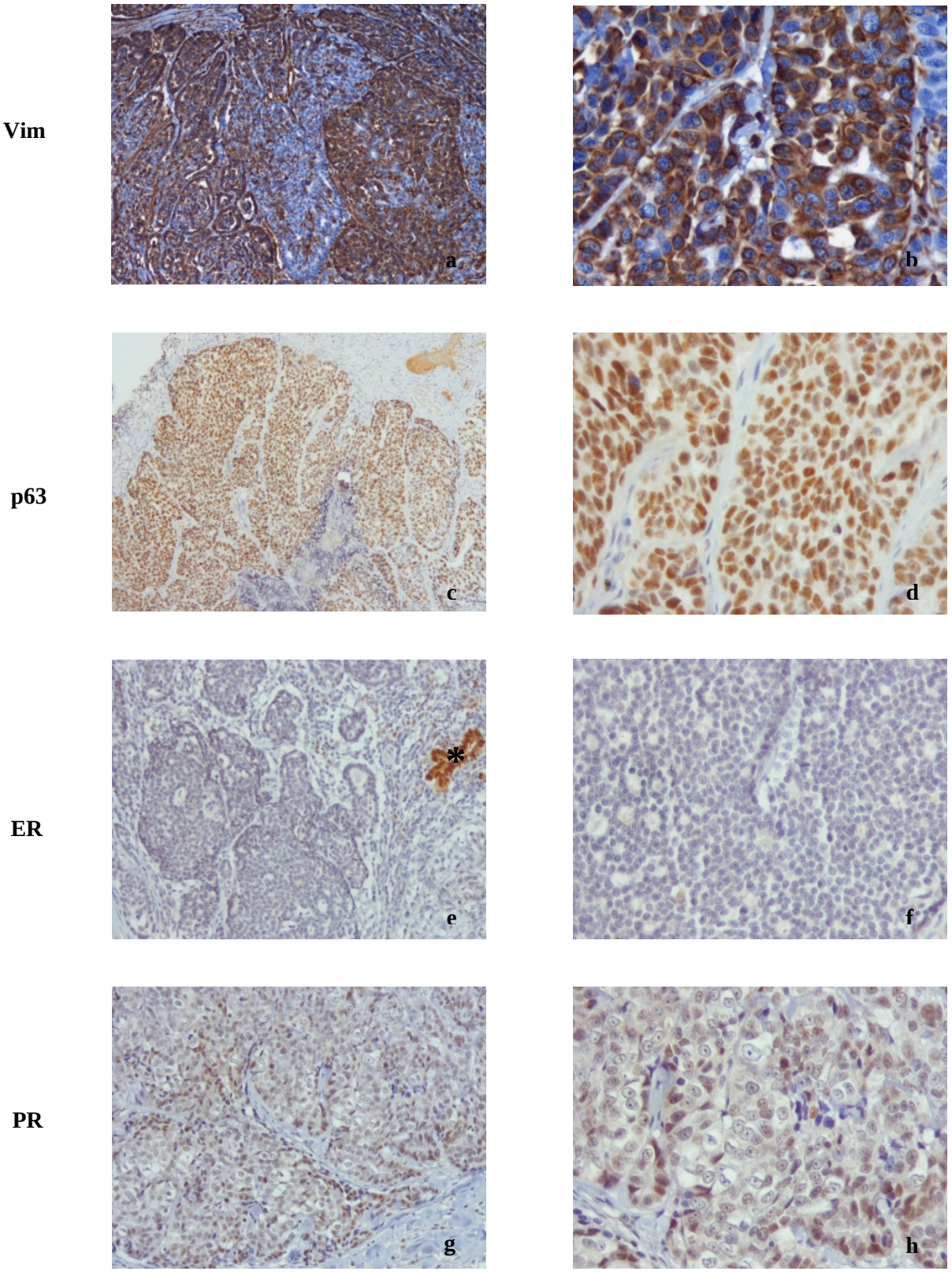

Figure 1

Figure 1 continued..

Ki-67

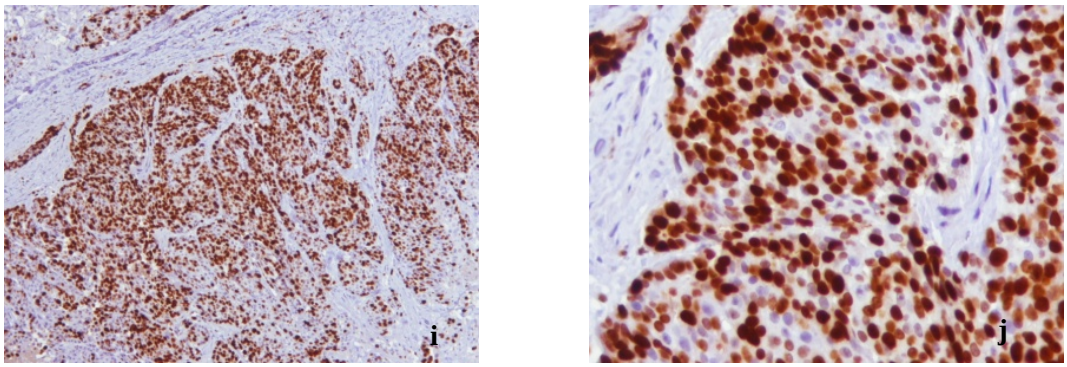

Figure 1. Vimentin, p63, estrogen receptor, progesterone receptor and Ki-67 expression in feline mammary carcinomas. (a) Vimentin expression in more than 50% of the epithelial neoplastic cells of a grade III tubulopapillary carcinoma (IHC, x100); (b) Detailed view of the increased vimentin expression (IHC, x400); (c) p63 expression in the epithelial cells of a grade III mucinous carcinoma (IHC, x100); (d) Detailed view of the p63 overexpression (IHC, x400); (e) Estrogen receptor-negative grade II cribriform carcinoma (*positive internal control) (IHC, x200); (f) Detailed view of the negative estrogen receptor carcinoma (IHC, x400); (g) Progesterone receptor reduction in a grade III solid carcinoma (IHC, x200); (h) Detailed view of the PR expression (IHC, x400); (i) High expression of Ki-67 in a grade III solid carcinoma (IHC, x100); (j) Detailed view of the Ki-67 expression (IHC, x400).

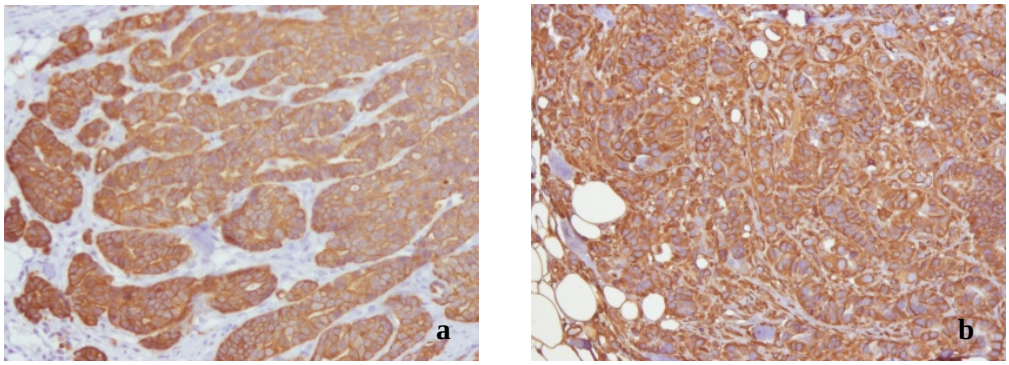

Figure 2. Pan-cytokeratin AE1/AE3 (a) and vimentin (b) co-expression in a grade III tubulopapillary carcinoma (IHC, x200).

four cribriform carcinomas, six mucinous carcinomas and two carcinosarcomas) were analysed. Seven (11.67%) malignant tumours were grade I; 25 (41.67%), grade II and 28 (46.67%), grade III. Neoplastic intravascular emboli were observed in 21 (36.21%) carcinomas, while lymph node metastases were identified in 18/35 (51.43%) cases with available lymph nodes. The expression of P- and E-cadherins in this series was previously described in [18].

AE1/AE3

In normal mammary tissues, pan-cytokeratin AE1/AE3 was expressed in the membrane and cytoplasm of luminal epithelial cells, with a lighter staining in myoepithelial cells. A similar pattern was observed in hyperplastic/dysplastic lesions, benign and malignant tumours, with the exception of a reduced expression in five (8.33%) carcinomas. Both intravascular emboli and lymph node metastases were positive for

AE1/AE3, with only one lymph node metastasis revealing less than 75% of cytokeratin-positive metastatic cells.

Vimentin

In normal mammary tissues, the expression of vimentin was observed in myoepithelial and mesenchymal cells as well as in the luminal compartment of the ducts. Similar patterns were observed in hyperplastic/dysplastic lesions and benign tumours, with the exception of the fibroadenomatous change cases where we observed a cytoplasmic expression in more than 10% of the ductal and acinar epithelial cells. In the two carcinosarcomas, the sarcomatous component was vimentin-positive and cytokeratin-negative.

An overexpression of vimentin was observed in the cytoplasm of neoplastic epithelial cells in 35 (58.33%) carcinomas (Figure 1a and 1b), a significant difference when compared with benign tumours ($p < 0.0001$) (Figure 2). Moreover, a significant association was found between the overexpression of vimentin and the histological grade of carcinomas ($p = 0.0318$), with no immunoreactivity observed in most low-grade carcinomas ($n = 6$; 85.71%) and positivity in nearly two-thirds of moderate and high-grade carcinomas ($n = 34$; 64.15%) (Table 1). There was a significant direct association between the overexpression of vimentin and P-cadherin in the epithelial cells of malignant tumours ($p < 0.0001$) (Table 2), and those that expressed both proteins were significantly associated with the presence of intravascular neoplastic emboli ($p = 0.0307$). Although there was no direct association between the expression of vimentin and E-cadherin, the combined expression of P- and E-cadherins was statistically related to the expression of vimentin, with P-cad$^+$ and E-cad$^-$ being the most predominant pattern ($n = 20$; 57.14%) amongst the vimentin-positive tumours. Vimentin expression in primary tumours was not always consistent with the expression observed in the intravascular neoplastic emboli and in corresponding lymph node metastases (Table 3).

p63

In normal mammary tissues, hyperplastic/ dysplastic lesions and benign tumours, the expression of p63 was restricted to the nuclei of myoepithelial cells, while the luminal epithelium was consistently negative.

In malignant carcinomas, p63-positive myoepithelial cells were found to be surrounding some areas of epithelial tumour cells. p63 immunostaining was also found in the squamous metaplastic (basal and parabasal) carcinoma cells. In three malignant tumours, p63 immunoexpression revealed the existence of a myoepithelial cell phenotype not easily identified by routine HE evaluation.

p63 expression was also observed, albeit at a lesser intensity than that in myoepithelial cells, in the nuclei of epithelial cells in 23 (38.33%) carcinomas (Figure 1c and 1d), which is significantly different when compared with benign tumours ($p = 0.0245$). There was a significant direct relationship between p63's aberrant expression and the histological grade of carcinomas ($p = 0.0072$), with all grade I tumours being p63-negative and more than half ($n = 15$; 53.57%) of the grade III carcinomas being p63-positive (Table 1). Furthermore, significant differences in p63 expression were observed between different histological types ($p = 0.0026$), with both carcinosarcomas, most mucinous carcinomas, and half of the solid carcinomas being p63-positive, while all cribriform and three-quarters of the tubulopapillary carcinomas were p63-negative (Table 1). The vast majority of P-cadherin-negative tumours were also p63-negative ($p = 0.0205$), but there was no significant association between the expression of p63 and E-cadherin. When the P- and E-cadherins' combined expression was considered, P-cad$^+$ and E-cad$^-$ was the most predominant pattern ($n = 13$; 56.52%) amongst the p63-positive tumours.

In the vast majority of cases, p63 was similarly expressed by primary tumours and their intravascular neoplastic emboli and lymph node metastases (Table 3).

ER

As expected, all normal mammary gland tissues expressed ER, in the nuclei of more than 60% of the epithelial cells, with moderate to strong intensity and a heterogeneous lobular distribution. A similar pattern was observed in hyperplastic/ dysplastic lesions and benign tumours, with the exceptions of one fibroadenomatous change and

Table 1. Association between vimentin, p63, PR, ER expression, Ki-67 index and clinicopathological parameters in feline malignant mammary tumours.

Clinicopathological parameters	***n***	**Vimentin**		**p63**		**ER***		**PR**		**Ki-67 index***			
		Positive	**Negative**	**Positive**	**Negative**	**Negative**	**Positive**	**Negative**	**Positive**	**<47.46%**	**47.46-61.1%**	**61.1-74.8%**	**>74.8%**
Histological type													
Tubulopapillary carcinoma	32	17 (53.13%)	15 (46.88%)	8 (25%)	24 (75%)	21 (65.63%)	11 (34.38%)	8 (25%)	24 (75%)	10 (31.25%)	7 (21.88%)	7 (21.88%)	8 (25%)
Solid carcinoma	16	9 (56.25%)	7 (43.75%)	8 (50%)	8 (50%)	12 (80%)	3 (20%)	4 (25%)	12 (75%)	3 (20%)	4 (26.67%)	5 (33.33%)	3 (20%)
Cribriform carcinoma	4	3 (75%)	1 (25%)	0	4 (100%)	3 (75%)	1 (25%)	0	4 (100%)	1 (25%)	2 (50%)	0	1 (25%)
Mucinous carcinoma	6	5 (83.33%)	1 (16.67%)	5 (83.33%)	1 (16.67%)	6 (100%)	0	0	6 (100%)	0	1 (16.67%)	3 (50%)	2 (33.33%)
Carcinosarcoma	2	1 (50%)	1 (50%)	2 (100%)	0	1 (50%)	1 (50%)	2 (100%)	0	0	1 (50%)	0	1 (50%)
p		NS		**0.0026**		NS		**0.0243**		NS			
Histological grade													
Grade I	7	1 (14.29%)	6 (85.71%)	0	7 (100%)	1 (14.29%)	6 (85.71%)	2 (28.57%)	5 (71.43%)	7 (100%)	0	0	0
Grade II	25	17 (68%)	8 (32%)	8 (32%)	17 (68%)	21 (84%)	4 (16%)	4 (16%)	21 (84%)	2 (8%)	9 (36%)	7 (28%)	7 (28%)
Grade III	28	17 (60.71%)	11 (39.29%)	15 (53.57%)	13 (46.43%)	21 (77.78%)	6 (22.22%)	8 (28.57%)	20 (71.43%)	5 (18.52%)	6 (22.22%)	8 (29.63%)	8 (29.63%)
p		**0.0318**		**0.0072**		**0.0018**		NS		**0.0003**			
Mode of growth													
Expansive	3	0	3 (100%)	0	3 (100%)	1 (33.33%)	2 (66.67%)	0	3 (100%)	1 (33.33%)	1 (33.33%)	0	1 (33.33%)
Infiltrative	56	34 (60.71%)	22 (39.29%)	23 (41.07%)	33 (58.93%)	42 (76.36%)	13 (23.64%)	13 (23.21%)	43 (76.79%)	12 (21.82%)	14 (25.45%)	15 (27.27%)	14 (25.45%)
p		NS		NS		NS		NS		NS			
Tumour's largest diameter													
<2 cm	33	17 (51.52%)	16 (48.48%)	11 (33.33%)	22 (66.67%)	21 (65.63%)	11 (34.38%)	7 (21.21%)	26 (78.79%)	6 (18.75%)	12 (37.5%)	8 (25%)	6 (18.75%)
2-3 cm	10	7 (70%)	3 (30%)	5 (50%)	5 (50%)	8 (80%)	2 (20%)	2 (20%)	8 (80%)	2 (20%)	2 (20%)	3 (30%)	3 (30%)

Table 1 continued..

Clinicopathological parameters	***n***	**Vimentin**		**p63**		**ER***		**PR**		**Ki-67 index***			
		Positive	**Negative**	**Positive**	**Negative**	**Negative**	**Positive**	**Negative**	**Positive**	**<47.46%**	**47.46-61.1%**	**61.1-74.8%**	**>74.8%**
>3 cm	17	11 (64.71%)	6 (35.29%)	7 (41.18%)	10 (58.82%)	14 (82.35%)	3 (17.65%)	5 (29.41%)	12 (70.59%)	6 (35.29%)	1 (5.88%)	4 (23.53%)	6 (35.29%)
p		NS		NS		NS		NS		NS			
Ulceration													
Absent	48	26 (54.17%)	22 (45.83%)	16 (33.33%)	32 (66.67%)	33 (70.21%)	14 (29.79%)	9 (18.75%)	39 (81.25%)	12 (25.53%)	13 (27.66%)	11 (23.4%)	11 (23.4%)
Present	12	9 (75%)	3 (25%)	7 (58.33%)	5 (41.67%)	10 (83.33%)	2 (16.67%)	5 (41.67%)	7 (58.33%)	2 (16.67%)	2 (16.67%)	4 (33.33%)	4 (33.33%)
p		NS		NS		NS		NS		NS			
Necrosis													
Absent	12	5 (41.67%)	7 (58.33%)	1 (8.33%)	11 (91.67%)	5 (45.45%)	6 (54.55%)	3 (25%)	9 (75%)	5 (45.45%)	1 (9.09%)	4 (36.36%)	1 (9.09%)
Present	48	30 (62.50%)	18 (37.50%)	22 (45.83%)	26 (54.17%)	38 (79.17%)	10 (20.83%)	11 (22.92%)	37 (77.08%)	9 (18.75%)	14 (29.17%)	11 (22.92%)	14 (29.17%)
p		NS		**0.0205**		NS		NS		NS			
Neoplastic intravascular emboli													
Absent	37	18 (48.65%)	19 (51.35%)	12 (32.43%)	25 (67.57%)	25 (67.57%)	12 (32.43%)	10 (27.03%)	27 (72.97%)	11 (29.73%)	8 (21.62%)	7 (18.92%)	11 (29.73%)
Present	21	16 (76.19%)	5 (23.81%)	10 (47.62%)	11 (52.38%)	16 (80%)	4 (20%)	4 (19.05%)	17 (80.95%)	3 (15%)	7 (35%)	6 (30%)	4 (20%)
p		NS		NS		NS		NS		NS			
Lymph node metastases													
Absent	17	10 (58.82%)	7 (41.18%)	5 (29.41%)	12 (70.59%)	12 (70.59%)	5 (29.41%)	2 (11.76%)	15 (88.24%)	5 (29.41%)	2 (11.76%)	6 (35.29%)	4 (23.53%)
Present	18	13 (72.22%)	5 (27.78%)	9 (50%)	9 (50%)	13 (76.47%)	4 (23.53%)	6 (33.33%)	12 (66.67%)	3 (17.65%)	6 (35.29%)	4 (23.53%)	4 (23.53%)
p		NS		NS		NS		NS		NS			

p: probability value. NS: not significant. *One case was excluded because there was no positive staining in the internal positive control of the sample.

Table 2. Association between vimentin, p63, PR, ER expression and Ki-67 index and P- and E-cadherins in feline malignant mammary tumours.

		P-cadherin			E-cadherin			P-cadherin/E-cadherin				
		Positive	**Reduced**		**Reduced**	**Positive**		**P-cad$^+$ and E-cad$^+$**	**P-cad$^+$ and E-cad$^-$**	**P-cad$^-$ and E-cad$^+$**	**P-cad$^-$ and E-cad$^-$**	
Vimentin	**Positive**	34 (97.14%)	1 (2.86%)	**<0.0001**	20 (57.14%)	15 (42.86%)	NS	14 (40%)	20 (57.14%)	1 (2.86%)	0	**0.0002**
	Negative	14 (56%)	11 (44%)		8 (32%)	17 (68%)		9 (36%)	5 (20%)	8 (32%)	3 (12%)	
p63	**Positive**	22 (95.65%)	1 (4.35%)	**0.0205**	13 (56.52%)	10 (43.48%)	NS	9 (39.13%)	13 (56.52%)	1 (4.35%)	0	**0.0421**
	Negative	26 (70.27%)	11 (29.73%)		15 (40.54%)	22 (59.46%)		14 (37.84%)	12 (32.43%)	8 (21.62%)	3 (8.11%)	
ER*	**Negative**	38 (88.37%)	5 (11.63%)	**0.0114**	25 (58.14%)	18 (41.86%)	**0.0027**	15 (34.88%)	23 (53.49%)	3 (6.98%)	2 (4.65%)	**0.0013**
	Positive	9 (56.25%)	7 (43.75%)		2 (12.5%)	14 (87.5%)		8 (50%)	1 (6.25%)	6 (37.5%)	1 (6.25%)	
PR	**Negative**	11 (78.57%)	3 (21.43%)	NS	9 (64.29%)	5 (35.71%)	NS	4 (28.57%)	7 (50%)	1 (7.14%)	2 (14.29%)	NS
	Positive	37 (80.43%)	9 (19.57%)		19 (41.3%)	27 (58.7%)		19 (41.3%)	18 (39.13%)	8 (17.39%)	1 (2.17%)	
Ki-67 index*	**<47.46%**	7 (50%)	7 (50%)	**0.0258**	4 (28.57%)	10 (71.43%)	NS	5 (35.71%)	2 (14.29%)	5 (35.71%)	2 (14.29%)	NS
	47.46-61.1%	13 (86.67%)	2 (13.33%)		5 (33.33%)	10 (66.67%)		8 (53.33%)	5 (33.33%)	2 (13.33%)	0	
	61.1-74.8%	13 (86.67%)	2 (13.33%)		8 (53.33%)	7 (46.67%)		6 (40%)	7 (46.67%)	1 (6.67%)	1 (6.67%)	
	>74.8%	14 (93.33%)	1 (6.67%)		10 (66.67%)	5 (33.33%)		4 (26.67%)	10 (66.67%)	1 (6.67%)	0	

NS: not significant. *One case was excluded because there was no positive staining in the internal positive control of the sample.

Table 3. Association between vimentin, p63, PR, ER expression in primary tumour and in the intravascular emboli and lymph node metastases.

			Neoplastic intravascular emboli			Lymph node metastases		
			Positive	**Negative**		**Positive**	**Negative**	
Primary tumour	**Vimentin**	**Positive**	13 (86.67%)	2 (13.33%)	NS	9 (69.23%)	4 (30.77%)	NS
		Negative	1 (33.33%)	2 (66.67%)		1 (20%)	4 (80%)	
	p63	**Positive**	5 (71.43%)	2 (28.57%)	***p* = 0.0210**	7 (77.78%)	2 (22.22%)	***p* = 0.0023**
		Negative	0	6 (100%)		0	9 (100%)	
	ER*	**Negative**	0	14 (100%)	***p* = 0.0015**	2 (16.67%)	10 (83.33%)	NS
		Positive	3 (100%)	0		3 (75%)	1 (25%)	
	PR	**Negative**	0	2 (100%)	***p* = 0.0074**	2 (33.33%)	4 (66.67%)	***p* = 0.0063**
		Positive	15 (100%)	0		11 (100%)	0	

p: probability value. NS: not significant. *One case was excluded because there was no positive staining in the internal positive control of the sample.

one fibroadenoma, both ER-negative. The majority of malignant tumours (n = 43; 72.88%) were ER-negative (Figure 1e and 1f), a significant difference when compared with benign tumours ($p < 0.0001$). One carcinoma was excluded from the study because we were unable to stain its internal positive control. The staining intensity was also heterogeneous in carcinomas with neighbouring areas of poor and strong nuclear expression. There was a significant inverse relationship between the expression of ER and the histological grade of carcinomas (p = 0.0018), with most of grade I (n = 6; 85.71%) being ER-positive, while the majority of grades II and III (n = 42; 80.77%) were ER-negative (Table 1). ER expression was also statistically related to P-cadherin (p = 0.0114) and E-cadherin (p = 0.0027) expressions. The majority of ER-negative tumours (n = 38; 88.37%) were P-cadherin-positive, while the majority of ER-positive tumours (n = 14; 87.5%) were also E-cadherin-positive (Table 2). ER positivity was significantly related to the combined expression of P- and E-cadherins (p = 0.0031), with P-cad$^+$ and E-cad$^-$ being the most predominant pattern (n = 23; 53.49%). The ER expression in primary tumours was coincident with the expression in the corresponding intravascular emboli in all cases, while in lymph node metastases there were some discrepancies (Table 3).

PR

Nuclear expression of PR was identified in more than 60% of the epithelial cells of all normal mammary gland tissues, with moderate to strong intensity. The distribution of positive nuclei was heterogeneous amongst lobules and/or ducts. The same pattern was observed in hyperplastic/ dysplastic lesions and benign tumours, with the exception of one fibroadenoma that was PR-negative.

Less than one-quarter of the malignant tumours (n = 14; 23.33%) were PR-negative (Figure 1g and 1h), which is not significantly different from

benign tumours. The staining intensity in carcinomas was heterogeneous, with neighbouring areas of poor and strong nuclear expression. All cribriform and mucinous carcinomas and three-quarters of tubulopapillary and solid carcinomas were PR-positive, while both carcinosarcomas were PR-negative, suggesting that PR staining is associated with the histological sub-type of carcinomas (p = 0.0243) (Table 1). There were no significant associations between PR and other clinicopathological parameters, or P- and E-cadherin expression. The ER expression in primary tumours was coincident with the expression in the corresponding intravascular emboli in all cases, albeit some discrepancies with lymph node metastases were observed (Table 3).

Ki-67

Ki-67 immunostaining in normal mammary gland tissues was nuclear, with a mean Ki-67 index of 14.6%, ranging from 0.2% to 52.4%. In hyperplastic/dysplastic lesions, the mean Ki-67 index was 30.83%, ranging from 3.42% to 67.38%. A marked difference was observed between the mean Ki-67 indexes in fibrocystic disease (15%, range: 3.42% to 30.75%) and fibroadenomatous change (49.29%, range: 27.32% to 67.38%).

The mean Ki-67 index of benign tumours (16.08%, range: 3.48% to 43.35%) was lower than that of malignant ones (58.28%, range: 13.7% to 86.61%) and when indexing the Ki-67 by quartiles, a significant association (p < 0.0001) was observed with the neoplastic lesions (benign and malignant tumours) (Figure 1i and 1j). One carcinoma was excluded from the study because no staining in the internal positive control of the sample was achieved. When malignant tumours were grouped by Ki-67-index quartiles, a significant relationship between the Ki-67 index and the histological grade (p = 0.0003) was observed (Table 1). All grade I tumours were included in the first quartile (Ki-67 index < 47.46%) while more than half of the tumours of grades II and III belonged to the higher two quartiles (Ki-67 index > 61.1%). It was possible to observe an association between Ki-67-index quartiles and the expression of P-cadherin (p = 0.0258), ER (p = 0.0069) and p63 (p = 0.0181) (Table 2). The first quartile was equally distributed between P-cadherin-negative and -positive tumours, while more than 90% of tumours in the highest quartile (Ki-67 > 74.8%) were P-cadherin-positive. Most of the ER-negative carcinomas (n = 38; 84.44%) were in the higher quartiles, while the majority of p63-negative tumours (n = 13; 99.86%) were in the lowest Ki-67 quartile. No association of Ki-67 with the expression of E-cadherin, vimentin and PR was observed.

Analysis of the combined expression of molecular markers and clinicopathological parameters in carcinomas justified the conclusion that P-cad$^+$, vim$^+$ and ER$^-$ tumours were of moderate to high histological grades (n = 25; 100%) (p = 0.0038), and that the majority of tumours with intravascular neoplastic emboli were P-cad$^+$ and vim$^+$ (n = 16; 76.19%) (p = 0.0307). Moreover, P-cad$^+$ and vim$^+$ tumours were associated with a 3.76 odds ratio (confidence interval (CI) 95%: 1.14-12.43) for vascular invasion. On the other hand, tumours simultaneously P-cad$^-$, vim$^-$, ER$^+$ and E-cad$^+$ and with the lowest Ki-67 index were all low-grade carcinomas.

DISCUSSION

Spontaneous feline mammary carcinomas have been proposed by the World Health Organization as a good model for human breast cancer, based on the similarities in the age of onset, incidence, histopathological features, biological behaviour, and poor prognosis of the malignant mammary tumours in both species [1, 2]. Consequently, it is important to evaluate feline mammary tumours at the molecular level in order to identify prognostic markers and develop targeted therapies, thus enhancing their value as models of human breast cancer and the approach towards treating feline mammary tumours. On this basis, the expression of three phenotypic markers: pan-cytokeratin AE1/AE3 (an epithelial cell marker), vimentin (a mesenchymal cell marker), and p63 (a myoepithelial cell marker) was evaluated both in normal mammary tissues and in mammary lesions.

In normal mammary glands, AE1/AE3 was expressed by luminal epithelial and myoepithelial cells (although with less intensity) and p63 was

restricted to myoepithelial cells. Besides being present in myoepithelial and stromal cells, vimentin was co-expressed with AE1/AE3, by ductal luminal epithelial cells. This co-expression was described for the first time by Caliari *et al*., 2014 [21], and interpreted as a hallmark of a non-terminally differentiated luminal component, diffusely distributed in the mammary ducts. Although the cat seems to be the only species where the mammary non-neoplastic luminal epithelium is described to express vimentin, the phenomenon has been described in the so-called "cap cells" of mice and the "side-population" of human breast containing the progenitor cell compartment [21].

p63 is a member of the p53-family of transcription factors found in the basal cell layer, including cap cells and myoepithelium, of the mammary gland [34]. In both benign and malignant human breast tumours, the expression of p63 is restricted to myoepithelial cells [35] although some authors described a less intensive nuclear p63 expression in epithelial cells of some particular breast carcinomas [35, 36]. More than one-third of the carcinomas of our series presented epithelial p63-expression and, although the staining intensity was weaker than that in myoepithelial cells, it was related to a higher histological grade. This expression may be due to the p63 expression in the early stages of epithelial differentiation [37] or may reflect a potential myoepithelial differentiation in a subset of neoplastic cells [35, 36]. p63 [38, 39] and P-cadherin [40] are both considered stem-cell markers, with p63 being known to act as a transcription factor of the P-cadherin promoter [41, 42]. In fact, the relationship found between these two molecules in our carcinomal series can be in part justified by the existence of an association at the genetic level.

In more than half of the malignant tumours in our series, epithelial cells revealed an aberrant co-expression of vimentin and AE1/AE3, a pattern that has been previously described in human breast cancer [27] and feline malignant mammary tumours [8, 21]. Such a phenomenon may be a hallmark of the direct myoepithelial histogenesis, a sign of the epithelial-mesenchymal transition (EMT) reflecting the end-stage of tumour dedifferentiation, or derive from breast progenitor cells with a bilinear (glandular and myoepithelial) differentiation potential [43]. The expression of vimentin by epithelial breast cancer cells has been associated with increased drug resistance, cancer's invasive behaviour and metastatic potential [44, 45].

Epithelial-mesenchymal transition is a multistep program characterized by the loss of epithelial characteristics, such as downregulation of epithelial markers (e.g., E-cadherin), and acquisition of a mesenchymal phenotype, namely through the upregulation of mesenchymal markers (e.g., vimentin) [40, 46, 47]. These changes result in the loss of cell-cell adhesion and polarity, and the acquisition of migratory and invasive properties as well as metastatic potential [40, 46, 48]. More than half of the P-cad$^+$ and vim$^+$ carcinomas in our series revealed a reduction in E-cadherin expression, suggesting the acquisition of mesenchymal features while losing epithelial characteristics. However, some P-cad$^+$ and vim$^+$ carcinomas were simultaneously E-cad$^+$ (n = 14; 41.17%), possibly due to an incomplete EMT. This intermediate state is described as a partial EMT, in which the cells retain some characteristics of epithelial cells albeit acquiring migratory ability, a feature of mesenchymal cells [40]. It has been proposed that P-cadherin may be considered an EMT marker, able to identify intermediate and transient EMT states associated with metastatic phenotypes, by interfering with epithelial cell-cell adhesion in the presence of E-cadherin [40].

Although hormone receptors are not currently considered to be prognostic markers in feline mammary tumours, they may represent key regulators of other important molecular markers in mammary carcinogenesis [7, 19]. In the current series, taking into account the expression of ER and PR in benign tumours, we can infer a reduction in the expression of these markers in malignant tumours, corroborating previous studies [1, 5, 7, 49] and suggesting that the role of steroid hormones in carcinogenesis is more pronounced at the early stages of tumour development, with a more autonomous growth in the advanced stages of malignancy [49]. In fact, an inverse association was found, in carcinomas, between the expression of ER and the histological grade.

The downregulation of ER was, however, more evident than that of PR, which is in agreement with previous reports of consistent loss of ER in carcinomas [5, 7, 50]. Although it has been proposed that the presence of PR is a good indicator of the integrity of the ER molecular pathway in human breast cancer [51], the high number of ER^- and PR^+ feline carcinomas, similar to previous observations [5, 7], and an absent association between ER and PR expression in our series, suggests the presence of other non-estrogen-dependent regulators of PR [2].

It has been postulated that estrogens stimulate the expression of E-cadherin in mice and that there are progesterone-responsive elements in the promoter region of the mouse E-cadherin gene [52], although the association between E-cadherin expression and ER/PR status in breast cancer is not consensual [12, 53, 54]. In feline mammary carcinomas, we observed that the majority of ER-positive tumours were also E-cadherin-positive, reinforcing the concept of an estrogen-regulated expression of E-cadherin. Several studies revealed a negative association between the overexpression of P-cadherin and the ER and PR status of breast cancer [11, 12, 13, 55, 56]. The lack of ER signalling is responsible for P-cadherin overexpression, categorizing the P-cadherin gene/*CDH*3 as an estrogen-repressed gene [57, 58]. Our results support this association, with the vast majority of ER-negative carcinomas being P-cadherin-positive. Thus, in feline mammary tumours, the ER expression seems to be associated with both cadherins, suggesting a regulatory role in their expression.

Basal-like human breast carcinomas (BLBCs) are a molecular sub-group of breast cancer, that have been considered the end-result of EMT and associated with poor prognosis [48, 59, 60, 61]. The immunohistochemical profile of this subtype of human breast cancer is still not consensual [60]. It is recognized that BLBCs are usually triple-negative cancers (ER^-, PR^- and $HER2^-$) and several different panels have been used to identify them, including basal cytokeratins, P-cadherin, vimentin, epidermal growth factor receptor (EGFR), and p63 [28, 37, 59, 62-65]. Several studies approached the molecular classification of feline mammary tumours [20, 21, 29] similar to what has been done in human breast cancer [22, 23], albeit with some differences. Caliari *et al*., 2014 described the feline mammary carcinomas as mainly aggressive hormone receptors-negative tumours [21], with basal cytokeratin and vimentin expression. Brunetti *et al*., 2013 classified one-tenth of the carcinomas as basal [29], while Wiese *et al*., 2013 characterized almost half of feline mammary tumours as triple-negative basal-like, proposing that they are a valuable, naturally occurring animal model for the study of human triple-negative breast cancer with a basal-like subtype [20]. In our series, although the majority of carcinomas was P-cadherin-positive (n = 48; 80%), ER-negative (n = 43; 72.88%), PR-negative (n = 46; 76.66%), p63-negative (n = 37; 61.66%) and vimentin-positive (n = 35; 58.33%), only five tumours (8.47%) were simultaneously $P\text{-}cad^+$, ER^-, PR^-, $p63^+$ and Vim^+, representing those that could be considered similar to BLBC.

In agreement with previous studies, the Ki-67 proliferative index showed a substantial increase in malignant tumours when compared with benign tumours and non-neoplastic lesions of the feline mammary gland [3, 4, 32, 50]. The use of Ki-67 as a prognostic factor in cats is however, controversial [3, 4, 32, 49] and its use in clinical decisions is still unsubstantiated [4]. Interestingly, in our series, higher Ki-67 indexes were related to P-cadherin overexpression, higher histological grades, and ER-negative status, supporting its association with tumour aggressiveness. However, the cut-off values from our study and others, [3, 4] hamper the proposal of useful cut-off limits with prognostic significance. To compare the results from different studies, it is mandatory that standard methodologies are followed.

When the combined expression of molecular markers was compared with the clinicopathological tumour-features, we observed that tumours simultaneously $P\text{-}cad^-$, vim^-, ER^+ and $E\text{-}cad^+$ and with the lowest Ki-67 index were all well-differentiated carcinomas, which suggests a molecular profile characteristic of carcinomas with low aggressiveness. On the other hand, $P\text{-}cad^+$, vim^+, ER^- tumours with a high Ki67 index had higher histological grades. Moreover, $P\text{-}cad^+$ and vim^+ tumours were 3.76 times more likely to invade vessels, corroborating the concept associated

with human breast cancer, that both P-cadherin [63] and vimentin [45] are invasion-associated molecules. Taken together, our results suggest that the immunohistochemical classification of feline carcinomas based on P-cadherin, ER, vimentin and Ki-67 expression may represent a reliable prognostic indicator. Furthermore, the large number of P-cad$^+$ tumours suggests that this protein may be a good therapeutic target [66, 67].

CONCLUSION

This study suggests that the overexpression of P-cadherin and vimentin, and the negative expression of ER can be considered hallmarks of feline mammary carcinomas. Moreover, it suggests that P-cadherin, ER and vimentin in association with high histological grade and high proliferative activity can be used as markers of poor prognosis, allowing a better assessment of the aggressive behaviour of feline mammary tumours.

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ETHICAL APPROVAL

This article does not contain any experimental studies with animals, performed by any of the authors. In fact, all procedures (surgical excision and necropsy examination) were performed in a clinical context. The surgical excision, attempting to treat the animals, was based on the best clinical judgment of the attending practitioners. Considering the authors had no influence on the selection and execution of such procedures, there was no reason to obtain Committee approval. The use of the excised tissues for research was explained to the owners and informed consents were obtained for all individual participants included in the study.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest in regard to this study.

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