Immunohistochemical Expression of CCR2, CSF1R and MMP9 in Canine Inflammatory Mammary Carcinomas

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Abstract. *Background: Canine inflammatory mammary cancer (IMC) and its human counterpart, inflammatory breast cancer, are extremely aggressive types of cancer. Our aim was to characterize immunohistochemical expression of C-C chemokine receptor 2, colony stimulating factor 1 receptor and metalloproteinase-9 in canine IMC versus non-IMC and to analyze associations with clinicopathological variables. Materials and Methods: Immunohistochemical staining of CCR2, CSF1R and MMP9 was performed in a series of 25 IMC and 15 non-IMC tumors. Results: No differences in the expression of these biomarkers between IMC and non-IMC were observed. Distinct nuclear subcellular expression of CCR2 was observed in IMC ($p<0.001$). For IMC, higher CCR2 expression was associated with increased nuclear grade ($p=0.037$), and higher neoplastic MMP9 expression was associated with fewer mitoses ($p=0.022$), higher nuclear grade ($p=0.047$) and increased CSF1R expression ($p=0.025$). Conclusion: Expression of CCR2, CSF1R and MMP9 in canine IMC could contribute to increased nuclear pleomorphism, but the biological mechanisms involved warrant further investigation.*

Canine inflammatory mammary cancer (IMC) is the most aggressive type of mammary tumor in dogs and has similar pathological and clinical characteristics to human inflammatory breast cancer (IBC) (1-5). The diagnosis of canine IMC as a clinical entity comprises both the histopathological detection of the hallmark characteristic, invasion of dermal lymphatic vessels by tumor emboli (1), and the presence of clinical signs associated with an inflammatory phenotype, including sudden presentation, erythema, edema, firmness, warmth and thickness of the mammary glands, with or without presence of an underlying tumor mass and not necessarily associated with dense inflammatory cell infiltration (1, 2, 4). In both humans and dogs, the disease rapidly progresses to a metastatic state and leads to short overall survival (6, 7). Despite intensive research and improvement of outcomes for human breast cancer, the prognosis for patients with IBC has remained poor, with an increased (43%) risk of death for patients with locally advanced non-IBC (8). Even in canine IMC, after palliative therapy alone or with chemotherapeutic treatments, survival times have not improved (9).

Tumor-associated macrophages (TAMs) are associated with increased tumor aggressiveness and worse prognosis of malignant canine mammary tumors (CMTs) (10-12). However, the implications of TAM infiltration in canine IMC are yet to be explored. In this study, we used immunohistochemistry to study the expression in IMC of two macrophage surface receptors, C-C chemokine receptor 2 (CCR2) (13) and macrophage colony-stimulating factor-1 receptor (CSF1R) (14), in addition to the expression of matrix metalloproteinase-9 (MMP9), which is mainly produced by macrophages (15).

The chemokine C-C motif ligand 2 (CCL2) and its main receptor CCR2 have a major role in monocyte chemotaxis, including TAM recruitment to the breast tumor microenvironment, where they participate in the process of metastasis, as demonstrated by Qian *et al.* in a mouse model of metastatic mammary tumor (16). To our knowledge, there is one report on the role of TAMs in IBC pathogenesis, where it was demonstrated that TAMs isolated from patients

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with IBC, secreting high levels of CCL2, stimulated IBC cell invasiveness *in vitro* (17). The significance of CCL2–CCR2 signaling in CMTs, including IMC is still to be determined.

CSF1R signaling, through binding of its ligand CSF1, is a determinant aspect of macrophage biology (18). In addition to its participation in normal mammary gland development, CSF1R signaling is also known to affect mammary carcinomas, as a *CSF1* gene signature has been associated with higher tumor grade, decreased expression of estrogen receptor, decreased expression of progesterone receptor, and an increase in identified p53 mutations (18, 19). In canine mammary tumors, CSF1R expression has also been associated with a more aggressive histological grade of malignancy (12) and *in vitro* promotion of proliferation, migration and invasion (20). In IBC, *CSF1R* gene expression levels are upregulated relatively to non-IBC tumors (21), but in canine IMC no information is presently available on CSF1R expression.

The MMPs are a family of proteolytic enzymes that degrade the extracellular matrix (ECM). Besides invasion of tissues adjacent to the tumor mass, MMPs can also modulate neoplastic cell differentiation, proliferation, apoptosis and angiogenesis (22). Upon activation, MMP9 can degrade gelatin and type IV collagen in the ECM and affect the adhesion of tumor cells, playing a role in tumor growth and angiogenesis (23). The role of MMPs has not been well studied in IBC. There is one report showing overexpression of MMP9 in IBC relative to non-IBC tumors and suggesting a role for MMP9 in the release of proangiogenic factors from the ECM (24).

Expression of MMP9 (25-27) and CSF1R (12) have been explored in CMTs but there is no information on the expression of these biomarkers in IMC. Therefore, our aim was to characterize the pattern of immunoreactivity of these biomarkers and explore their potential role in canine IMC.

Materials and Methods

Tumor specimens. From the histopathology archives of INNO Laboratories (Braga, Portugal), 25 formalin-fixed paraffin-embedded samples of IMC were obtained. These samples had been obtained from dogs by large excisional biopsies (n=25), as this is the general procedure for CMTs and there was no initial diagnosis of IMC. Animals presented with clinical signs of MC (edema, erythema, pain, warmth, firmness, thickening) and the characteristic invasion of dermal lymphatic vessels by tumor emboli was confirmed by histopathological analysis. Samples were retrieved between the years of 2010 and 2012. This tumor series had been partially used previously in another study by our group (28). Other non-inflammatory malignant CMTs (n=15), were obtained from the archives of INNO Laboratories to compare against the IMC series.
Histopathological evaluation. The tumor samples were fixed in 10% buffered formalin for at least 48 h and embedded in paraffin. For diagnosis, 3-μm sections were cut and processed for routine hematoxylin-eosin staining (H&E). Histopathological diagnosis was performed on H&E-stained slides according to the most recent classification of CMTs (29). For the diagnosis of IMC cases, besides the histological hallmark of the invasion of dermal lymphatic vessels, the presence of characteristic clinical presentation (edema, redness, warmth and pain) was confirmed in the medical records.

Several clinicopathological characteristics were analyzed: ulceration, necrosis, lymph-node metastasis, presence of vascular mimicry, mitotic count, tubular differentiation grade, nuclear grade and histological grade of malignancy. Vascular mimicry was determined by the presence of endothelial-like cells, following previously published criteria (30). The number of mitoses was counted in 10 high-power fields and classified into three grades according to the methodology proposed by Peña and colleagues (31). Tubular differentiation, nuclear grade and histological grade of malignancy were also evaluated according to recent recommendations for CMTs grading (31) The anonymity of the clients was maintained throughout this study. Client consent for use of patient samples in research studies was given at the time of sample submission for diagnosis.

Immunohistochemical technique. For the immunohistochemical staining, 3-μm-thick sections of each tumor sample were cut and mounted on silane-coated slides. The sections were deparaffinized in xylene and rehydrated in alcoholic solutions of decreasing concentration, ending in tap water. All the washes and dilutions were made in PBS (pH 7.4) at room temperature. Incubation steps were carried out in a wet chamber (BioOptica, Milan, Italy).

The detection of CCR2, CSF1R and MMP9 was carried out win the streptavidin–biotin peroxidase complex method, using a commercial detection system (Ultra Vision Detection System; Lab Vision Corporation, Fremont, CA, USA) following the manufacturer's instructions. All the washes and dilutions were made in PBS (pH 7.4).

For CSF1R and CCR2, heat-induced antigen retrieval was performed by proteinase K treatment (20 μg/ml in TE buffer, pH 8.0) for 10 min at 37˚C. After cooling the slides at room temperature, endogenous peroxidase was blocked, through incubation with 3% H_2O_2 for 30 min. Slides were then dried and sections outlined with a hydrophobic pen (Liquid Blocker; Daido Sangyo Co., Tokyo, Japan), washed in PBS for 5 min and blocking serum applied for 15 min (Ultra V Block; Lab Vision Corporation). Subsequently the sections were incubated overnight at 4˚C, with antibodies to CCR2 and CSF1R (both produced at the Roslin Institute, Edinburgh, UK), both supplied as mouse anti-dog monoclonal hybridoma supernatants, or with rabbit polyclonal antibody to MMP9 (Rb-1539-1; Neomarkers, Fremont, CA, USA) for 1 h at room temperature at 1:200 dilution, as previously reported (32). After incubation with primary antibody, sections were washed in PBS for 5 min, at room temperature, and then biotinylated serum applied, followed by streptavidin peroxidase for 15 min each (both included in theUltravision Detection System kit; Lab Vision Corporation), with intermediate washes in PBS, for 5 min. Immunolabeling was revealed by incubation with 3,3-diaminobenzidine tetrahydrochloride (DAB; SIGMA, St. Louis, MO, USA) 0.05% with 0.01% H_2O_2 for 5 min. After washing in distilled water, the sections were counterstained with Gill hematoxylin, dehydrated, cleared and mounted. For the negative controls, mouse IgG1 replaced monoclonal mouse anti-canine CCR2 and CSF1R antibodies, PBS replaced polyclonal anti-MMP9. The positive controls used were a section of canine lymph node for CCR2 (33) and CSF1R (14), and bronchiolar epithelium for MMP9 (32).

Immunolabeling evaluation. The expression of CCR2 and CSF1R staining, characterized by both brown intracellular and membrane staining, was scored using a semi-quantitative method. For CCR2, two parameters were considered: extent and intensity of expression, adapting a previously used methodology (34). Extension, defined as the percentage of the tumor showing expression of CCR2 was classified into: grade 0: no expression (0%); grade 1: <10%; grade 2: ≥10% and ≤25%; grade 3: >25% and ≤50%; grade 4: >50% and ≤75% and grade 5: >75%. Intensity was evaluated by visual assessment on a scale of 0-5 into negative: 0, weak: 1, moderate low: 2, moderately high: 3, high: 4, and very high: 5. A score using the product of extent and intensity was stratified by the mean value on these tumor series (cut-off value =15) in order to classify CCR2 immunohistochemical expression as high (≥15) or low (<15).

For CSF1R, evaluation was performed considering only the extent parameter, since no significant changes were observed in the intensity of the samples. Extension of expression of CSF1R was classified according to a previously described method (35) into: grade 0: ≤1% of expression; grade 1: >1% and ≤33%; grade 2: >33% and ≤66%; grade 3: >66%. Expression of CSF1R was considered positive if >1% and low or negative if ≤1%.

MMP9 staining was quantified in the stromal and neoplastic component. Stromal expression of MMP9 in >50% of the tumor was classified as high, or as low if ≤50%. The neoplastic expression of MMP9 in the cytoplasm was rated as high if >25% or low if ≤25%, according to a previously published method (27). The intensity of MMP9 expression for each component was also graded as negative: 0, weak: 1, intermediate: 2 or strong: 3. A combined score of extent and intensity of MMP9 expression was obtained by the multiplication of each component. Tumors with a score of 4 or more were considered to have high overall MMP9 expression, and those with a score below 4 were graded as having low expression.

Agreement between three independent observers (TPR, IP, FLQ) was reached for the attribution of extent and intensity grades of the three biomarkers. The classification was performed without previous knowledge of the diagnosis.

Statistical analysis. Analysis of associations of markers with clinicopathological variables was performed by using Pearson Chi-square statistical test. The statistical analysis was performed using statistical software SPSS v.17.0 (Statistical Package for the Social Sciences, IBM, Armonk, NY, USA). The acceptance statistical significance value was considered to be $p<0.05$.

Results

Tumor samples. A total of 25 IMCs (62.5%) and other 15 non-IMCs (37.5%) were included in these series. All canine IMCs were classified histologically as anaplastic carcinomas of histological grade of malignancy III. The non-IMCs were classified histologically as tubulopapillary carcinoma (n=8), complex carcinoma (n=4), solid carcinoma (n=1), carcinosarcoma (n=1) and anaplastic carcinoma (n=1).

Immunohistochemical expression of CCR2. Immunohistochemical expression of CCR2 was observed as a uniform brown labeling of the cytoplasm, or localized on the nuclear envelope or the cytoplasmic membrane. In the group of IMCs, CCR2 expression was classified as high in 56% (14/25) of the cases and in the group of non-IMCs, CCR2 expression was considered high in 60% (9/15) of the cases.

No statistically significant differences were obtained in the Pearson chi-square statistical analysis of expression of CCR2 between the two different groups of tumors ($p=0.804$). For the IMC group, only one statistically significant association was found, for CCR2 expression with the nuclear grade ($p=0.037$). Tumors of high nuclear pleomorphism (grade 3) demonstrated increased CCR2 expression and tumors of nuclear grade 2 had lower CCR2 expression.

Interestingly, of the different patterns of subcellular localization observed for CCR2, a nuclear pattern was predominant in the IMC group. By performing chi-square analysis of the distribution of CCR2 expression over the two tumor types a statistically significant difference was obtained, with nuclear and membranous expression patterns observed only in IMC and a predominantly cytoplasmic type expression on non-IMC, as shown in Figure 1. Examples of the different subcellular CCR2 expression types can be seen in Figure 2A-C. However, distinct patterns of CCR2 subcellular expression were not significantly associated with any clinicopathological variable in the IMC group.

Immunohistochemical expression of CSF1R. Immunohistochemical expression of CSF1R was observed mostly in the cytoplasm, for both IMC and non-IMC cases, without the differential patterns observed for CCR2 (Figure 2D and E). The score of CSF1R expression was evaluated as high in 64% (16/25) of IMC cases and 86.7% (13/15) of non-IMC cases.

No differences were observed in CSF1R expression between IMC and non-IMC groups using the Pearson chi-square statistical test ($p=0.120$). No statistically significant associations with clinicopathological variables were found for the immunohistochemical expression of CSF1R.

Immunohistochemical expression of MMP9. Immunoreactivity for MMP9 was observed in stromal and neoplastic cells of both IMC and non-IMC cases and the extent and intensity were recorded for each tumor component. MMP9 expression was located in the cytoplasm of neoplastic cells (Figure 2F and G). The stromal component exhibited an increased intensity of MMP9 expression relative to neoplastic cells.

Stromal MMP9 was classified with high scores in 88% (22/25) of IMCs and 80% (12/15) of non-IMC cases. Neoplastic MMP9 expression had high scores in 52% (13/25) of IMC and 46.6% (7/15) of non-IMC cases.

No differences were found between expression of MMP9 in canine IMC and non-canine IMC cases, considering both stromal ($p=0.909$) and neoplastic ($p=0.741$) MMP9 scores.

For the IMC group, associations with clinicopathological variables were explored for both stromal and neoplastic MMP9

Table I. *Association of neoplastic metalloproteinase-9 (MMP9) score with clinicopathological variables and C-C chemokine receptor 2 (CCR2) and colony stimulating factor 1 receptor (CSF1R) immunohistochemical expression in canine inflammatory mammary carcinomas. A value of p<0.05 was considered statistically significant and is indicated in bold.*

	Neoplastic MMP9 score		*p*-Value*
Clinicopathological variable	Low	High	
Ulceration			0.570
Present	5	4	
Absent	7	9	
Necrosis			0.568
Present	4	3	
Absent	8	10	
Lymph-node metastasis			0.302
Present	8	6	
Absent	4	7	
Vascular mimicry			0.789
Present	4	5	
Absent	8	8	
Mitotic grade			**0.022**
2	2	8	
3	10	5	
Nuclear grade			**0.047**
2	5	1	
3	7	12	
Tubular differentiation grade			0.930
2	2	2	
3	10	11	
CCR2 grade			0.165
Low	7	4	
High	5	9	
CSF1R grade			**0.025**
Low	7	2	
High	5	11	

*Pearson Chi-square.

scores. The stromal MMP9 score did not present any statistically significant associations with the clinicopathological variables studied.

The neoplastic MMP9 score (Table I) was significantly associated with the mitotic count (p=0.022), with tumors with high mitotic counts (grade 3) having lower MMP9 expression than those with intermediate mitotic counts (grade 2). The association between neoplastic MMP9 expression and nuclear grade was the opposite, with tumors of increased nuclear pleomorphism having higher MMP9 expression in neoplastic cells (p=0.047).

Another significant association was observed between the neoplastic MMP9 score and CSF1R expression (p=0.025): tumors with higher CSF1R expression had increased MMP9 expression in the neoplastic component.

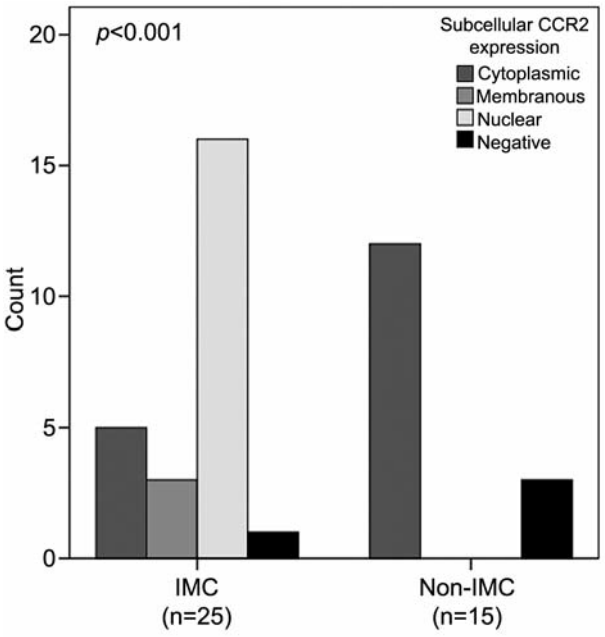

Figure 1. *Patterns of subcellular localization of C-C chemokine receptor 2 in canine inflammatory (IMC) and non- inflammatory mammary carcinoma (non-IMC).*

Discussion

IBC and IMC have an invariably poor prognosis and represent the most aggressive and lethal types of breast cancer in women and dogs, respectively (2, 3). Research performed in IBC has aimed to define a panel of biomarkers responsible for the features of high aggressiveness observed in IBC relative to non-IBC and consequently find therapeutic targets able to improve the outcome of the disease (36).

One of the main limitations of our study is the lack of characterization of macrophage infiltration in IMC to complement the immunohistochemical expression of CCR2 and CSF1R in mammary tumor tissue. Unfortunately this was not possible due to limitations in the amount of formalin-fixed paraffin-embedded tissue available, since this tumor series had already partially been used in another study (28).

CCR2–CCL2 signaling has been recognized for its role in breast cancer angiogenesis (37,38) and involvement in the metastatic process (16, 39) *via* recruitment of metastasis-facilitating $CCR2^+$ TAMs. In patients with breast cancer, overexpression of CCL2 was shown to contribute to tumor progression to be associated with a poor prognosis and earlier relapse after treatment (40, 41). Regarding IBC and IMC as highly metastatic breast cancer types, it is not unlikely that CCR2 and CCL2 might have a role in their metastatic phenotype.

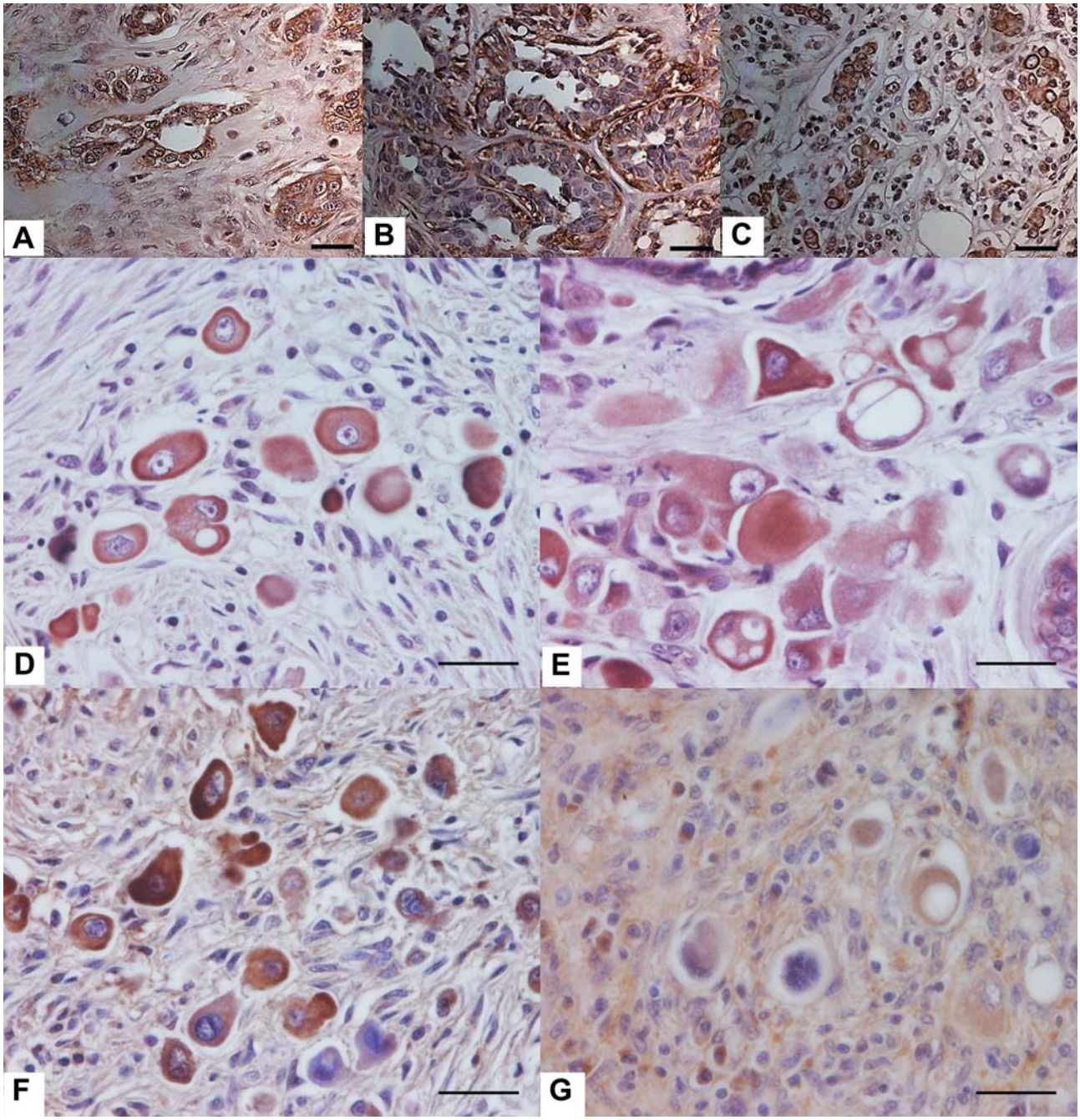

Figure 2. *Predominant patterns of C-C chemokine receptor 2 expression in canine inflammatory mammary carcinomas: A: cytoplasmic; B : membranous; C: nuclear. Cytoplasmic immunoreactivity for colony stimulating factor 1 receptor in inflammatory mammary carcinoma cells (D and E). Immunoreactivity for metalloproteinase-9 in the cytoplasm of neoplastic cells (F) and adjacent stromal tissue (G) of canine inflammatory mammary carcinomas. Scale bars represent 40 μm.*

Immunohistochemical expression of CCR2 was detected in both IMC and non-IMC groups without any differences in the intensity and extent of CCR2 expression being noted. However, different patterns of subcellular localization were observed in the immunohistochemical analysis of CCR2, with a predominance of nuclear expression in the IMC group and cytoplasmic expression in the non-IMC group. A nuclear pattern might indicate transportin-1-dependent translocation of CCR2 from the membrane to the nucleus, where G protein-coupled receptor signaling continues (42). The mechanism of

CCR2 internalization can be regulated by lipopolysaccharide activation and toll-ilike receptor 4 signaling, which triggers G-protein kinase phosphorylation and subsequently inhibits CCR2 movement away from the membrane, controlling monocyte migration in response to CCL2 (43). CCR2 internalization occurs together with CCL2 cycling from the plasma membrane to endosomal structures (44). The fact that nuclear localization of CCR2 was more frequent in the IMC group suggests there is increased CCR2 translocation to the nucleus in these cases, where it could be involved in the regulation of transcriptional events (45). Additionally, we show that increased CCR2 expression is significantly associated with higher nuclear grade (p=0.037), as tumors presenting increased pleomorphism had higher CCR2 expression.

Similarly, for CSF1R expression, no differences were observed between IMC and non-IMC tumors, which might also suggest a more relevant role for CSF1R at earlier stages of tumor progression. However, to verify this, a larger tumor series including normal and benign mammary tumors would be required.

No associations of CSF1R expression with clinico-pathological variables were observed in this tumor series. In a malignant CMT series, immunohistochemical expression of CSF1R was found to be increased in tumors with higher histological grade of malignancy, but no IMC cases were included in that study (12). Regarding IBC studies, increased *CSF1R* gene expression in IBC compared to non-IBC cases has also been reported (21). In non-IBC tumors, immunohistochemical expression of CSF1R has been associated with lymph node metastasis, larger tumor size and a poorer prognosis (46). Metastatic non-IBC tumors have also been demonstrated to have increased CSF1R expression relative to non-metastatic tumors (47). Variation in the antibodies used, methods of immunohistochemical analysis and the tumor series itself might account for the discordance observed with our results.

MMP9 is a gelatinase involved not only in the invasion and metastasis of cancer through the destruction of collagen IV in the basal membrane and ECM, but also in angiogenesis and cancer cell growth (48). MMP9 expression and its significance have been extensively explored in human breast cancer (49-54) and CMTs, excluding canine IMC (25-27, 55, 56). In IBC the expression of MMP9 and other MMPs has already been studied (24). Since information on MMP9 expression in canine IMC is lacking, we decided to determine if the increased invasiveness of canine IMC was related to an increase in MMP9 and how would MMP9 expression altered with CCR2 or CSF1R expression.

In our results, neither stromal nor neoplastic MMP9 expression was increased in IMC *versus* non-IMC cases. Using gelatin zymography, slightly higher activity of MMP9 was demonstrated in IBC tumor samples *versus* non-IBC tumors, however this was not a significant difference (24).

Within canine IMC, no statistically significant associations were observed between the stromal MMP9 score and the clinicopathological variables studied. In malignant CMTs, excluding IMCs, associations have been found between a high level of stromal MMP9 staining and the presence of lymph-node and distant metastases (27). In this study, the finding of an elevated neoplastic MMP9 score associated with a lower mitotic count (p=0.022) corroborates a study on human invasive breast carcinoma which reported an inverse correlation between MMP9 and Ki-67 proliferative index (57). In CMTs, an association between increased expression of MMP9 and Ki-67 has been reported (25), but another study, by Santos and colleagues did not confirm this association (27). For canine IMC, however, there are no results comparable to ours and thus further studies are required.

Another significant association was observed between neoplastic MMP9 and CSF1R expression (p=0.025). Tumors with high CSF1R expression had increased MMP9 expression in the neoplastic component. This association might be explained by the fact that macrophages, that express CSF1R, are also producers of MMP9 (58). Invasion of breast carcinoma cells *in vitro* and *in vivo* has been shown to be enhanced by both CSF1R autocrine and paracrine feedback loops involving TAMs (59). This observation might explain the association of MMP9-mediated invasion with increased CSF1R expression, but to confirm this in our results, determination of macrophage infiltration would need to be performed.

This study suggests that CCR2, CSF1R and MMP9 are not differentially expressed between IMC and non-IMC tumors. However, a differential nuclear pattern of CCR2 expression was detected in IMC. A larger tumor series including normal and benign CMTs would be necessary to confirm the relevance of these biomarkers at earlier stages of neoplastic progression. The different patterns of CCR2 subcellular expression in IMC and the association of MMP9 and CSF1R deserve to be further explored. Since IMC has been suggested as a model for IBC, it is possible that the present findings might also have impact in the study of IBC.

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Conflicts of Interest

The Authors declare no conflicts of interest.

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