Veterinary Immunology and Immunopathology 227 (2020) 110075

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Chemokine gene expression influences metastasis and survival time of female dogs with mammary carcinoma

Harsha Ariyarathna*, Neroli Thomson, Danielle Aberdein, John S. Munday

School of Veterinary Science, Massey University, Palmerston North, 4442, New Zealand

ARTICLE INFO

Keywords:
Breast cancers
Canine mammary tumours
Chemokines and receptors
Tumour behaviour

ABSTRACT

Chemokines are signaling proteins secreted by immune cells which regulate leukocyte trafficking. The aberrant expression of chemokines and their receptors by neoplastic cells influences the behaviour of many human cancers. This study evaluated gene-expression of the chemokines: CCL5, CXCL10, CXCL12 and the chemokine receptors: CXCR3, CXCR4, CXCR7, CCR4, CCR9 in 41 histologically-malignant, outcome-known, canine mammary tumours. These chemokines and chemokine receptors were selected as all were previously shown to influence the behaviour of human breast cancers. The expression of chemokines CCL5 and CXCL12 were significantly higher in tumours which subsequently metastasised than tumours that did not metastasise ($p < 0.05$). Increased expression of these chemokines was also correlated with shorter survival times of the dogs (CCL5: $r_s = -0.40$, $p = 0.02$, CXCL12: $r_s = -0.40$, $p = 0.03$) while CCL5 was independently prognostic of survival times ($p = 0.026$). A significantly higher proportion of tumours that subsequently metastasised expressed CXCR3 ($p = 0.037$), CXCR4 ($p = 0.026$), CXCR7 ($p = 0.025$) and CCR9 ($p = 0.039$) receptors while the survival times of the dogs with tumours that expressed CXCR4 ($p = 0.045$) and CCR9 ($p = 0.039$) receptors were significantly shorter than dogs with tumours that did not express these receptors. Chemokine and chemokine receptor gene-expression has not been previously correlated with disease outcome of canine mammary tumours. These findings indicate that altered expression of chemokines and their receptors influences the behaviour of canine mammary tumours suggesting a potential role of them as prognostic markers or therapeutic targets.

1. Introduction

Chemokines are small molecular weight signaling proteins secreted by immune cells that control migration and positioning of immune and inflammatory cells within the body (Hembruff and Cheng, 2009). Although the primary function of chemokines is leukocyte trafficking, recent research suggests they also influence growth, progression and metastasis of many human cancers (Rollins, 2006). Some human cancer cells have been shown to produce chemokines while other studies have identified abnormal expression of chemokine receptors by neoplastic cells (Müller et al., 2001).

Breast cancer is one of the most common cancers of women worldwide (Ghoncheh et al., 2016). Breast cancer cells have been shown to express chemokine receptors and increased expression of some chemokine receptors has been associated with a worse disease outcome (Müller et al., 2001). Additionally, some breast cancer cells have also been reported to produce chemokines with increased expression of some chemokines associated with a more aggressive clinical course and a worse disease outcome (Soria and Ben-Baruch, 2008).

Mammary gland tumours are the most common neoplasm of intact female dogs (Karayannopoulou et al., 2005). Many similarities have been identified between human breast cancers and canine mammary gland tumours (CMGTs) including a hormonal influence on development, histopathologic features, expression patterns of some molecular markers, and an unpredictable clinical course (Pinho et al., 2012). As in humans, gene expression of chemokines and chemokine receptors has been evaluated in CMGTs with two studies finding higher expression of some chemokines and chemokine receptors in CMGTs that were histologically classified as malignant compared to CMGTs that were histologically classified as benign (Andaluz et al., 2016; Ettlin et al., 2017) or normal mammary gland tissue adjacent to the mammary neoplasm (Klopfleisch et al., 2011). However, whether or not the expression of chemokine or chemokine receptors influence the biological behaviour of CMGTs has not been previously investigated. Therefore, the aim of the present study was to evaluate gene expression of five chemokine receptors, CXCR3, CXCR4, CXCR7, CCR4, CCR9 and three chemokines CXCL10, CXCL12, CCL5 in a series of 41 malignant and 12 benign CMGTs. These chemokines and chemokine receptors were selected

* Corresponding author.
E-mail address: H.Ariyarathna@massey.ac.nz (H. Ariyarathna).

https://doi.org/10.1016/j.vetimm.2020.110075
Received 16 February 2020; Received in revised form 25 April 2020; Accepted 15 May 2020
Available online 13 June 2020

because they have been extensively studied in humans and have been shown to influence the biological behaviour of human breast cancers (Salvucci et al., 2006; Miao et al., 2007; Ma et al., 2009; Mirisola et al., 2009; Olkhanud et al., 2009; Johnson-Holiday et al., 2011; Mulligan et al., 2013; Zhang et al., 2018). As the disease outcome of the CMGTs included in the present study was known, chemokines and chemokine receptor gene expression could be compared in neoplasms that subsequently metastasised with those that did not. Additionally, it could be determined if chemokine and chemokine receptor expression was associated with survival times of these dogs. The identification of an association between chemokine and chemokine receptor gene expression and disease outcome would suggest that chemokine and chemokine receptor gene expression may, as in human breast cancers, influence tumour behaviour.

2. Material and method

2.1. Case selection and assessment of survival times

Canine mammary gland tumour cases submitted to IDEXX diagnostic laboratory, New Zealand, for histopathology between 2012 and 2015 were sourced from the laboratory surgical biopsy archive. In all cases, tumour surgical excision had been performed with a curative intent. Details regarding the patient signalment including age and reproductive status were identified from the surgical biopsy archive database. A questionnaire was sent to the submitting veterinarians to obtain other information. The information requested in the questionnaire included previous history of mammary gland disease, other concurrent disease conditions, the number of mammary neoplasms present, abscessation or ulceration on the tumour surface, pre-surgical clinical exam findings, type of surgical procedure performed, additional treatments that had been used, evidence of mammary tumour metastasis, diagnostics used to detect tumour metastasis, and the cause of death for dogs that died. In addition, the submitting veterinarians were asked to provide the post-surgical clinical records of the dogs at least for three years from the date of surgery. Cases were excluded if adjunct therapies including anti-inflammatory drugs or steroids were used to alter the neoplasm behavior or if the tumour surface was reported to be ulcerated or contained abscesses. Using the post-surgical clinical records provided by the submitting veterinarians, the disease-specific overall survival time for each case was calculated retrospectively from the date of tumour excision to the date of the dog's death or euthanasia due to clinically-diagnosed mammary tumour metastasis.

2.2. Histological classification and grading

Formalin-fixed paraffin-embedded (FFPE) CMGT tissue blocks of the selected cases were retrieved from IDEXX laboratory archives, prepared into thin sections and stained with haematoxylin-eosin (H&E). The H&E-stained sections were examined to determine the histological subtypes and grades of the tumours, following the guidelines of Goldschmidt et al. and Pena et al. respectively (Goldschmidt et al., 2011; Peña et al., 2013). Accordingly, simple carcinomas were graded according to three criteria: percentage tubule formation, nuclear pleomorphism, and mitoses/ 10 high power fields. In heterogeneous carcinomas, tubular scoring was assessed in the most representative malignant area. In addition, in complex and mixed tumors, the percentage of tubular formation was scored considering only epithelial areas with nuclear pleomorphism evaluated in all malignant components.

Tumours were classified into three groups; *malignant-metastatic* if the neoplasm was classified as malignant using histological criteria and had a clinical diagnosis of mammary tumour metastasis based on the development of radiographic lesions of pulmonary metastasis with other suggestive clinical signs of tumour metastasis, *malignant non-metastatic* if the neoplasm was classified as malignant using histological criteria but no clinical or radiographic evidence of metastases developed during the follow-up period, and *benign* if the neoplasm was histologically consistent with a benign neoplasm.

2.3. RNA extraction

For RNA extraction, three 10 μm tissue sections were cut from each FFPE mammary tumour tissue block and placed on glass slides. In the tissue sections where > 20 % of the mammary neoplasm contain intervening stroma, the sections were placed on glass slides but left unstained and unfixed. They were then viewed under a dissecting microscope and neoplastic cells were carefully scraped off using a clean scalpel blade into 1.5 mL microtubes for RNA extraction, using the HE stained section of the same specimen as a guide. This step was used to ensure that all tissue sections used for RNA extraction contain at least 80 % tumour tissue. When neoplastic cells of various origins were present on a single section, all neoplastic cells were included for RNA extraction without discrimination. Before RNA extraction, all the equipment used, as well as the surface of the laboratory bench, was cleaned with RNAse decontamination solution (RNAseZap, Sigma-Aldrich, MO, USA). Total RNA was extracted from the samples using the Nucleospin totalRNA FFPE XS kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions and nucleic acid concentrations were quantified using a Qubit 2.0 fluorometer and assay kit (Life Technologies, Carlsbad, CA, USA). For the cases which had more than one FFPE block for a single tumour, RNA extraction was performed from each block separately and the extracts were mixed together prior to further assessments. From cases that had more than one mammary neoplasm, tissue block from the malignant tumour was used for RNA extraction if the multiple neoplasms included only a single malignant neoplasm. To remove any residual DNA, post-extraction DNAse digestion was performed using Ambion Turbo DNA-free DNAse following the manufacturer's instructions (Life Technologies). Complementary DNA synthesis was carried out with the Transcriptor first strand cDNA synthesis kit (Roche Applied Science, Mannheim, Germany) using 0.5 μg total RNA, and both random hexamer and oligo-dT primers.

2.4. RT-PCR

Five chemokine receptors including CXCR3, CXCR4, CXCR7, CCR4, CCR9 and three chemokines CXCL10, CXCL12, and CCL5 were selected for gene expression analysis. To normalize the gene expression between CMGT samples, HPRT and RPL32 reference genes were used. For all the selected genes except CXCR7, previously published primer sequences were used (Table 1) (Brinkhof et al., 2006; Im et al., 2017; Maeda et al., 2012; Nascimento et al., 2013). For CXCR7, new primers were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and mfold (http://unafold.rna.albany.edu/?q=mfold) and the newly developed assay was validated (Supplementary file 1).

All real-time PCR assays (RT-PCR) were performed using Mic qPCR Cycler (Bio Molecular System, Upper Coomera, Australia). The RT- PCR reactions were performed using AccuMelt HRM SuperMix (Quanta Biosciences, Gaithersburg, MD), using 10 ng of cDNA with 0.5 μM forward and reverse primer concentration in a total volume of 10 μL reaction mix. All reactions were performed in duplicates and each plate included a positive control and a no template control. Residual genomic DNA was excluded on the basis of the melting temperature and/or minus-RT controls. Reference gene stability was analysed using geNorm software.

2.5. Relative quantification of the chemokine and chemokine receptor gene expression in mammary tumour samples

For each gene, the melting temperatures and the shapes of the melting curves of the samples were compared with the corresponding positive controls. The samples which had melting temperatures within the range of ± 1.5 °C of the melting temperature of the positive controls

Table 1
Primer sequences of the chemokines and chemokine receptors used in this study.

Gene	Gene bank Accession No:	Primers (5′ - 3′)	bps	Tm (°C)	Reference
CCL5	NM_001003010.2	F: AAGGGCTGACTGATAAATGTGA R: AGCGAGAATTTTAATGGAAAGC	52	51	Nascimento et al., 2013
CXCL10	AB183191.1	F: CACATGTTGAGATCATTGCCA R: TTCAGACATCTTTTCTCCCCA	62	54	Nascimento et al., 2013
CXCR4	NM_001048026.1	F: GAGCGGTTACCATGGAAGAG R: CGGTTGAAGTGAGCATTTTCC	108	54	Im et al., 2017
CXCR7	NM_001003281.2	F: TTGGAGCAAAACGCCAAGTG R: TCTTGGAGACGATGCAACCC	92	56	Designed primers
CXCL12	NM_001128097.1	F: TCTTCGAGAGCCACAT TGC R: TTCAGTCTTGCCACGAT CTG	82	57	Im et al., 2017
CCR9	XM_541909	F: CACTTCCTCCCACCCTTGTA R: TGGTCTTGACTCTGGTGCAG	100	56	Maeda et al., 2012
CXCR3	AB185149.1	F: TTCTTTGCCATCCCAGATTTC R: ATGCATGGCATTTAGGCG	67	53	Nascimento et al., 2013
CCR4	NM_001003020.1	F: TTTGGACTAGGTCTCTGCAAGA R: AAAAGCCCACCAGGTACATC	52	55	Nascimento et al., 2013
RPL32	XM_848016.1	F: TGGTTACAGGAGCAACAAGAA R: GCACATCAGCAGCACTTCA	100	54	Maeda et al., 2012
HPRT	AY283372	F: AGCTTGCTGGTGAAAAGGAC R: TTATAGTCAAGGGCATATCC	114	56	Brinkhof et al., 2006

were selected for the analyses. When multiple runs were included for a single gene, the CV of the cycle threshold (Cts) of the positive controls were calculated and only considered appropriate for analysis if the CVs were < 20 %.

Relative expression of genes of interest in each CMGT was analysed using ΔΔCt method. Briefly, for each sample, the difference (ΔCt) between the Ct value of the gene of interest and the average Ct value of the two reference genes were calculated. Then a ΔΔCt value was calculated by taking the difference between the calculated ΔCt value of each sample and the average ΔCt value of the control group, considering the non-metastatic malignant mammary gland tumours as the control group.

2.6. Statistical analysis

The correlation between the relative expression of genes of interest and the survival times of the dogs was analysed by Spearman rank-order correlation test. Kruskal-Wallis H test was used to compare the gene expression between malignant-metastatic and malignant non-metastatic CMGTs, different histological sub-types, and histological grades of CMGTs. Samples with detectable reference gene expression but without detectable expression of the gene of interest were considered as negative for the particular gene. When a gene had a high number of negative samples, gene expression data was converted to a binary positive or negative result. Pearson Chi-Squared test was used for the group comparisons in these genes instead of Kruskal-Wallis H test as the former allowed inclusion of negative samples into the statistical analysis. The differences of survival times between gene expression positive and gene expression negative groups were compared using Kaplan-Maier survival curves and Log-rank test. A hierarchal multivariate analysis was performed to identify which tumour-related variables and chemokine, or chemokine receptor genes independently predict the survival times of dogs with malignant mammary gland tumours. The tumour-related variables tested in multivariate analysis included tumour size, tumour histological grade, and the presence of intra-vascular or intra-lymphatic tumour emboli. The chemokines or chemokine receptors included in the multivariate analysis were selected considering the significant correlation between their expression and survival times of dogs identified in the present study. All statistical analyses were performed using the SPSS version 25 program (SPSS Inc., Chicago, IL, USA). *P* values less than < 0.05 were considered to be indicative of statistically significant differences.

3. Results

3.1. Selected cases

From a total of 100 CMGTs included in the archives, additional information was available for 63 cases. Ten cases were excluded; the cause of death was not known in five dogs, there was insufficient follow-up time in three dogs, and two dogs had received anti-inflammatory drugs. Tumour surface ulceration or abscessation was not reported in any of the cases. Therefore, a total of 53 cases were included in the study. Of the selected 53 dogs, 36 (67.9 %) were 5–10 years old, 14 (26.4 %) were older than 10 years and age was unknown in three (5.5 %) dogs. Forty-three (81.1 %) dogs were intact female dogs while 10 (18.9 %) dogs were spayed females. Of the selected 53 dogs, 51 (96.2 %) dogs had solitary mammary neoplasms and two dogs had multiple neoplasms. Ten dogs had been diagnosed with other concurrent disease conditions at the time of diagnosis of mammary neoplasia including lipoma in two dogs and single cases of alopecia, hindlimb weakness, heart failure, dental diseases, ocular cyst, intervertebral disc disease, ceruminal cyst and debility due to old age. Except one dog which was reported to have had a benign neoplasm that had been previously surgically removed, none of the dogs had any history of previous mammary gland disease. Simple mastectomies had been performed in 51 (96.2 %) dogs while regional mastectomies had been performed in two dogs (3.8 %).

3.2. Tumour size, histological sub-types and grades

The 53 CMGTs included 7 cases from 2012, 13 from 2013, 21 from 2014, and 12 cases from 2015. There were 10 (18.9 %) small tumours (< 3 cm), 31 (58.5 %) medium-sized tumours (3 − 5 cm) and 12 (22.6 %) large tumours (> 10 cm). Forty-one (77.3 %) CMGTs were histologically classified as malignant while 12 (22.7 %) were benign. The two dogs which had multiple neoplasms had two neoplasms each: one histologically-benign neoplasm and one histologically-malignant neoplasm. The malignant mammary tumours were classified into eight different histological sub-types with simple carcinomas further subclassified into tubular, tubulopapillary, cribriform, and cystic papillary carcinomas. Grading of the malignant tumours revealed that 13 were Grade I, 23 were Grade II and 5 were Grade III. Of the 53 neoplasms, 21 subsequently metastasised (therefore were classified as malignant-metastatic), 20 were malignant with no clinical evidence of metastasis (malignant non-metastatic), and 12 were benign. Intra-vascular or

intra-lymphatic tumour emboli were observed in the HE stained histological sections of five malignant mammary gland tumours all of which were in the malignant-metastatic group. Except in three benign neoplasms, an inflammatory cell infiltrate predominantly present in the stromal tissues was observed in all other mammary gland tumours.

3.3. Risk of tumour metastasis

In this study, 21/41 (51.2 %) dogs with malignant mammary tumours died after developing evidence of tumour metastasis within three years of surgical excision of the neoplasms. The presence of tumour metastasis was confirmed by postmortem examination in two dogs and by cytological examination of thoracic masses in two dogs. The diagnosis of metastasis in other dogs was a clinical diagnosis mainly based on the detection of multiple solid masses suggestive of metastatic mammary tumour masses in thoracic radiographs, and the absence of other disease that could produce similar radiographic lesions. All 21 dogs had moderate to severe lymphadenopathy in draining lymph nodes with these lymph nodes observed to be fixed to the underlying tissues in six dogs. However, the lymphadenopathy was not further investigated using other diagnostics including cytological or histological examination due to the wishes of the owners. The pre-surgical and post-surgical clinical exam findings of regional lymph nodes are provided in the Supplement file 2.

At the end of the follow-up period, 16 dogs were alive. None of these dogs had lymphadenopathy in draining lymph nodes or any other clinical evidence suggestive of mammary tumour metastasis. Due to a lack of clinical evidence of metastasis, thoracic radiography was only performed in three dogs. These radiographs did not reveal any evidence of pulmonary metastases.

Four dogs that had histologically diagnosed malignant mammary gland tumours died before the end of the follow-up period due to unrelated causes. None of these dogs had any clinical evidence suggestive of mammary tumour metastasis prior to death. None of the 12 dogs with histologically benign mammary neoplasms died within the study period. Neither of the dogs which had multiple neoplasms had any clinical evidence of tumour metastasis and therefore both were classified as malignant non-metastatic. The patient and tumour characteristics of the cases included in malignant-metastatic, malignant non-metastatic and benign groups are summarized in Table 2.

Table 2
Clinical characteristics of the patients and gross and histological characteristics of the neoplasms included in the malignant-metastatic, malignant non-metastatic and benign groups.

	Malignant-metastatic	Malignant non-metastatic	Benign
N	21	20	12
Age			
5 – 10 yrs	15	10	11
> 10 yrs	6	7	1
Unknown	0	3	0
Reproductive status			
Intact	16	16	11
Spayed	5	4	1
Number of tumours			
Single	19	20	12
Multiple	2	0	0
Tumour size			
Small (< 3 cm)	0	0	10
Medium (3 – 5 cm)	11	18	2
Large (> 5 cm)	10	2	0
Histological type			
	Simple carcinoma (5)	Intra-ductal papillary carcinoma (6)	Complex adenoma (7)
	Adenosquamous carcinoma (5)	Simple carcinoma (6)	Simple adenoma (3)
	Ductal carcinoma (3)	Complex carcinoma (3)	Papillary adenoma (2)
	Carcinoma - Mixed type (3)	Ductal carcinoma (3)	
	Carcinoma - solid (2)	Adenosquamous carcinoma (1)	
	Comedo carcinoma (1)	Carcinoma - Anaplastic (1)	
	Carcinoma - Anaplastic (1)		
	Intra-ductal papillary carcinoma (1)		
Histological grade			
Grade I	3	10	N/A
Grade II	14	9	N/A
Grade III	4	1	N/A
Presence of tumour emboli	5	0	0

3.4. Assay validation of CXCR7 and reference gene stability

The newly developed CXCR7 gene expression assay was linear within the tested range from 10^2 to 10^7 target copies with an efficiency of 1.035 and r^2 value of 99.85 %. The CV of the mean linearized Ct values obtained with five replicates of different dilutions of the standard in a single test run ranged from 10.1 to 17.9%. The CV of the mean linearized Ct values obtained in three separate runs of the test ranged from 7.4 to 19.5%. This indicates adequate precision and reproducibility of the assay.

The average geNorm M value was ≤ 0.2 indicating a high reference gene stability. The geNorm V value was < 0.15 suggesting that the pairwise variation between the two reference genes HPRT and RPL32 is minimal and these two reference genes can be reliably used to normalise the gene expression between samples.

3.5. Relative gene expression of chemokines CCL5, CXCL12 and CXCL10 in CMGTs

In the 41 malignant CMGTs included in the study, reference gene expression was positive in 40 tumours and undetectable in one. This mammary tumour was excluded from further analyses. Analysis of the relative quantities of gene expression was only performed for genes in which a high proportion of the tumours had detectable expression of the gene of interest. This included three chemokines: CCL5, CXCL10 and CXCL12. For CXCL12 target gene expression was observed in 39 (97 %) CMGTs. One sample was excluded due to absence of CXCL12 expression. CCL5 expression was observed in 38 (97.5 %) mammary tumours. One tumour did not have detectable expression and the other was excluded due to inappropriate melting peak. Detectable CXCL10 expression was observed in 26 (66.7 %) mammary tumours and undetectable in six. Eight tumours were excluded from CXCL10 gene expression analysis due to other reasons; the melting temperatures were not appropriate in six tumours, and in two tumours only one replicate was positive.

For CCL5, the ΔΔCt values in malignant CMGTs ranged from -3.97 to 7.08. The mean ranks of ΔΔCt of CCL5 in malignant-metastatic tumours was significantly different from that of malignant non-metastatic tumours (Kruskal-wallis H test, $Z = 4.3$, $p = 0.038$). For CXCL12, the ΔΔCts in malignant CMGTs ranged from -3.61 to 1.13 and the mean ranks of ΔΔCt values between metastatic and non-metastatic mammary tumour groups was significantly different (Kruskal-wallis H test, $Z = 12.4$, $p < 0.005$). In contrast, expression of CXCL10 was not different between the two groups ($p = 0.76$). There were no significant differences in relative gene expression of CCL5, CXCL12 and CXCL10 genes between different histological sub-types or different grades of CMGTs.

Table 3
Gene expression analysis for chemokine receptors. *For each gene, 1–5 samples were excluded for inappropriate melting peaks. Six mammary tumors could not be included in CCR9 assay due to insufficient sample volumes.

Chemokine receptor	Proportion of malignant CMGTs with positive target gene expression			
	Total*	Metastatic	Non-metatstatic	Chi-sq p value
CXCR3	23/39 (56 %)	15/20 (75 %)	8/19 (42 %)	0.037
CXCR4	20/38 (55 %)	14/20 (70 %)	6/18 (33 %)	0.026
CXCR7	19/38 (50 %)	13/19 (68 %)	6/19 (32 %)	0.025
CCR9	12/33 (36 %)	8/14 (57 %)	4/19 (21 %)	0.039
CCR4	20/36 (55 %)	7/16 (45 %)	13/20 (65 %)	0.17

3.6. Gene expression of chemokine receptors: CXCR3, CXCR4, CXCR7, CCR4 and CCR9 in CMGTs

Gene expression data for chemokine receptors was considered positive when target gene expression was detected in the tumour, or negative when target gene expression was not detected in the tumour despite adequate expression of the reference genes. Although CXCR3, CXCR4, CXCR7 and CCR4 genes had an adequate number of mammary tumours with detectable target gene expression, the data could not be reliably quantified as the Ct values of some of the samples were not within the linear range of the assay. For CCR9 gene, the number of positive tumours was low. Therefore, relative gene quantification was not performed for these genes and tumours were considered on positive or negative basis for statistical analysis.

Chemokine receptor CXC 3 (CXCR3) gene expression was detected in 23/39 malignant CMGTs, with no CXCR3 expression detected in 16 tumours. One tumour was excluded due to an inappropriate melting peak. Of the 20 CMGTs that subsequently metastasised, 15 (75 %) were positive for CXCR3 expression while CXCR3 expression was detected only in 8/19 (42 %) of the CMGTs which did not develop metastases (Table 3). Therefore, CMGTs with positive CXCR3 expression metastasised significantly more frequently in this study than CMGTs without CXCR3 expression (Chi-squared test, $p = 0.037$). Positive CXCR4 expression was identified in 20/38 malignant CMGTs samples and 18 samples were negative for CXCR4. Two samples were excluded due to inappropriate melting peaks. Fourteen (70 %) metastatic tumours had positive expression of CXCR4 while only 6/18 (33 %) non-metastatic samples showed positive expression of CXCR4. Therefore, a significantly higher proportion of metastatic malignant CMGTs were positive for CXCR4 expression compared to the non-metastatic malignant CMGTs (Chi-squared test, $p = 0.026$). Positive CXCR7 expression was observed in 19/38 mammary tumours while it was negative in 19 tumours. Two samples were excluded due to inappropriate melting peaks. The proportion of metastatic malignant CMGTs which had positive CXCR7 expression (13/19, 68 %) was significantly higher than the proportion of CXCR7 positive malignant tumours which did not subsequently metastasise (6/19, 32 %) (Chi-squared test, $p = 0.025$). Only 33 CMGTs could be included in the CCR9 assay. Six samples had insufficient sample and therefore could not be assayed. One sample was excluded due to an inappropriate melting peak. Of the 14 metastatic mammary tumours included, 8 (57 %) tumours had positive CCR9 expression while only 4/19 (21 %) non-metastatic CMGTs had positive CCR9 expression. Therefore, a significantly higher proportion of CMGTs which had developed tumour metastases had positive CCR9 expression ($p = 0.039$) compared to those that did not develop metastasis during the follow-up period. Twenty samples were positive for CCR4 expression while 17 samples were negative. Four samples were excluded due to inappropriate melting temperatures. In contrast to other chemokines and chemokine receptors, there was no significant difference between the proportions of CCR4 positive tumours in the metastatic (7/16, 45 %) and non-metastatic (13/20, 65 %) CMGT groups (Chi-squared test, $p = 0.17$).

3.7. Gene expression and survival times of dogs

Analysis of the correlation between chemokine gene expression (relative quantity) and survival time in the dogs with malignant CMGTs found a statistically significant, moderate negative correlation between CXCL12 gene expression and survival times (Spearman's rank-order correlation $r_s = -0.40$, $p = 0.03$). There was also a moderate, negative correlation between CCL5 expression and survival times of dogs (Spearman's rank-order correlation, $r_s = -0.40$, $p = 0.02$). However, there was no significant correlation between CXCL10 expression in CMGTs and survival time (Spearman's rank-order correlation, $r_s = 0.27$, $p = 0.38$).

The overall mean survival time (MST) of the 41 dogs with malignant CMGTs was 721 days: (95 % CI 609–833). When dogs were grouped according to the chemokine receptor gene expression, the mean survival time differed significantly between groups of some chemokine receptors but not others. The mean survival time (MST) of the dogs with CXCR4 positive tumours was significantly lower (623 days, 95 % CI 455–793) than that of the dogs with CXCR4 negative mammary gland tumours (Log-rank test, 845 days, 95 % CI 688–1002, $p = 0.045$, Fig. 1). Similarly, the MST of the dogs with CCR9 positive tumours was 686 days (95 % CI 488–885) which was significantly lower than the MST of CCR9 negative dogs (Log-rank test, 817 days: 95 % CI 750–1039, $p = 0.039$). In contrast, the differences of MSTs between the chemokine receptor positive and negative groups were not significant for CXCR3, CXCR7 and CCR4 chemokine receptors. The hierarchal multivariate analysis included chemokines and chemokine receptors CCL5, CXCL12, CXCR4 and CCR9. The selected chemokines or chemokine receptors for analysis either had significant correlation between their expression and survival times of the dogs (CCL5 and CXCL12) or had significantly shorter survival times in dogs with neoplasms with detectable gene expression than those of the dogs with neoplasms without detectable gene expression (CXCR4 and CCR9). In addition to the chemokines and chemokine receptors, tumour size, tumour grade and presence of tumour emboli in histological section were also included as independent variables. Of these variables, histological grade ($\Delta F = 4.3$, $p = 0.048$) and CCL5 ($\Delta F = 5.7$, $p = 0.026$) were identified as independently predicting the survival times of the dogs with malignant mammary neoplasms (Table 4).

3.8. Gene expression in benign samples

In regard to chemokine gene expression in the 12 benign CMGTs, expression of CCL5 was identified in 6/12 (50 %) mammary tumours, CXCL10 expression in 7 (64 %), and CXCL12 expression in only one benign neoplasm (10 %). However, even when chemokine gene expression was detected in benign tumours, this expression was very low and outside the linear ranges of the assays. This is in contrast with the malignant CMGTs where chemokine gene expression was present in the majority of samples and was higher, being within the linear range of the assays. Regarding chemokine receptor gene expression (CXCR3, CXCR4, CXCR7, CCR4 and CCR9) only two benign tumours from each gene had detectable gene expression. Further, for all the chemokine

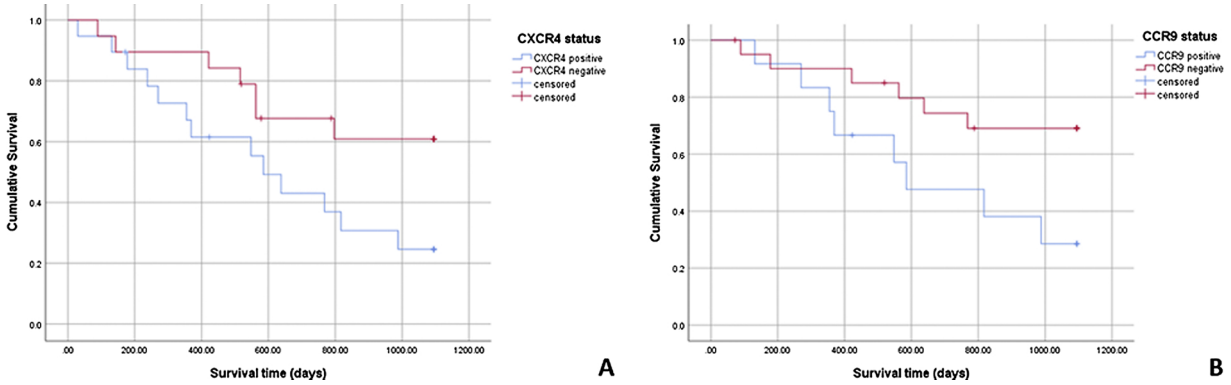

Fig. 1. Kaplan-Meier survival curves of the dogs. A: Dogs with mammary gland tumours positive and negative for CXCR4. The mean survival times between dogs which had malignant mammary tumours which were positive for CXCR4 was significantly different from that of tumours which were negative for CXCR4 ($p = 0.039$) B: Dogs with mammary gland tumours positive and negative for CCR9. The mean survival times between dogs which had malignant mammary tumours which were positive for CCR9 was significantly different from that of tumours which were negative for CCR9 ($p = 0.018$).

Table 4
Hierarchical multivariate analysis. The three tumour-related variables were selected considered their significant association with survival time shown in previous studies. The selected chemokines or chemokine receptor genes have either showed significant correlation with survival times (CCL5 and CXCL12) or showed significantly shorter survival times in dogs which had neoplasms with detectable gene expression than those of the dogs which had neoplasms without detectable gene expression (CXCR4 and CCR9) in this study. The variability of the survival times predicted by each variable is denoted by ΔF and p values indicate the significance of ΔF.

Independent variables	ΔF	p
Tumour size	0.365	0.551
Tumour histological grade	4.301	0.048**
Presence of tumour emboli	2.528	0.124
CCL5	5.665	0.026**
CXCL12	0.522	0.477
CXCR4	1.293	0.168
CCR9	0.251	0.89

**$p < 0.05$

receptor genes Chi-squared analysis showed that the proportion of benign CMGTs with positive gene expression was significantly lower than the proportion of malignant mammary tumours which had positive gene expression (all p values < 0.05).

4. Discussion

All the chemokines and chemokine receptors analysed in the present study, except CXCL10 and CCR4, had higher gene expression in malignant CMGTs that subsequently metastasised than in malignant CMGTs that did not metastasise. In addition, higher expression of CXCR4, CCR9, CCL5 and CXCL12 was associated with shorter survival times of the dogs. Of the tested chemokines and chemokine receptors, CCL5 was identified to predict the survival times of the dogs with malignant mammary neoplasms independent of tumour size, histological grade, presence of tumour emboli in histological sections and gene expression of other included chemokines and chemokine receptors. Therefore, the present findings suggest that the expression of chemokines and chemokine receptors influences tumour behaviour. To the authors' knowledge, this is the first time that chemokine and chemokine receptor gene expression in CMGTs has been associated with disease outcome in dogs.

The present results are consistent with many human breast cancer studies which have also revealed expression of chemokines and chemokine receptors by neoplastic cells influence tumour behaviour and patient survival (Salvucci et al., 2006; Miao et al., 2007; Ma et al., 2009; Mirisola et al., 2009; Olkhanud et al., 2009; Johnson-Holiday et al., 2011; Mulligan et al., 2013; Zhang et al., 2018). These human studies have identified several mechanisms to explain how the expression of chemokines or chemokine receptors may influence tumour behaviuor (Mollica Poeta et al., 2019). Firstly, the chemokine network in a tumour has been shown to influence the extent and phenotypic composition of the inflammatory cell infiltrate within the tumour. Some inflammatory cells are pro-tumourigenic while others have anti-tumourigenic properties (Mollica Poeta et al., 2019; Defourny et al., 2019). Therefore, altered chemokine gene expression may indirectly influence the tumour behaviour by changing the intra-tumoural inflammation in a way that promotes tumour growth and spread. Secondly, aberrant expression of chemokine receptors on the surface of neoplastic cells can promote migration of these cell towards distant organs that contain higher concentration of the corresponding chemokine (Mollica Poeta et al., 2019). For example, human breast cancers with high CXCR4 expression were shown to metastasise to lung, liver and bone more frequently than breast cancers with low CXCR4 expression. This is hypothesized to be because lung, liver and bone tend to contain a high concentration of CXCL12 which binds to the CXCR4 receptors (Mollica Poeta et al., 2019).

The use of chemokine expression to predict prognosis has been investigated for many different human cancer types (Jiang et al., 2006; Akashi et al., 2008; Palacios-Arreola et al., 2014). For example, chemokine panels consisting of 7–12 chemokines were shown to accurately predict the behaviour of breast cancers (Prabhakaran et al., 2017; Thomas et al., 2019). In dogs, it is difficult to accurately predict the behaviour of malignant mammary neoplasms using existing conventional prognostic tools (Santos et al., 2013). The inability to predict which neoplasms are likely to metastasise can delay the therapeutic interventions aimed at preventing mammary tumour metastasis. In the present study, relative expression of chemokines CCL5 and CXCL12 was significantly higher in mammary tumours which subsequently developed metastasis compared to the tumours which did not develop metastasis. In addition, chemokine receptors CXCR3, CXCR4, CXCR7 and CCR9 were detected more frequently in the tumours that subsequently metastasised compared to those which did not develop clinical evidence of metastasis. Chemokine CCL5 was identified to predict the survival times of the malignant canine mammary neoplasms independent of tumour size, tumour grade and presence of tumour emboli in histological sections. Therefore, the present findings suggest these chemokines and chemokine receptors may be useful to predict which CMGTs are more likely to metastasise. However, while measuring the expression of chemokines and chemokine receptors may help to predict prognosis, the methods are technically challenging and additional method development would be required before adopting measuring gene expression as a routine diagnostic tool.

As well as their potential use as prognostic markers, chemokines and chemokine receptors represent potential targets for cancer immunotherapy (Wu et al., 2009; Mizejewski, 2018; Mollica Poeta et al., 2019). For example, if increased chemokine receptor activity leads to a more aggressive clinical behaviour of a neoplasm, blocking this receptor using a chemokine antagonist or a monoclonal antibody could reduce the likelihood of tumour metastasis and metastasis related death (Mizejewski, 2018; Mollica Poeta et al., 2019). Recently, a monoclonal antibody and several synthetic and natural chemokine receptor antagonists have been approved by the United States Food and Drug Administration to treat several human cancers including some types of breast cancers (Mizejewski, 2018). However, considering the extent of research being carried out in the field of chemokine-targeted cancer immunotherapy, only a limited number of chemokine-targeted novel therapeutics have been introduced for clinical use. The lack of an appropriate animal model which mimics the characteristics and behaviour of human breast cancers contributes to the difficulties in developing novel chemokine related target therapies (Mizejewski, 2018). Recently, dogs have been proposed as a better animal model than mouse or rat models for pre-clinical testing of cancer therapeutics (Pinho et al., 2012; Overgaard et al., 2018). The findings of the present study show chemokine and chemokine receptor gene expression patterns in CMGTs resemble those of human breast cancers. Additionally, these results show that expression of chemokines and their receptors influence the behaviour of mammary gland neoplasms in dogs. Therefore, mammary gland neoplasms in dogs may provide an appropriate animal model of human breast cancers for testing chemokine-targeted cancer therapeutics. Furthermore, the similarity of chemokine expression between CMGTs and human breast cancers may suggest the possibility of using already available chemokine receptor-targeted cancer therapeutics intended for human use to treat CMGTs.

In the present study, gene expression of CXCR3, CXCR4, CXCR7, CCR9, CCL5 and CXCL12 was associated with tumour behaviour in a way that was consistent with studies of human breast cancers. In contrast, expression of CXCL10 and CCR4 has been shown to predict tumour behaviour in humans but was not significantly associated with metastasis or survival time in the present study of CMGTs. The reason for this difference was unclear; however, only a relatively small number of samples were able to be analysed for CXCL10 due to inappropriate melting temperatures observed in some samples. The smaller than anticipated sample size may have contributed to the lack of significant findings observed for this chemokine. It is also possible that the mechanism by which CXCL10 and CCR4 influence the behaviour of human cancers does not apply to CMGTs due to differences in tumour biology between dogs and humans.

The present study did not identify any significant differences in chemokine gene expression between different histological types and histological grades of malignant CMGTs. Although it is possible that chemokine expression in CMGTs is not affected by histological type and grade, it is necessary to further investigate this matter with a larger sample size including sufficiently high number of samples in different histological types and tumour grades.

In the present study, tumour histological grade was identified to be independently predictive of survival times of the dogs with malignant canine mammary neoplasms. Tumour grade was shown to be prognostic of disease outcome or survival times of dogs with malignant mammary neoplasms previously in univariate (Rasotto et al., 2017) and multivariate analyses (Karayannopoulou et al., 2005; Peña et al., 2013). Unlike tumour histological grade, tumour size or presence of tumour emboli were not independently predictive of the survival times of the dogs in this study. Tumour size has been identified to be prognostic of CMGTs in previous studies by univariate (Santos et al., 2013; Hellmén et al., 1993) and multivariate analysis (Rasotto et al., 2017). The lack of significance in the present study could be due to small number of large-sized neoplasms in this study. Although the presence of intra-lymphatic or intra-vascular tumour emboli is generally suggestive of more aggressive disease, detection of tumour emboli in histological sections is dependent on several factors such as number of histological sections prepared from a neoplasm. Therefore, the lack of significance identified in the present study could be a result of that.

In this study, significantly lower proportions of benign CMGTs had positive gene expression for chemokine and chemokine receptor genes. This is consistent with previous canine studies and indicated that expression of chemokine and chemokine receptor genes increases as the differentiation of a neoplasm decreases (Andaluz et al., 2016; Ettlin et al., 2017). However, the ideal comparison of chemokine and chemokine receptor expression should be performed between the neoplastic mammary gland and the non-neoplastic mammary gland adjacent to the neoplastic mammary gland. This could not be fullfilled in the present study due to insufficient non-neoplastic mammary gland available in the tissue sections and therefore benign mammary tumours were used as an alternative for non-neoplastic mammary gland. Although the benign mammary neoplasms are closely representative of the chemokine expression of the non-neoplastic mammary gland, more studies are necessary to confirm these findings.

One limitation of the present study was the lack of tumour staging at diagnosis. This suggests it is possible that some CMGTs could have metastasised prior to the neoplasm being excised. Additionally, due to the retrospective nature of the study, it was impossible to definitively exclude that some of the CMGTs that were classified as non-metastatic had not developed clinically silent metastatic disease in the 3-year period of the study.

Except three benign mammary gland tumours, all other mammary neoplasms contained an inflammatory cell infiltrate which was predominantly present in the tumour stroma. Most of these inflammatory cells were not included for RNA extraction as the stromal tissues were removed prior to RNA extraction. However, absolute exclusion of inflammatory cells or tumour stromal tissues from RNA extraction was practically impossible and this is another limitation of the present study. Further studies using immunohistochemistry are needed to confirm that chemokines and chemokine receptor proteins are expressed in neoplastic cells and to identify which neoplastic cells produce them in tumours where more than one cell type exist such as mixed mammary tumours.

In summary, the present study is the first reported investigation of chemokine and chemokine receptor gene expression in CMGTs with known clinical outcome. The results showed that expression of some of the chemokine and chemokine receptor genes was significantly associated with the development of metastases and survival times in these dogs. As well as providing insight into the factors that influence behaviours of CMGTs, these results suggest that chemokines and chemokine receptors have future uses as prognostic markers or therapeutic targets in these common life-threatening neoplasms of dogs.

Cell line validation statement

Cell lines have not been used in the present study and therefore it was not necessary to perform cell line validation.

Funding

This work is funded by Massey University, New Zealand, Postgraduate Research Fund (Grant No: 20194) and Healthy Pets NZ (Grant No: 21685).

Declaration of Competing Interest

The authors declare that there is no conflict of interest

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2020.110075.

References

Akashi, T., Koizumi, K., Tsuneyama, K., Saiki, I., Takano, Y., Fuse, H., 2008. Chemokine receptor CXCR4 expression and prognosis in patients with metastatic prostate cancer. Cancer Sci. 99, 539–542.

Andaluz, A., Yeste, M., Rodríguez-Gil, J.E., Rigau, T., García, F., del Álamo, M.M., 2016. Pro-inflammatory cytokines: Useful markers for the diagnosis of canine mammary tumours? Vet. J. 210, 92–94.

Brinkhof, B., Spee, B., Rothuizen, J., Penning, L.C., 2006. Development and evaluation of canine reference genes for accurate quantification of gene expression. Anal. Biochem. 356, 36–43.

Defourny, S.V., Romanucci, M., Grieco, V., Quaglione, G.R., Santolini, C., Della Salda, L., 2019. Tumor–microenvironment interaction: analysis of mast cell populations in normal tissue and proliferative disorders of the canine prostate. Vet. Sci. 6, 16.

Ettlin, J., Clementi, E., Amini, P., Malbon, A., Markkanen, E., 2017. Analysis of gene expression signatures in cancer-associated stroma from canine mammary tumours reveals molecular homology to human breast carcinomas. Int. J. Mol. Sci. 18, 1101.

Ghoncheh, M., Pournamdar, Z., Salehiniya, H., 2016. Incidence and mortality and epidemiology of breast cancer in the world. Asian Pac. J. Cancer Prev. 17, 43–46.

Goldschmidt, M., Peña, L., Rasotto, R., Zappulli, V., 2011. Classification and grading of canine mammary tumors. Vet. Pathol. 48, 117–131.

Hellmén, E., Bergström, R., Holmberg, L., Spångberg, I.B., Hansson, K., Lindgren, A., 1993. Prognostic factors in canine mammary tumors: a multivariate study of 202 consecutive cases. Vet. Pathol. 30, 20–27.

Hembruff, S.L., Cheng, N., 2009. Chemokine signaling in cancer: implications on the tumor microenvironment and therapeutic targeting. Cancer Ther. 7, 254–267.

Im, K., Graef, A., Breen, Lindblad-Toh, K., Modiano, J.F., Kim, J.H., 2017. Interactions between CXCR4 and CXCL12 promote cell migration and invasion of canine hemangiosarcoma. Vet. Comp. Oncol. 15, 315–327.

Jiang, Y.P., Wu, X.H., Shi, B., Wu, W.X., Yin, G.R., 2006. Expression of chemokine CXCL12 and its receptor CXCR4 in human epithelial ovarian cancer: an independent prognostic factor for tumor progression. Gynecol. Oncol. 103, 226–233.

Johnson-Holiday, C., Singh, R., Johnson, E., Singh, S., Stockard, C.R., Grizzle, W.E., Lillard, J.W., 2011. CCL25 mediates migration, invasion and matrix metalloproteinase expression by breast cancer cells in a CCR9-dependent fashion. Int Oncol. 38, 1279–1285.

Karayannopoulou, M., Kaldrymidou, E., Constantinidis, T., Dessiris, A., 2005. Histological grading and prognosis in dogs with mammary carcinomas: application of a human grading method. J. Comp. Pathol. 133, 246–252.

Klopfleisch, R., Lenze, D., Hummel, M., Gruber, A.D., 2011. The metastatic cascade is reflected in the transcriptome of metastatic canine mammary carcinomas. Vet. J. 190, 236–243.

Ma, X., Norsworthy, K., Kundu, N., Rodgers, W.H., Gimotty, P.A., Goloubeva, O., Lipsky, M., Li, Y., Holt, D., Fulton, A., 2009. CXCR3 expression is associated with poor survival in breast cancer and promotes metastasis in a murine model. Mol. Cancer Ther. 8, 490–498.

Maeda, S., Ohno, K., Tsukamoto, A., Nakashima, K., Fukushima, K., Goto-Koshino, Y., Fujino, Y., Tsujimoto, H., 2012. Molecular cloning and expression analysis of the canine chemokine receptor CCR9. Vet. Immunol. Immunopathol. 145, 534–539.

Miao, Z., Luker, K.E., Summers, B.C., Berahovich, R., 2007. CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature. Proc. Natl. Acad. Sci. U. S. A. 104, 15735–15740.

Mirisola, V., Zuccarino, A., Bachmeier, B.E., Sormani, M.P., Falter, J., Nerlich, A., Pfeffer, U., 2009. CXCL12/SDF1 expression by breast cancers is an independent prognostic marker of disease-free and overall survival. Eur. J. Cancer 45, 2579–2587.

Mizejewski, G.J., 2018. Breast Cancer, chemokines, and metastasis: a search for decoy ligands of the CXCR4 receptor. J. Neoplasms. 1, 1–9.

Mollica Poeta, V., Capucetti, A., Massara, M., Bonecchi, R., 2019. Chemokines and chemokine receptors: new targets for cancer immunotherapy. Front. Immunol. 10, 379.

Müller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M.E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S.N., 2001. Involvement of chemokine receptors in breast cancer metastasis. Nature 410, 50.

Mulligan, A.M., Raitman, I., Feeley, L., Pinnaduwage, D., Nguyen, L.T., O'Malley, F.P., Ohashi, P.S., Andrulis, I.L., 2013. Tumoral lymphocytic infiltration and expression of the chemokine CXCL10 in breast cancers from the Ontario Familial Breast Cancer registry. Clin. Cancer Res. 19, 336–346.

Nascimento, M.S., Albuquerque, T.D., Do-Valle-Matta, M.A., Caldas, I.S., Diniz, L.F., Talvani, A., Bahia, M.T., Andrade, C.M., Galvão, L.M., Câmara, A.C., Guedes, P.M., 2013. Naturally Leishmania infantum-infected dogs display an overall impairment of chemokine and chemokine receptor expression during visceral leishmaniasis. Vet. Immunol. Immunopatol. 153, 202–208.

Olkhanud, P.B., Baatar, D., Bodogai, M., Hakim, F., Gress, R., Anderson, R.L., Deng, J., Xu, M., Briest, S., Biragyn, A., 2009. Breast cancer lung metastasis requires expression of chemokine receptor CCR4 and regulatory T cells. Cancer Res. 69, 5996–6004.

Overgaard, N.H., Fan, T.M., Schachtschneider, K.M., Principe, D.R., Schook, L.B., Jungersen, G., 2018. Of mice, dogs, pigs, and men: choosing the appropriate model for Immuno-oncology research. ILAR J. 59, 247–262.

Palacios-Arreola, M.I., Nava-Castro, K.E., Castro, J.I., García-Zepeda, E., Carrero, J.C., Morales-Montor, J., 2014. The role of chemokines in breast cancer pathology and its possible use as therapeutic targets. J. Immunol. Res., 849720. https://doi.org/10.1155/2014/849720.

Peña, L., Andrés, P.D., Clemente, M., Cuesta, P., Perez-Alenza, M., 2013. Prognostic value of histological grading in noninflammatory canine mammary carcinomas in a prospective study with two-year follow-up: relationship with clinical and histological characteristics. Vet. Pathol. 50, 94–105.

Pinho, S.S., Carvalho, S., Cabral, J., Reis, C.A., Gärtner, F., 2012. Canine tumors: a spontaneous animal model of human carcinogenesis. Transl. Res. 159, 165–172.

Prabhakaran, S., Rizk, V.T., Ma, Z., Cheng, C.H., Berglund, A.E., Coppola, D., Khalil, F., Mulé, J.J., Soliman, H.H., 2017. Evaluation of invasive breast cancer samples using a 12-chemokine gene expression score: correlation with clinical outcomes. Breast Cancer Res. 19, 71.

Rasotto, R., Berlato, D., Goldschmidt, M.H., Zappulli, V., 2017. Prognostic significance of canine mammary tumor histologic subtypes: an observational cohort study of 229 cases. Vet. Pathol. 54, 571–578.

Rollins, B.J., 2006. Inflammatory chemokines in cancer growth and progression. Eur. J. Cancer 42, 760–767.

Salvucci, O., Bouchard, A., Baccarelli, A., Deschênes, J., Sauter, G., Simon, R., Bianchi, R., Basik, M., 2006. The role of CXCR4 receptor expression in breast cancer: a large tissue microarray study. Breast Cancer Res. Treat. 97, 275–283.

Santos, A.A., Lopes, C.C., Ribeiro, J.R., Martins, L.R., Santos, J.C., Amorim, I.F., Gärtner, F., Matos, A.J., 2013. Identification of prognostic factors in canine mammary malignant tumours: a multivariable survival study. BMC Vet. Res. 9, 1.

Soria, G., Ben-Baruch, A., 2008. The inflammatory chemokines CCL2 and CCL5 in breast cancer. Cancer Lett. 267, 271–285.

Thomas, J.K., Mir, H., Kapur, N., Bae, S., Singh, S., 2019. CC chemokines are differentially expressed in Breast Cancer and are associated with disparity in overall survival. Sci. Rep. 40, 14.

Wu, X., Lee, V.C., Chevalier, E., Hwan, S.T., 2009. Chemokine receptors as targets for cancer therapy. Curr. Pharm. Des. 15, 742–757.

Zhang, Y., Xu, L., Peng, M., 2018. CXCR3 is a prognostic marker and a potential target for patients with solid tumors: a meta-analysis. OncoTargets Ther. 11, 1045.