Exploring new biomarkers in the tumour microenvironment of canine inflammatory mammary tumours

Original Article

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Abstract

Human inflammatory breast cancer (IBC) and Canine inflammatory mammary cancer (CIMC) are the most aggressive forms of mammary cancer. Current research aims to identify new therapeutic targets. Here, we investigated gene expression levels of biomarkers associated with the inflammatory microenvironment.

A total of 32 formalin-fixed paraffin-embedded samples of canine mammary carcinoma (CIMC=26; non-CIMC =6) were used and their cDNA subjected to quantitative PCR (qPCR) to establish gene expression levels for mediators commonly implicated in linking carcinogenesis with inflammation. Gene expression differences between CIMC and non-CIMC types were obtained for COX-2 (P=0.004), synuclein gamma (P=0.006), tribbles1 (P=0.025), VEGF (P=0.017) and CSF1R (P=0.045). Among these biomarkers correlations were found, particularly between SNCG and tribbles1 (r=0.512, P=0.001).

The efficient metastasis of CIMC is intimately linked to components in the tumour microenvironment. This study suggests that upregulation and correlation of SNCG and tribbles1 deserves to be further explored.

Keywords: inflammation, inflammatory breast cancer, canine inflammatory mammary carcinomas

Introduction

Inflammatory breast cancer (IBC) in humans and the corresponding canine disease, designated as canine inflammatory mammary carcinoma (CIMC), represent the most aggressive type of mammary cancer in both species with invariably short survival times after diagnosis.[1-3]

Inflammatory mammary carcinomas in companion animals were firstly described in dogs [4] and more recently in cats.[5] This highly metastatic type of breast cancer presents unique histopathological features and clinical signs, similar for both humans and canines. In face of these resemblances, the possibility of using CIMC as model of study for human IBC has been purposed by several authors.[6-9] In both species, the histological hallmark of inflammatory carcinomas is the formation of tumour emboli in dermal lymphatic vessels resulting in profuse oedema, due to the obstruction of lymph drainage.[10,11]

During the initiation step of carcinogenesis, malignant transformation is triggered by the accumulation of DNA mutations.[12] The inflammatory conditions may provide the necessary genetic instability to the activation of DNA damage leading to these initiating events of carcinogenesis.[13,14] Considering human IBC or CIMC as types of cancer with chronic inflammation, expression of diverse cytokines and inflammatory mediators has been explored in search for an explanation for the severe aggressiveness of the disease.

Dogs bearing CIMC have been shown to have increased IL-10 (interleukin-10) and IL-8 (interleukin-8) serum concentrations compared to dogs diagnosed with other malignant or benign mammary tumours.[15] In human patients with IBC, analysis of a cytokine prolife of monocytes from breast draining veins has shown that TNF-α (tumour necrosis factor alpha), IL-10 and IL-8 have significantly increased motility and invasion of IBC cancer cell lines *in vitro*.[16] These observations contribute to the growing body of evidence suggesting the value

of CIMC as a model for the corresponding human disease,[6] and also strengthen the concept of tumour-associated macrophages as determinant elements in the establishment of a tumour-favourable microenvironment.

For both species, the high aggressiveness and fatality of this disease comparatively to other breast cancer types, demands a wider range of therapeutic options and prognostic factors to help categorize patients and provide the better treatment. Due to the metastatic success of human IBC and CIMC, knowledge obtained for this cancer type could be applied to the prevention of other cancer types where an inflammatory microenvironment is present. Also, the discovery of new therapeutic targets may provide new opportunities for the treatment of this aggressive disease in both humans and canines.

Expression of COX-2 (cyclooxygenase 2) and VEGF (vascular endothelial growth factor) has been shown to have prognostic value for both IBC and CIMC.[6,17] Signalling of macrophage proliferation and differentiation occurs through the receptor of CSF-1 (macrophage colony stimulating factor 1) , CSF1R.[18] The recruitment of blood monocytes into the tumour is governed mainly by the chemokine CCL2 (monocyte chemotactic protein 1) and its receptor CCR2.[19] Overall, the presence of a macrophage infiltrate in breast cancer has been shown to correlate with features of malignancy and a worse prognosis.[20,21]synuclein gamma, in its turn, has been investigated for a role in invasion *in vitro* and metastasis of breast cancer *in vivo* in a mouse model,[22] showing also a prognostic value.[23] Tribbles1 is governed by Snail and Twist, important transcription factors during the EMT (epithelial to mesenchymal transition),[24] when vimentin is expressed as a mesenchymal marker.

In this study, it was our aim to characterize the genetic expression of biomarkers known to be involved in several aspects of the tumour microenvironment of CIMC: angiogenesis (COX-2 and VEGF), macrophage infiltration (CCR2, CSF1R, CCL2), invasion and metastasis (synuclein gamma - SNCG) and epithelial to mesenchymal transition (Tribbles1,

vimentin). Simultaneously, by investigating new tumour biomarkers in CIMC, we try to identify new therapeutic targets that could be used to increase our knowledge on this disease and improve overall survival times of canine patients with CIMC.

Materials and methods

Tumour samples

From the histopathology archives of INNO Laboratories, (Braga, Portugal) 26 formalin fixed paraffin embedded samples of canine inflammatory mammary tumours were obtained. These samples had been taken from of dogs by large incisional biopsy (n=26). Animals presented to consult with clinical signs of inflammatory mammary carcinoma (oedema, skin redness, pain, warmth) and by histopathologic analysis, the characteristic invasion of dermal lymphatic vessels by tumour emboli was confirmed. Samples were retrieved between the years of 2010 and 2012. Other non-inflammatory mammary tumours, classified histologically as tubulopapillary carcinomas (n=6), were obtained to compare against the CIMC tumour series. These tumour samples have been partially used in another study by our group, currently submitted for publication.

Histopathologic evaluation

All specimens had been fixed in 10% neutral buffered formalin for 2 to 7 days, and after macroscopic analysis, sections representative of the tumour lesions were dehydrated and embedded in paraffin. A 3μm thick section was processed for routine haematoxylin and eosin (HE) staining for diagnostic purposes. Tumours were independently evaluated by two veterinary pathologists (J.P. and I.P.) who then agreed on the established diagnoses, according to the criteria defined by World Health Organization (WHO) for the histological

classification of mammary tumours of domestic animals.[25] The histopathologic diagnosis stated the presence of dermal lymphatic invasion and through investigation of the medical records, clinical signs of CIMC were confirmed for a definite diagnosis. The clinicopathological characteristics observed included: ulceration, necrosis, lymph node metastasis, vascular mimicry, mitotic grade, 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.[26] The number of mitosis was counted in 10 high-power fields and classified in 3 grades according to the recommended guidelines.[11] Tubular differentiation, nuclear grade and histological grade of malignancy were also evaluated according to the recent recommendations for CMT grading. [11] The anonymity of the patients was maintained throughout this study. Client consent for use of patient samples was obtained for all the cases.

RNA extraction and cDNA synthesis

The extraction of RNA from formalin-fixed paraffin-embedded tumour samples was carried out using PureLink FFPE Total RNA Isolation Kit (K1560-02, Invitrogen, Carslbad, CA, USA), according to the manufacturers recommendations. For this study, 26 formalin fixed paraffin embedded tumour samples of CIMC and 6 tubulopapillary carcinomas were analysed. Briefly, five 10μm tissue sections were dewaxed and lysed with proteinase K. The lysate was purified through binding and washing steps. Finally, the eluted RNA was digested with DNase I (Invitrogen, Carslbad, CA, USA) eliminate genomic DNA contamination. The RNA quantity and 260/280 ratios were verified on the spectrophotometer Nanodrop 1000 (ThermoScientific, Waltham, MA, USA) and integrity was checked by examining the presence of mRNA subunits after running a 5μL sample in a 2% agarose gel. The presence of clear bands, without a vertical smear indicated good integrity RNA.

Since most samples did not have mRNA integrity of the required quality, cDNA synthesis was carried out by an alternative method employing Single Primer Isothermal Amplification (SPIA) technology as recommended by the manufacturer of Ovation FFPE WTA System (3403, Nugen, Leek, Netherlands). The complete process of cDNA synthesis by SPIA consists in the generation of two strands of cDNA in two separate steps which are amplified by SPIA at the end of the process. Firstly, RNA demodification was carried out to eliminate the formation of secondary structure RNA, followed by the primer annealing and then the synthesis of hybrid cDNA strands.

For the purification of the cDNA obtained, the Agencourt RNAClean XP purification beads, supplied with the Ovation FFPE WTA system were applied and the purified DNA used in the final step of SPIA amplification. A step of cDNA purification followed using the Qiagen QIAquick PCR Purification Kit (28104, Qiagen, Limburg, Netherlands).

The quality of cDNA was verified as previously described, by performing amplification of reference genes (HPRT, GAPDH or RSP19) by standard polymerase chain reaction, using a *Taq* polymerase, before processing the samples for qPCR.

Quantitative Real Time PCR (qPCR)

Sequences of canine mRNA for the target genes were obtained from the Ensembl website (www.ensembl.org/Canis_familiaris). Primers were designed using PrimerPlus online software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) applying the optimal qPCR settings, and checked for the presence of hairpins, primer dimer formation at low entropy with the Oligo DT analyzer web tool (https://www.idtdna.com/calc/analyzer).

To assure the amplification of coding regions and not any remainder genomic cDNA, the location of at least one of the primers spanning exon-exon junctions was confirmed by

using the online tool Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer sequences and relevant parameters are listed on table 1.

In agreement with recommendations for good practices in qPCR experiments,[27] the geometric mean of RNA gene expression of three reference genes (GAPDH, RPL32, RSP19) was used to determine the relative expression of target genes.

The concentration of the primers used was optimized beforehand using serial dilutions of cDNA synthesized from mRNA obtained from REM 134 cells. For each sample three technical replicates were run and dissociation curves analysed individually to rule out the formation of non-specific PCR products, such as primer dimers and background amplification. The presence of a single peak at the known product melting temperature and the absence of any primer- dimers that might have generated a peak at a lower temperature was the criteria used to assess the purity of the PCR product.[28] Standard curves were produced from serial dilutions of samples to evaluate the PCR efficiency.

For the qPCR reaction, primers were diluted at concentrations of 100-300nM, according to previous optimization results, and cDNA was diluted 1:10. The components of a master mix were set according to the instructions of the manufacturers of SYBR green UDG (11733-046, Invitrogen, Carslbad, CA, USA). The qPCR thermal cycler (Agilent, MxPro 3005, USA) was programmed for : UDG incubation at 50^0C for 2 mins, Taq activation at 95ºC for 2 min; 45 cycles of 95ºC for 15 seconds, 60ºC for 30 seconds; and finally 95ºC for 1 min, 60ºC for 30seconds, 95ºC for 15 seconds and 25ºC for 30seconds. The extended 45 cycle program was set to determine low levels of transcript levels, registered from Ct=21 up till Ct=38. Data was analysed in the MxPro software (Agilent, USA). According to Schmittgen and Livak (2008),[29]the target gene expression relative to the reference gene was calculated by applying the following formula to the mean Ct values for each sample: $2^{-_{\Delta}Ct}=2^{-Ct\ (ref\ gene)\ -\ Ct(target\ gene)}$ where Ct designates the cycle threshold at which the fluorescent light overcomes the

background noise and the result is expressed in arbitrary units. The geometric mean of the Ct values for the reference gene was considered.

Statistical analysis

To compare means of two groups with non-parametric distributions, Wilcoxon rank-sum test was used. For the analysis of correlations between non-parametric distribution groups, a Kendal tau B statistical test was used. Analysis of associations with clinicopathological variables was performed by using Chi-Square Pearson statistical test. The statistical analysis was performed using the software SPSS v.17.0 (Statistical Package for the Social Sciences, IBM). To divide values of relative gene expression into low and high, the cut off value was applied by the median values of RGE for each group. The statistical significance value was considered to be $P<0.05$.

Results

Tumour samples

The tumours used in this study were obtained from a population of dogs of varied breeds (Table 2). In the group of inflammatory carcinomas, mongrels are the largest group represented (38.5%). The mean age of the animals from both groups was comparable (10.8 years old in both groups). The information on the status of lymph node metastasis was not available for 53.9% (n=14) of the cases of inflammatory carcinomas and 33.6% (n=2) of the non-inflammatory carcinomas group. All the tumour samples of inflammatory carcinomas had arisen as primary tumours. The histopathologic analysis of malignancy grade confirmed all these tumours had high histopathologic grade of malignancy (grade 3).

Relative gene expression of canine mammary Inflammatory versus non-inflammatory carcinomas

In the group of inflammatory carcinomas, relative gene expression expressed as mean±SEM was 0.566±0.018 for COX-2, 0.050±0.016 for VEGF, 0.516±0.321 for tribbles1, 0.138±0.049 for synuclein gamma, 0.0168±0.009 for CCR2, 0.010±0.003 for CSF1R, 1.845±0.578 for vimentin and 40.136±9.517 for CCL2. In the tubulopapillary carcinomas group, expression for VEGF and CSF1R was positive in only one of the six cases, expression for COX-2, tribbles1, SNCG and CCR2 was negative in all cases, expression 17.967±17.521 for vimentin, and 22.465±4.727 for CCL2.

Relative gene expression (RGE) of COX-2, VEGF-A, SNCG, tribbles 1 and CSF1R was higher in inflammatory than in non-inflammatory carcinomas and these differences are statistically significant (Figure 1). The differences observed on the RGE values for the other biomarkers (CCL2, CCR2 and vimentin) were considered non-significant.

Regarding correlations between the RGE of biomarkers studied within the group of inflammatory carcinomas (Table 3), it is noteworthy a moderate and positive correlation between tribbles 1 and synuclein gamma (r=0.512, P=0.001), which might indicate co-expression in inflammatory carcinomas and suggests an interaction between the two proteins. Other weaker positive and statistically significant correlations were obtained between the RGE of VEGF-A and tribbles (r=0.396, P=0.008), VEGF-A and COX-2 (r=0.326, P=0.024), VEGF-A and SNCG (r=0.314, P=0.031) and CSF1R and tribbles 1 (r=0.310, P=0.042). A tendency was observed for a correlation between COX-2 and tribbles 1 (r=0.296, P=0.051) with borderline values of statistical significance.

Association between RGE grade and clinicopathological variables

Within the group of canine inflammatory carcinomas (N=26), the presence of associations with the clinicopathological variables studied and the relative gene expression

grade of COX-2, VEGF, tribbles1, SNCG, CCR2, CSF1R, vimentin and CCL2 was analysed by Pearson Chi-Square test.

Between CCR2 relative gene expression grade and the mitotic grade, a statistically significant association was observed (P=0.005). Most of the tumours classified with mitotic grade 3 (11/13) had high grade CCR2 relative gene expression and conversely, in the majority of tumours presenting mitotic grade 2 (moderate) a low grade of CCR2 relative gene expression was obtained. For the other biomarkers studied (CSF1R, COX-2, VEGF, CCL2, tribbles1, vimentin and SNCG) no statistically significant associations were obtained with clinicopathological variables in this study.

Discussion

In this retrospective study, a comparative approach was used to investigate the expression of genes that might serve as new biomarkers for canine mammary tumours, and in particular, inflammatory carcinomas. One of the main limitations of this study was the reduced tumour series size, including only 6 control samples, and limited biological material preserved in formalin and embedded in paraffin which we used to extract RNA. The reliability of the mRNA extracted from FFPE tissues has been corroborated by other authors [30-32] and was here verified by previous amplification of reference genes. To our best knowledge, the determination of gene expression levels of tribbles 1, CSF1R, CCL2, CCR2, synuclein gamma and vimentin constitutes a novelty in dog mammary tumours and therefore in inflammatory carcinomas.

COX-2 is an enzyme converting arachidonic acid into different prostaglandins that serves as an important inflammatory mediator.[33] The role of COX-2 in breast cancer carcinogenesis of both humans and dogs has been studied for its association with formation of

metastasis and angiogenesis [34-36] and a worse prognosis.[37] In our results COX-2 overexpression was verified in CIMC comparatively to non-CIMC tumours, contrary to results of COX-2 mRNA expression obtained by comparing a set of IBC with non-IBC samples. [38] By ELISA, COX-2 expression has been investigated in tissue homogenates of CIMC,[7] and higher levels of this enzyme were found relatively to malignant canine mammary tumours. In this study we also found a positive correlation between COX-2 and VEGF expression, which is in agreement with previous studies in humans [34] and dogs.[35]

Vascular endothelial growth factor (VEGF) is a biomarker of angiogenesis associated with poor outcome in IBC patients and overexpressed in CIMC relatively to non-CIMC tumours, as confirmed in our results. [39,40] Also, statistically significant correlations were obtained between VEGF and tribbles1 and between VEGF and synuclein gamma, both unreported until now in canine mammary tumours. However, since these are weak correlations, a study comprising an enlarged population would be necessary to achieve firm conclusions on these correlations.

Tribbles 1 is a gene of the pseudokinase family which has been identified as a myeloid oncogene involved in acute myeloid leukaemia,[41] although its role as a tumour suppressor gene has also been described for the same disease.[42] Tribbles 1 is an intervenient the regulation of mitosis and cell movements during morphogenesis, controlled by Snail and Twist, both involved in epithelial to mesenchymal transition (EMT).[24] However, this is not the preferred pathway for migration and metastasis in IBC, since downregulation of TGFβ [43] and a predominance of E-cadherin has been shown in these cases.

In our results, both tribbles 1 and synuclein gamma appear upregulated in CIMC cases and present a statistically significant correlation between them, although its meaning requires mechanistic studies to be fully understood. Although it is not likely that synuclein gamma and tribbles 1 are cooperating in the EMT process in IBC, both have been studied for the

participation in steps of carcinogenesis. Tribbles 1 expression is not well studied in breast cancer yet, but it has been reported to be involved in a breast carcinogenesis mechanism by ephrin receptor B6 silencing, as a target of several miRNAs.[44] Tribbles 1 has also been described to be an independent predictor of prognosis in ovarian cancer in a study of primary tumour cultures derived from ascitic fluids.[45] Tribbles 1 is recognized as an essential factor for prostate carcinoma growth and survival in 3D culture environments, overexpressed in clinical samples of prostatic cancer.[46]

Synuclein gamma, also designated breast cancer specific gene 1, is a biomarker of poor prognosis in triple negative human breast cancer,[23] a characteristic of 30-40% of IBC cases. [1] Synuclein gamma is expressed in neoplastic mammary gland, but not in normal epithelial mammary glandular cells.[47] In vitro overexpression of synuclein gamma in the breast cancer cell line MDA-MBA-435 caused increased cell motility and invasiveness, which was confirmed *in vivo,* by injecting these cells in the mammary fat pads of nude mice, producing enhanced metastasis to the lymph nodes and increased tumour cell growth.[22] Later on, the involvement of synuclein gamma with ERK and Rho GTPases signalling was suggested to be responsible for the increased motility and migration observed in breast cancer cell lines *in vitro.*[48] In MDA-MB-231 mammary cancer cells, targeting synuclein gamma with siRNA has been shown to reduce proliferation and migration dowregulating phosphorylation of ERK and AKT, and also induce apoptosis by cell cycle arrest.[49] Moreover, in a colon cancer cell line it has been demonstrated that induced synuclein gamma overexpression increased cell migration, invasion and adhesion to endothelial cells.[50] The overexpression of synuclein gamma we observed in CIMC could be related with the prominent role of cell migration and invasion in this tumour type.

Increased RGE of CSF1R was observed in CIMC, similarly to a study of IBC analysing NF-κB related genes where CSF1R was found to be upregulated relatively to non-IBC

tumours.[51] A statistically significant correlation is also observed between tribbles1 and CSF1R relative gene expression, which could reflect tribbles1 involvement in macrophage migration [52] which is also partly governed by CSF-1 signalling.[18]

Overall, relative gene expression of COX-2, VEGF, tribbles1, SNCG and CSF1R was found to be increased in CIMC cases relatively to non-CIMC, which could be implicated in the pathogenesis of this disease, but could not be associated with prognosis nor metastasis due to limited population and follow up data.

Relatively to the associations found between CCR2 relative gene expression and the mitotic grade (P=0.005), no comparable reports have been found in the literature. However, experiments performed in a prostate carcinoma cell line indicated that inhibition of CCR2 has caused decreased proliferation and augmented apoptosis,[53] which relates to our results.

Due to the fact that all CIMC tumours had high histological grade of malignancy (grade 3) no significant associations were established with this clinicopathological variable.

Surprisingly, in our results no associations with clinicopathological variables were observed for COX-2 relative gene expression in CIMC, in agreement with observations for other malignant CMT,[35] but opposed to what has been verified in other reports [7,54,55] with observation of associations to tumour size and histological grade of malignancy.

Also for VEGF relative gene expression, no association with clinicopathological variables was registered here in agreement with some authors,[35,56] but contrary to other reports.[57] These disparate results from a comparative aspect demonstrate the need to continue investigating this neoplasia at clinical and molecular levels to better understand its pathophysiology.

Conclusions

With this work we aimed to contribute to the study of the tumour microenvironment of canine inflammatory mammary carcinomas. To the best of our knowledge this is the first report of elevated gene expression levels of synuclein gamma, tribbles 1 and CSF1R in CIMC versus other malignant canine mammary tumours. Moreover, significant correlations between these markers were found, in particular between synuclein gamma and tribbles 1, which might have a role in the aggressive behaviour of CIMC and possibly also in IBC. In IBC the role of tribbles1 and synuclein gamma expression has not been evaluated yet, and the possibility for their implication in the pathogenesis of this disease will require further research. The discovery of novel tumour biomarkers allows an enhanced knowledge on the biological behaviour of these aggressive mammary tumour type and suggests potential therapeutic targets.

Conflict of interest statement

None of the authors has any personal or financial relationships with other people or organizations that could inappropriately influence or bias this work

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Table 1 - Sequence and characteristics of qPCR primers used in this study.

qPCR primers	Sense	Sequence 5'-3'	Lenght (bp)	GC (%)	Melting temperature (°C)
COX-2	Forward	TATCAGGTAATTGATGGAGAGGTG	24	41.67	57.57
	Reverse	CAGCAAACTGCAGGTGTTCA	20	50.00	59.26
CCL2	Forward	TAAAAGAGTCACCAGCAGCAA	21	42.90	54.50
	Reverse	TTTAGGACGGTCTTGAAGATCA	21	40.90	53.40
CCR2	Forward	TCCTTCTCACCATCCCATTC	20	50.00	56.6
	Reverse	AGAAGGTCCCGCCAAAATAC	20	50.00	57.88
CSF1R	Forward	AACAAGACCTGGACAAAGAGGA	22	45.45	59.22
	Reverse	TTCTTGGATGCAAGGAAGG	19	47.37	55.05
Synuclein gamma	Forward	GTGGTCACCAGCATCAACAC	20	55.00	59.41
	Reverse	GTTGTTCTAGGTCCTCCTTTCG	22	50.00	58.41
Tribbles	Forward	CATCGCCGACTACCTGCT	18	61.11	59.19
	Reverse	TGTTTAATAGGAAACACCTTGCAG	24	37.5	57.67
VEGFA	Forward	CGAAGTGGTGAAGTTCATGG	20	50.00	56.74
	Reverse	GCAGGATGGCTTGAAGATGT	20	50.00	58.24
Vimentin	Forward	CGGGAGAAGTTGCAAGAGGA	20	55.00	59.68
	Reverse	TCCACTTTCCGCTCAAGGTC	20	55.00	59.97
GAPDH	Forward	GCCTGGAGAAAGCTGCCAAA	20	55.00	61.18
	Reverse	CGTCGAAGGTGGAAGAGTGG	20	60.00	60.39
RPL32	Forward	CATCGATCGCTGGGCATCAT	20	55.00	60.67
	Reverse	CTGGTGCCGGATGAACTTCT	20	55.00	60.04
RSP19	Forward	CCTTCCTCAAAAAGTCTGGG	20	50.00	55.9
	Reverse	GTTCTCATCGTAGGGAGCAAG	21	52.38	58.2

Table 2 - Characteristics of the population studied

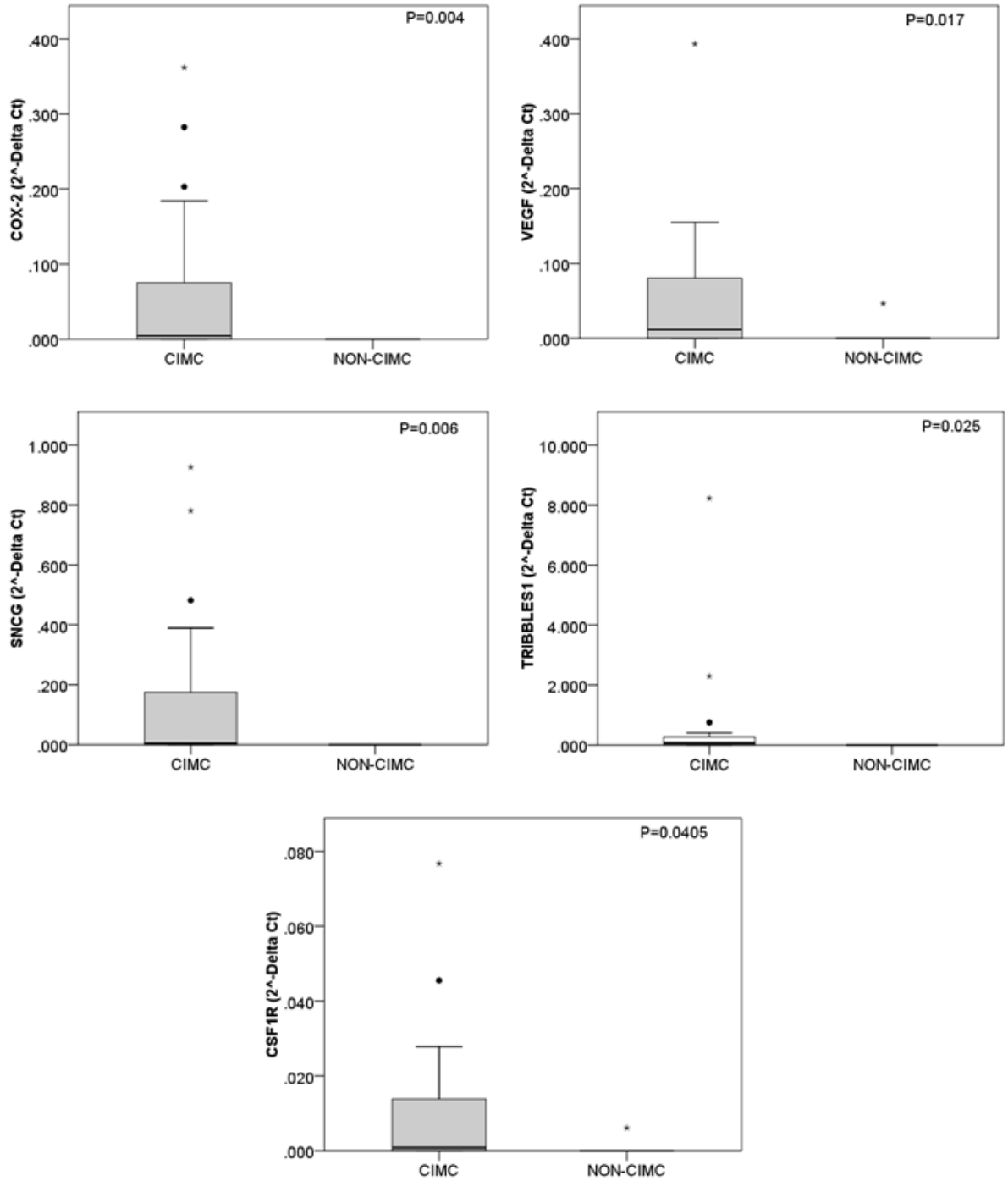
	Breed			Age		LN metastasis		Primary CIMC
		n	%				n; %	%
Inflammatory carcinomas (n=26)	Mongrel	10	38.5	Mean	10.8	Present	9; 34.6	100
	Poodle	2	7.7	Minimum	6	Absent	3; 11.5	
	Cocker spaniel	4	15.4	Maximum	17	Unknown	14; 53.9	
	Labrador Retriever	1	3.9	≤10 years old	n=12; 46%			
	Golden retriever	2	7.7	>10 years old	n=14; 54%			
	Boxer	3	11.5					
	Bichon Frisé	1	3.9					
	Pekingese	1	3.9					
	German Shepherd	1	3.9					
	Great Dane	1	3.9					
Non-inflammatory carcinomas (n=6)	Mongrel	1	16.6	Mean	10.8	Present	3; 50	
	Husky	1	16.6	Minimum	6	Absent	1; 16.7	
	Setter Spaniel	1	16.6	Maximum	16	Unknown	2; 33.3	
	Boxer	1	16.6	≤10 years old	n=3; 50%			
	Poodle	1	16.6	>10 years old	n=3; 50%			
	Pekingese	1	16.6					

LN - lymph node; CIMC - inflammatory carcinoma

Table 3: Matrix of correlations for the biomarkers studied, obtained by the Kendall's tau b statistical test

Kendall's tau b n=26	VEGF	Tribbles1	SNCG	CCR2	CSF1R	Vimentin	CCL2
COX-2	**r=0.326 P=0.024**	r=0.296 P=0.051	r=0.240 P=0.103	r=0.007 P=0.962	r=0.070 P=0.634	r=0.070 P=0.624	r=-0.127 P=0.373
VEGF	–	**r=0.396 P=0.008**	**r=0.314 P=0.031**	r=0.233 P=0.123	r=0.160 P=0.270	r=0.134 P=0.341	r=0.097 P=0.493
Tribbles1	–	–	**r=0.512 P=0.001**	r=0.071 P=0.654	**r=0.310 P=0.042**	r=0.279 P=0.590	r=0.100 P=0.500
SNCG	–	–	–	r=-0.033 P=0.829	r=0.253 P=0.088	r=0.119 P=0.407	r=-0.087 P=0.545
CCR2	–	–	–	–	r=0.055 P=0.719	r=0.124 P=0.408	r=-0.081 P=0.587
CSF1R	–	–	–	:–	–	r=0.177 P=0.218	r=-0.048 P=0.737
Vimentin	–	–	–	–	–	–	r=0.163 P=0.243

Figure 1


P=0.004
COX-2 (2^-Delta Ct)
.400
.300
.200
.100
.000
CIMC
NON-CIMC
P=0.017
VEGF (2^-Delta Ct)
.400
.300
.200
.100
.000
CIMC
NON-CIMC
P=0.006
SNCG (2^-Delta Ct)
1.000
.800
.600
.400
.200
.000
CIMC
NON-CIMC
P=0.025
TRIBBLES1 (2^-Delta Ct)
10.000
8.000
6.000
4.000
2.000
.000
CIMC
NON-CIMC
P=0.0405
CSF1R (2^-Delta Ct)
.080
.060
.040
.020
.000
CIMC
NON-CIMC