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Evaluation of VEGF-A and CCL2 in dogs with brachycephalic obstructive airway syndrome or canine idiopathic pulmonary fibrosis and in normocephalic dogs

I. Niinikoski [a,*], S. Kouki [a], N. Koho [a], M. Aromaa [a], S. Holopainen [a], H.P. Laurila [a], A. Fastrès [b], C. Clercx [b], L. Lilja-Maula [a], M.M. Rajamäki [a]

[a] Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine, P.O. Box 57, FI-00014, University of Helsinki, Finland
[b] Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Liège, Bd de Colonster 1, 4000 Liège, Belgium

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ABSTRACT

Brachycephalic obstructive airway syndrome (BOAS) and canine idiopathic pulmonary fibrosis (CIPF) of West Highland White Terriers (WHWTs) often cause intermittent or chronic hypoxemia. Our objective was to evaluate serum and bronchoalveolar lavage fluid (BALF) concentrations of hypoxemia-related proinflammatory mediators vascular endothelial growth factor A (VEGF-A) and chemokine (C—C motif) ligand 2 (CCL2) in brachycephalic dogs (BDs) and WHWTs with and without CIPF. Additionally, effects of BOAS severity and ageing on these mediators were assessed.

114 BDs (28 English Bulldogs (EBs), 37 French Bulldogs, 49 Pugs), 16 WHWTs with CIPF, 26 healthy WHWTs, and 39 normocephalic control dogs were included. Fifty-four BDs were re-examined after two to three years. Bead-based immunoassay was used for proinflammatory mediator measurements.

Compared with controls, significantly higher serum concentrations of VEGF-A were seen in EBs ($P = 0.009$) and of CCL2 in CIPF and healthy WHWTs ($P < 0.001$; $P = 0.002$). BALF samples were available from controls, EBs, and WHWTs. VEGF-A was significantly lower in EBs ($P < 0.001$) and in CIPF and healthy WHWTs ($P = 0.006$; $P = 0.007$) and CCL2 was higher in CIPF WHWTs ($P = 0.01$) compared with controls. Between visits, only serum VEGF-A significantly decreased in BDs ($P < 0.001$), but breed, BOAS severity, or its change had no significant effect.

In conclusion, in EBs with BOAS proinflammatory changes in VEGF-A were detected in both serum and BALF. Ageing reduced serum VEGF-A in BDs. In WHWTs, our results confirmed earlier findings of CCL2 as an important biomarker for CIPF.

1. Introduction

Hypoxemia has been associated with systemic inflammation in humans (Gozal et al., 2002; Uchiyama et al., 2019) and is partly described also in dogs (Glaus et al., 2004; Rancan et al., 2013; Roels et al., 2015b). Hypoxemia can occur for various reasons, including obstruction during ventilation in brachycephalic obstructive airway syndrome (BOAS) (Hendricks et al., 1987; Hoareau et al., 2012) and end-stage lung disease such as canine idiopathic pulmonary fibrosis (CIPF) in West Highland White Terriers (WHWTs) (Corcoran et al., 1999; Heikkilä et al., 2011; Holopainen et al., 2019). In this study, proinflammatory mediators vascular endothelial growth factor A (VEGF-A) and chemokine (C—C motif) ligand 2 (CCL2) were selected due to their key role in hypoxemia (Gozal et al., 2002; Hayashi et al., 2006). These proteins have remained predominantly unexplored in BOAS thus far, as has the effect of BOAS severity grade and ageing on inflammatory response.

BOAS arises from a congenital reduction of the cranio-facial length

Abbreviations: BOAS, Brachycephalic obstructive airway syndrome; CIPF, Canine idiopathic pulmonary fibrosis; WHWT, West Highland White Terrier; VEGF-A, Vascular endothelial growth factor A; CCL2, Chemokine (C-C motif) ligand 2; BD, Brachycephalic dog; EB, English Bulldog; SDB, Sleep-disordered breathing; OSA, Obstructive sleep apnea; IPF, Idiopathic pulmonary fibrosis; BALF, Bronchoalveolar lavage fluid; FB, French Bulldog.

* Corresponding author.
E-mail address: iida-maria.niinikoski@helsinki.fi (I. Niinikoski).

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without a concurrent reduction in the soft tissues, inducing varying degrees of upper airway obstruction (Oechtering, 2010; Roedler et al., 2013). Mild hypoxemia has been documented in awake brachycephalic dogs (BDs) (Hoareau et al., 2012; Canola et al., 2018; de Melo Dias et al., 2018). Intermittent hypoxemia during sleep has been described in English Bulldogs (EBs), as the result of sleep-disordered breathing (SDB), as in human obstructive sleep apnea (OSA) (Hendricks et al., 1987; Hendricks et al., 1991). In OSA, upper airway obstruction results in recurring cessations in breathing during sleep. In humans, the connection between OSA and systemic inflammation is widely recognized (Svensson et al., 2012; Li and Zheng, 2017; Imani et al., 2020), and observed, among others, as changes in VEGF-A and CCL2 concentrations (Gozal et al., 2002; Kim et al., 2010; Gileles-Hillel et al., 2014). In BDs, inflammatory changes have been detected, consisting of elevated plasma concentrations of tumour necrosis factor α, interleukin −10, −13, −17, and nitric oxide (Rancan et al., 2013) and higher leucocyte and neutrophil counts (Facin et al., 2020). However, no effect of BOAS on C-reactive protein (Planellas et al., 2012; Planellas et al., 2015; Gianella et al., 2019) or interleukin-6 (Rancan et al., 2013) has been found.

WHWTs with CIPF were included in the present study as a model of chronic hypoxemic lung disease to act as a reference disease group. CIPF is an interstitial pulmonary disease with progressive fibrosis, sharing multiple clinical features with human idiopathic pulmonary fibrosis (IPF) (Corcoran et al., 2011; Heikkilä et al., 2011). Previously, elevated serum and bronchoalveolar lavage fluid (BALF) CCL2 concentrations have been linked to the pathogenesis of CIPF in WHWTs (Krafft et al., 2013; Roels et al., 2015b). Furthermore, increased serum CCL2 concentrations found in healthy WHWTs may be related to breed predisposition to CIPF (Roels et al., 2015a). Serum VEGF-A has been previously investigated only in healthy WHWTs, showing concentrations below the ELISA kit detection limit in 89.3% of samples (Roels et al., 2015a).

The aims of this study were twofold. First, to evaluate serum and BALF concentrations of VEGF-A and CCL2 in BDs and in WHWTs with and without CIPF, and in healthy normocephalic dogs. Second, to investigate the effects of BOAS severity grade and ageing on these proinflammatory mediators.

2. Materials and methods

2.1. Ethics approval, study animals, and samples

All animals were privately owned pet dogs. Owners signed an informed consent form. The study protocol was approved by the Committee of Experimental Animals of Southern Finland (ESLH-2008-05403/Ym-23, ESAVI-2010-03587/Ym-23, ESAVI/1005/04.10.03/2011, ESAVI/7383/04.10.07/2013, ESAVI/11519/04.10.07/2014, ESAVI/10906/04.10.07/2017), the Ethical Committee of the University of Liège (no: 1435 and 2245) and by the University of Helsinki Viikki Campus Research Ethics Committee (5B/2008, 1/2014, 13/2020).

This cross-sectional study was performed at the Veterinary Teaching Hospitals of the University of Helsinki, Finland and the University of Liège, Belgium. The study population consisted of 114 BDs, 42 WHWTs, and 39 normocephalic (mesaticephalic or dolicocephalic) control dogs of breeds other than WHWTs. All dogs were examined at the University of Helsinki, except for healthy control dogs with BALF samples, which were examined at the University of Liège. VEGF-A and CCL2 had not been measured from these individuals previously, but the dogs have been included in other studies (Lilja-Maula et al., 2017; Aromaa et al., 2019; Fastrès et al., 2020; Laurila and Rajamäki, 2020; Aromaa et al., 2021). The demographics are shown in Table 1.

The inclusion criteria for the BD group were purebred EB, French Bulldog (FB), or Pug, age between one and five years at first visit, and no history of airway surgery or treatment due to BOAS. BDs were considered healthy by the owner, had no signs of disease barring BOAS-related issues, and had no significant changes on clinical examination. The severity grading of BOAS was performed as previously presented in Lilja-Maula et al. (2017) and based on Liu et al. (2015). The dogs were graded as having no (grade 0), mild (grade 1), moderate (grade 2), or severe (grade 3) BOAS signs. Based on this grading, the dogs were classified as BOAS-negative (BOAS -) (grades 0 and 1) or BOAS-positive (BOAS +) (grades 2 and 3) (Table 2). BOAS severity grade was reassessed for 54/112 dogs (EB $n = 8$, FB $n = 16$, Pug $n = 30$) two to three years after the initial study visit and was noted as remaining constant (31/54), deteriorating by one grade (15/54), or improving by one grade (8/54), as described in Aromaa et al. (2021).

The diagnosis of CIPF was based on all or most of the following: compatible history with characteristic signs, hypoxemia in arterial blood gas analyses, and characteristic findings in thoracic diagnostic imaging or post-mortem histopathology, as described in detail previously (Heikkilä et al., 2011; Holopainen et al., 2019). Healthy WHWTs exhibited no hypoxemia and no signs, clinical examination findings, or abnormal findings suggesting CIPF in thoracic diagnostic imaging.

Healthy normocephalic control dogs showed no signs of illness and had normal physical examination, hematology, and serum biochemistry findings.

Exclusion criteria included corticosteroid or cyclosporine treatment in the two weeks preceding sampling. Dogs with a previous diagnosis of atopy were not excluded.

Stored frozen (−80 °C) serum and BALF samples were used. In WHWTs and control dogs, BALF samples were collected by bronchoscopy as previously described (Krafft et al., 2011; Heikkilä et al., 2011). Briefly, one or two (1–2 mL/kg) aliquots of sterile saline (0.9% NaCl) were instilled into two different lung lobes via a bronchoscope (Olympus

Table 2
Clinical grading of severity of brachycephalic obstructive airway syndrome signs in all 114 brachycephalic dogs.

Class	Negative		Positive	
	$n = 75$		$n = 39$	
Grade	0 = none $n = 11$	1 = mild $n = 64$	2 = moderate $n = 24$	3 = severe $n = 15$

Table 1
Demographics of study dogs.

	EB ($n = 28$)	FB ($n = 37$)	Pug ($n = 49$)	WHWT CIPF ($n = 16$)	WHWT healthy ($n = 26$)	Control dogs with	
						Serum ($n = 25$)	BALF ($n = 14$)
Gender							
Female	13	23	30	8	14	9	10
Male	15	14	19	8	12	16	4
Age (years)							
Median	3.4	2.5	2.8	12.6	10.0	2.6	6.3
Range	2.1–5.3	1.2–5.6	1.0–5.6	9.1–14.9	4.3–14.0	1.3–5.6	1.3–11.3

EB, English bulldog; FB, French bulldog; WHWT CIPF, West Highland White Terrier with canine idiopathic pulmonary fibrosis; BALF, bronchoalveolar lavage fluid.

GIF type N30, Fujinon EB-530, Fujifilm). In BDs, BALF samples were collected only from EBs via catheter with an aliquot of 7–10 mL/lobe.

2.2. Analysis of proinflammatory mediators

Concentrations of VEGF-A and CCL2 were measured from duplicate samples with canine-specific Luminex multiplex immunoassay (ProcartaPlex, Thermo Fisher Scientific, Vienna, Austria). The samples were read on Bio-Plex 200 (Bio-Rad Laboratories, Hercules, California, USA) and analyzed with Bioplex Manager 6.2 software.

Reproducibility was evaluated with replicates in each plate and independent plates. The maximum intra-assay and inter-assay coefficients of variation (CV) for the VEGF-A assay were 15% and 12%, with a lower limit of quantification (LLOQ) of 2.85 pg/mL. The maximum intra-assay and inter-assay CVs for CCL2 were 15% and 4%, respectively, with LLOQ of 13.1 pg/mL. Correction coefficients between assays from different lots were calculated based on the replicates on each plate.

2.3. Statistical analysis

Differences in serum concentrations of VEGF-A and CCL2 at first visit and change between first and follow-up visit were analyzed with multivariate analysis of variance (MANOVA) models. The models included breed, BOAS class, and the interaction between breed and BOAS class as fixed effects. BD breeds were separately compared against controls with analysis of variance (ANOVA) models, with breed or BOAS severity grade as the sole fixed effect. The effect of change in BOAS severity grade on the change in VEGF-A and CCL2 was investigated with an ANOVA model, with the three-class BOAS severity change as a fixed effect. Differences in concentrations of VEGF-A and CCL2 between WHWTs and controls in serum and WHWTs, EBs, and controls in BALF were analyzed with ANOVA models, where group was the sole fixed effect.

For pairwise comparisons, Tukey-Kramer adjustment was used to control for multiplicity, excluding BOAS severity grade analyses, in which Dunnett's adjustment was used. Analyses were conducted in a log-transformed scale to comply with normality assumptions, excluding analyses regarding change between visits, which were conducted in absolute scale. For all statistical analyses, values below the lower limit of detection were replaced with half of LLOQ.

Statistical analyses were done using SAS System for Windows, version 9.4 (SAS Institute Inc., Cary, North Carolina, USA), GraphPad Prism for Macintosh, version 9.3.0 (GraphPad Software, San Diego, California, USA), and IBM SPSS Statistics for Macintosh, version 27.0.1 (IBM Corp., Armonk, New York, USA). *P*-values <0.05 were considered significant.

3. Results

3.1. Serum samples

In the analysis of serum VEGF-A for the first visit, EBs had significantly higher concentration than controls (Fig. 1A). FBs and Pugs did not significantly differ from controls ($P = 0.89$; $P = 0.17$). No differences were detected between BOAS-positive and -negative classes in all BDs ($P = 0.73$). No significant differences were detected between WHWT groups and controls (CIPF WHWTs vs. control $P = 0.08$; healthy WHWTs vs. control $P = 0.25$) or between WHWT groups ($P = 0.66$). No significant differences were detected in CCL2 concentration at first visit for brachycephalic breeds compared with controls ($P = 0.12$) or between BOAS-positive and -negative classes ($P = 0.59$). In the analysis of CCL2 concentration of WHWTs, significantly higher concentrations were detected for both WHWT groups than for controls. Also, within WHWTs, higher concentrations were seen in the CIPF group than in healthy WHWTs (Fig. 1B). Statistically significant differences between BOAS severity grades 0–3 were not found for VEGF-A ($P = 0.86$, Fig. 2A) or CCL2 ($P = 0.70$, Fig. 2B) for the first visit.

In all BDs, the concentration of VEGF-A significantly decreased from

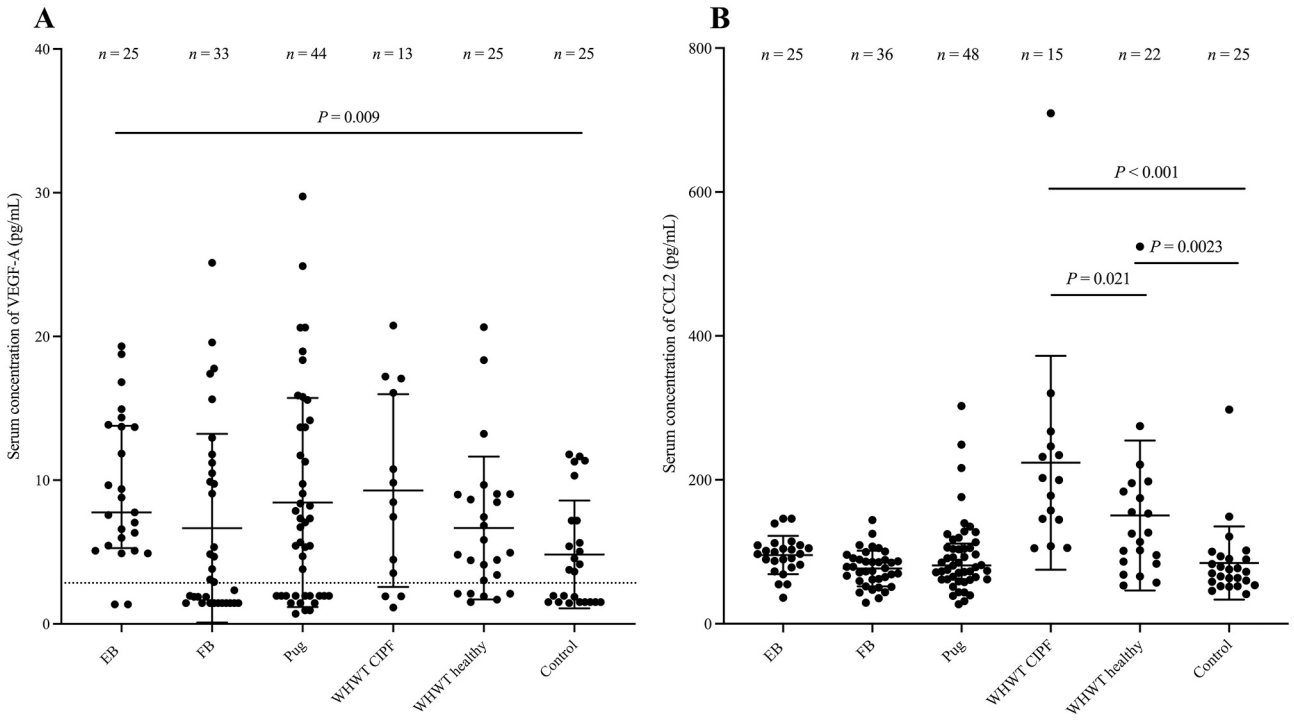

Fig. 1. Scatter plot with median and interquartile range of (A) vascular endothelial growth factor A (VEGF-A) and (B) chemokine (C—C motif) ligand 2 (CCL2) in serum samples from English bulldogs (EBs), French bulldogs (FBs), Pugs, and West Highland White Terriers (WHWTs) affected with canine idiopathic pulmonary fibrosis (CIPF) and healthy WHWTs, and control dogs of other breeds. The dashed line indicates the lower limit of quantification. Significant differences ($P < 0.05$) compared with healthy dogs are indicated.

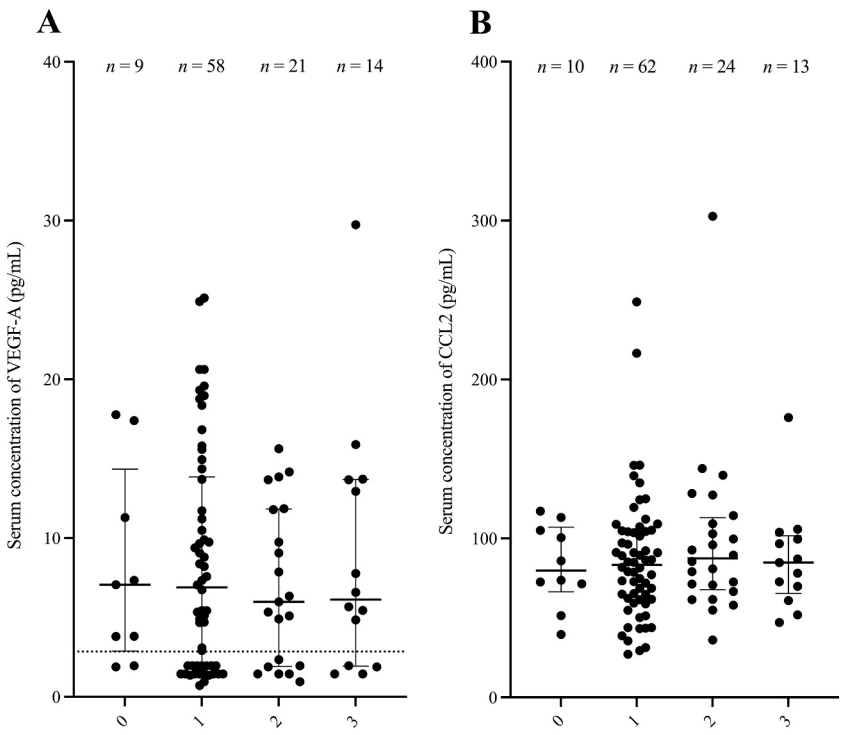

Fig. 2. Scatter plot with median and interquartile range of (A) vascular endothelial growth factor A (VEGF-A) and (B) chemokine (C—C motif) ligand 2 (CCL2) in serum samples from all brachycephalic dogs with brachycephalic obstructive airway syndrome (BOAS) severity grades 0–3 (0 = no; 1 = mild; 2 = moderate; 3 = severe). The dashed line indicates the lower limit of quantification. No significant differences between BOAS severity grades were found.

first to second visit (Fig. 3A). No differences for brachycephalic breeds ($P = 0.97$) or between BOAS-positive and -negative classes ($P = 0.37$) were seen. There was no significant change in CCL2 between visits ($P = 0.07$, Fig. 3B). There were no differences for brachycephalic breeds ($P = 0.79$) or between BOAS-positive and -negative classes ($P = 0.89$) in the change in CCL2 between visits. Change in BOAS severity grade did not have a significant effect on either VEGF-A ($P = 0.48$) or CCL2 ($P = 0.43$) between visits.

3.2. BALF samples

EBs and both WHWT groups had significantly lower BALF VEGF-A concentrations than controls (Fig. 4A). No significant differences emerged between CIPF or healthy WHWT groups and EBs ($P = 1.0$, $P =$

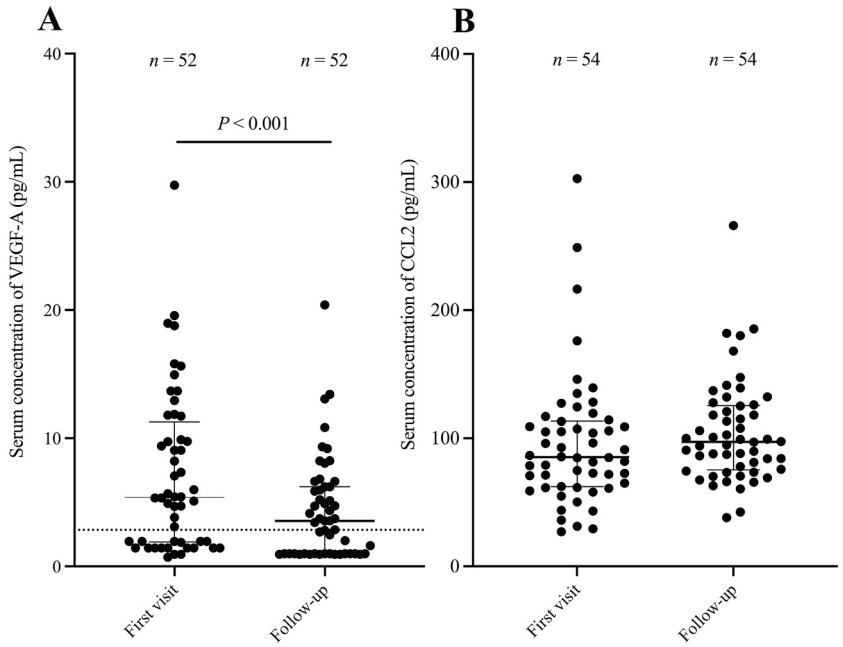

Fig. 3. Scatter plot with median and interquartile range of (A) vascular endothelial growth factor A (VEGF-A) and (B) chemokine (C—C motif) ligand 2 (CCL2) in serum samples between the first and the follow-up visit of brachycephalic dogs. The dashed line represents the lower limit of quantification. Significant differences ($P < 0.05$) are indicated.

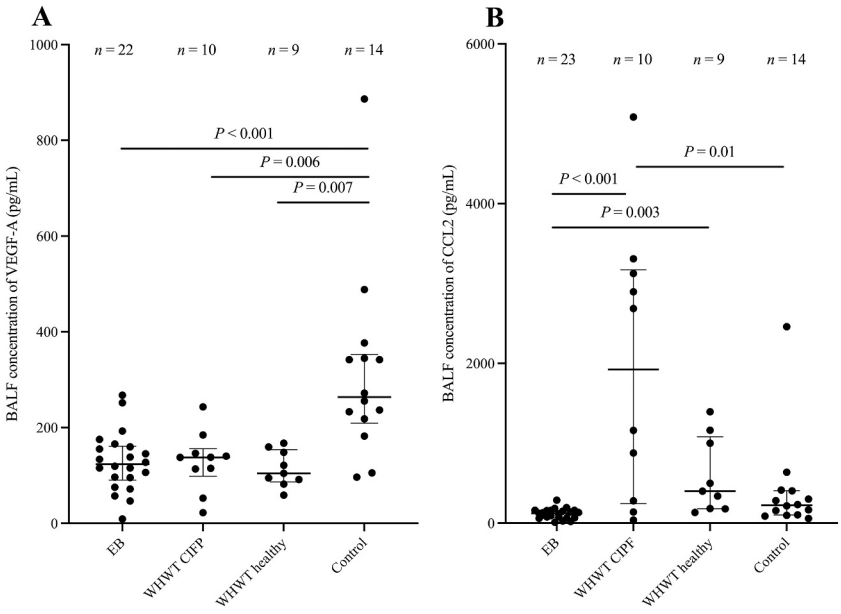

Fig. 4. Scatter plot with median and interquartile range of (A) vascular endothelial growth factor A (VEGF-A) and (B) chemokine (C—C motif) ligand 2 (CCL2) in bronchoalveolar lavage fluid (BALF) samples from English bulldogs (EBs), West Highland White Terriers (WHWTs) affected with canine idiopathic pulmonary fibrosis (CIPF) and healthy WHWTs, and control dogs of other breeds. No samples were below the lower limit of quantification. Significant differences ($P < 0.05$) are indicated.

1.0) or within WHWT groups ($P = 1.0$). In CCL2, WHWTs with CIPF had significantly higher concentrations than controls (Fig. 4B). EBs had significantly lower CCL2 concentrations than CIPF WHWTs and healthy WHWTs (Fig. 4B). There were no significant differences between EBs and controls ($P = 0.06$), between WHWT groups ($P = 0.34$), or between healthy WHWTs and controls ($P = 0.52$).

4. Discussion

We found higher serum VEGF-A and lower BALF VEGF-A concentrations to be suggestive of a proinflammatory condition in EBs with BOAS. Although BOAS severity class or its change had no effect, ageing decreased serum VEGF-A. High serum CCL2 concentrations in both healthy and CIPF WHWTs and high BALF CCL2 in CIPF WHWTs reinforced the perception of breed predisposition to CIPF and of CCL2 as a biomarker for the disease.

VEGF-A is considered an important proinflammatory mediator in hypoxemia, inducing vascular growth and increasing vascular permeability (Carmeliet et al., 1996; Raja et al., 2014). Increased serum VEGF-A values have been reported in chronically hypoxemic human patients, but also in patients with intermittent hypoxemia such as in OSA (Zhang et al., 2017). In our study, arterial blood gas analyses from BDs were not available, but previously mild decreases in healthy awake BDs in partial pressure of arterial oxygen have been described (Hoareau et al., 2012; Canola et al., 2018; de Melo Dias et al., 2018). Additionally, Hendricks et al. (1987) noted intermittent hypoxemia in EBs due to SDB resembling OSA. Unfortunately, the number of EBs in the different BOAS grades was too small to allow comparison between severity grades within the breed. When examining across all three BD breed groups, no differences in VEGF-A concentrations were seen between severity grades. Since BDs were owner-reported to be healthy, breeding-aged dogs mostly with mild or moderate signs related to BOAS, it may be that there was not enough variation in BOAS severity, in contrast to a study examining other biomarkers in dogs with severe BOAS (Planellas et al., 2015). Additionally, SDB in this population remains unexplored.

CIPF WHWTs were included as a model of chronic marked hypoxemia. Although no statistical difference between CIPF and control group was shown, a trend towards higher VEGF-A in CIPF, significant at 8%, was seen. Previously, elevated serum VEGF-A concentrations have been measured in healthy dogs with similar hypoxemia due to staying at high altitudes (Glaus et al., 2004). Roels et al. (2015a) showed no significant increase in VEGF in healthy WHWTs compared with healthy dogs of other breeds, but only 10.7% of samples in this breed predisposition study were above the ELISA detection limit. Additionally, no comparison to CIPF WHWTs was performed in this study. In human IPF, both unchanged (Meyer et al., 2000) and elevated (Ando et al., 2010; Barratt et al., 2017) VEGF concentrations have been reported. The reason for this may be differential expression of VEGF-A isoforms, resulting in a change in their ratio (Barratt et al., 2017). Since the immunoassay used in our study detected pan-VEGF-A and not just isoform VEGF-A_{165}b, which seems principally increased in IPF (Barratt et al., 2017), this may well apply also to our findings. However, VEGF plays a key role in IPF since nintedanib, a molecule with VEGF receptor antagonistic properties, is registered for IPF treatment (Lancaster et al., 2019).

Several confounders, such as age and physical activity, may influence results. Even short 10-minute activity increases serum VEGF-A (Wenk, 2004). Although all dogs were examined for other diseases, subclinical conditions, such as neoplasia (de Queiroz et al., 2013), cannot be completely ruled out. Atopic dermatitis, a common issue in the breeds in our study, has not however been shown to increase serum VEGF-A (Cobiella et al., 2020).

We found a significant decrease in VEGF-A concentrations in all BDs between visits, regardless of breed or BOAS severity class. This may be due to ageing, similarly to humans (Saldías et al., 2017).

Significant differences for serum CCL2 were not found between BDs and controls or between BOAS severity grades or classes. In hypoxemia, CCL2 is produced by endothelial cells to induce leucocyte migration to inflamed tissue and is thought to contribute to the development of cardiovascular disorders associated with OSA (Hayashi et al., 2006). However, CCL2 concentration reflects the length of hypoxemia in humans, as an association between CCL2 concentration and OSA severity has been reported (Kim et al., 2010; Gileles-Hillel et al., 2014). Since the BDs were mostly mildly affected by BOAS, it is possible that the length of BOAS-related hypoxemia was insufficient to elevate CCL2.

Confirming earlier findings (Krafft et al., 2013; Roels et al., 2015a; Roels et al., 2015b) with new cohorts, we found that serum CCL2 was higher in both healthy WHWTs and WHWTs with CIPF than in controls, but also in CIPF WHWTs relative to healthy WHWTs. A predisposing role for CCL2 in the development of CIPF as well as human IPF has been postulated (Roels et al., 2015a; Gui et al., 2019). CCL2 stimulates collagen secretion and promotes the generation of abundant extracellular matrix (Gharaee-Kermani et al., 2003; Moore et al., 2005; Inomata

et al., 2014).

CCL2 concentrations did not change between visits in BDs. In contrast to VEGF-A, ageing has not been shown to significantly affect serum CCL2 (Ishioka et al., 2013).

In BALF, we observed significantly lower VEGF-A concentrations in both EBs and WHWTs with and without CIPF than in controls, and no difference between EBs and WHWT groups. It must be noted that EBs had no findings of lung disease, and therefore, the mechanism behind this remains unknown. To the authors' knowledge, VEGF-A in canine BALF has not been explored previously. Lower VEGF BALF concentrations have been reported in human IPF and in other chronic lung diseases (Meyer et al., 2000; Koyama et al., 2002). VEGF is critical in maintaining normal lung physiology (Berse et al., 1999), and the mechanism underlying the decline in BALF may be related to an inflammatory state. Inflammation decreases the number of alveolar epithelial cells and increases the concentration of proteases, leading to degradation of VEGF-A (Koyama et al., 2002; Barratt et al., 2017). Contrarily, serum VEGF-A increases in hypoxemic conditions, possibly originating from hypoxemia-stimulated monocytes, which leads to the difference in concentrations between BALF and serum (Ando et al., 2010).

As in serum, in BALF WHWTs with CIPF had significantly elevated CCL2 concentrations compared with controls. Roels et al. (2015b) showed significantly increased BALF CCL2 in WHWTs with CIPF relative to healthy WHWTs, as has been detected in human IPF (Baran et al., 2007). Although in our study healthy WHWTs had significantly higher serum CCL2 than controls, no such difference was noted in BALF, possibly because of the limited group size.

Our study has some limitations. Due to the lack of arterial blood samples and objective sleep measurements evaluating possible hypoxemia during sleep in BDs, their oxidation status and the degree of SDB are unknown. Since the BDs were owner-reportedly healthy, breeding-aged dogs, the most severely affected dogs were few. Lastly, the number of BALF samples was low, and in BDs, BALF samples were collected only in EBs.

5. Conclusions

Increased concentration of VEGF-A in serum and decreased concentration in BALF suggest a proinflammatory condition in EBs that may be linked to BOAS. CCL2, however, seems to be unaffected in BOAS. Ageing may decrease serum VEGF-A concentrations but has no effect on CCL2. Serum and BALF concentrations in healthy WHWTs and WHWTs with CIPF confirm previous findings of CCL2 as an important biomarker for CIPF.

Declaration of Competing Interest

None.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2022.09.022.

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