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Highlights

Research in Veterinary Science xxx (2015) xxx–xxx

Evaluation of chemokines CXCL8 and CCL2, serotonin, and vascular endothelial growth factor serum concentrations in healthy dogs from seven breeds with variable predisposition for canine idiopathic pulmonary fibrosis

E. Roels [a,*], E. Krafft [a], N. Antoine [b], F. Farnir [c], H.P. Laurila [d], S. Holopainen [d], M.M. Rajamäki [d], C. Clercx [a]

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- Serum CXCL8 concentrations are elevated in healthy dogs from the WHWT breed.
- Serum CCL2 concentrations are elevated in healthy dogs from the WHWT and M breeds.
- No relevant interbreed differences exist for serum 5-HT concentrations regarding CIPF predisposition.
- Serum VEGF concentrations were below the kit detection limit in 89.3% of samples tested.

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Evaluation of chemokines CXCL8 and CCL2, serotonin, and vascular endothelial growth factor serum concentrations in healthy dogs from seven breeds with variable predisposition for canine idiopathic pulmonary fibrosis

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ABSTRACT

The West Highland white terrier (WHWT) is particularly prone to canine idiopathic pulmonary fibrosis (CIPF). We hypothesized that higher circulating concentrations of chemokines CXCL8, CCL2, serotonin (5-HT), or vascular endothelial growth factor (VEGF) could serve as predisposing factors for CIPF development in the WHWT breed. Serum samples from 103 healthy dogs of seven different breeds variably predisposed to CIPF were collected. Serum CXCL8 concentrations were higher in healthy WHWT compared with each of the other groups of healthy dogs. Serum CCL2 concentrations were higher in healthy WHWT and Maltese compared with King Charles spaniels and Malinois Belgian shepherds. No relevant inter-breed differences were observed for serum 5-HT concentrations regarding CIPF predisposition. VEGF values from 89.3% of samples tested were below the kit detection limit. In conclusion, high CXCL8 blood concentrations and possibly CCL2 concentrations might be related to the breed predisposition of the WHWT for CIPF and warrants further investigations.

1. Introduction

Canine idiopathic pulmonary fibrosis (CIPF) is a progressive parenchymal lung disease of unknown origin, which mainly affects older dogs of the West Highland white terrier (WHWT) breed (Heikkilä-Laurila and Rajamäki, 2014). Rare cases have also been described in other terrier breeds such as the Staffordshire terrier (Lobetti et al., 2001) and the Scottish terrier (Krafft et al., 2011). Pathogenesis of CIPF is currently unknown, although a genetic basis is strongly suspected due to the breed predisposition (Heikkilä-Laurila and Rajamäki, 2014). CIPF shares several clinical features with human IPF (Corcoran et al., 1999; Lobetti et al., 2001; Heikkilä et al., 2011); however, there are minor histopathological differences between these entities (Syrjä et al., 2013). In human IPF, chronic alveolar epithelial cell injuries and subsequent dysregulated tissue repair are considered to be the main pathological processes involved in the pathogenesis of this fibroproliferative disease (Coward et al., 2010; Raghu et al., 2011). The mechanisms of repair initiated by a tissue injury are complex and are determined by the presence of biological mediators such as growth factors and chemokines, which coordinate most aspects of the inflammatory and subsequent repair responses. Consequently, we hypothesized that higher circulating concentrations of pro-fibrotic molecules in dogs from the WHWT breed may serve as predisposing factors for CIPF development, by contributing to exacerbated tissue repair after an injury, leading subsequently to the development of fibrosis. This hypothesis is further supported by a recent publication focused on transforming growth factor beta 1 (TGF-β1) demonstrating higher serum TGF-β1 concentrations in healthy dogs from breeds predisposed to CIPF in comparison with breeds not predisposed to the disease (Krafft et al., 2014). Therefore, the aim of the present study was to compare basal circulating blood concentrations of four molecules of interest obtained in healthy dogs from seven breeds differently predisposed to CIPF. Selection of molecules studied was based on their potential implication into the pathogenesis of canine pulmonary fibrosis in view of data from either human or canine literature and were as follows: the chemokine (C-X-C motif) ligand 8 (CXCL8) (Cui et al., 2010; Ahn et al., 2011; Krafft et al., 2013), the chemokine (C-C motif) ligand 2 (CCL2) (Krafft et al., 2013; Moore, 2014), the serotonin (5-HT) (Konigshoff et al., 2010; Krafft et al., 2013), and the vascular endothelial growth factor (VEGF) (Ando et al., 2010; Woodcock et al., 2013).

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2. Materials and methods

2.1. Source of samples and ethics statement

A total of 103 healthy dogs from seven different breeds were included in this study. Ninety-three of these dogs were examined and sampled at the University of Liege, Belgium, and the remaining 10 at the University of Helsinki, Finland. Breeds selected were WHWT, Scottish terrier (ST), Jack Russell terrier (JRT), Maltese (M), King Charles spaniel (KCS), Labrador retriever (LR), and Malinois Belgian Shepherd (MBS). Those breeds were considered as highly (WHWT), possibly (ST, JRT), or not (M, KCS, LR, MBS) –CIPF predisposed breeds. ST and JRT were chosen as terrier breeds potentially predisposed to CIPF; given that one CIPF case has already been confirmed by histopathology in a dog from the ST breed (Krafft et al., 2011) and that CIPF has been clinically suspected in five JRT, but not histopathologically confirmed (personal observations). M breed was chosen as a non-terrier breed sharing similarities in weight and size with the WHWT breed. KCS breed was chosen as another small-size non-terrier breed predisposed for degenerative mitral valve disease (DMVD), another fibrotic disease (Borgarelli and Buchanan, 2012). LR and MBS breeds were chosen as large breeds definitively not predisposed to fibrotic lung disease. All dogs included were privately owned, and samples were obtained after acquiring the written consent of the owners. The study protocol was approved by the Committee of Experimental Animals of the University of Liège, Belgium (permit number: 1435, date of approval: 14 March 2013) and by the equivalent committee of Western Finland (permit number: ESLH-2008-05403, date of approval: 27 June 2008; ESAVI/7383/04.10.07/2013, date of approval: 13 November 2013). Detailed features of the study population are summarized in Table 1. Health status was based on complete history and physical examination in all dogs, in addition to routine hematologic and serum biochemical examinations in 87% and 79% of dogs, respectively. A thoracic high-resolution computed tomography (HRCT) was performed in nine out of 18 clinically healthy WHWT and did not reveal any abnormalities. Besides, all included WHWT were followed up at various time intervals after blood sampling. Thirteen of them were still alive at the time of writing, 1.1–6 years after sampling of the blood used in the present study. So far, none of them have developed any respiratory complaint as determined by telephone consultation with the owners. The remaining five dogs were euthanized or died within 6 months to 4.6 years after blood sampling, for reasons unrelated to the respiratory system; lung tissue samples were available in three of these dogs (2.6, 4.4, and 4.6 years after blood sampling, respectively). On lung histopathology, mild interstitial fibrosis was noticed in all three dogs, but none of them ever displayed any signs compatible with CIPF. Given the time interval between blood sampling and lung histopathology and the absence of clinical signs, blood samples from those three dogs were not discarded.

2.2. Samples processing

Blood samples were obtained in plain tubes from all dogs. Thirty minutes after blood collection, tubes were centrifuged at 4 °C for 15 min at 1300 × *g*. Serum was harvested and transferred into 1.5 mL plastic cryotubes, and samples were stored at −80 °C until analysis. Serum CXCL8, CCL2, and VEGF measurements were performed using commercial canine ELISA kits (R&D Systems) in duplicate, according to the manufacturer's instructions. Serum 5-HT measurement was performed using a commercial human ELISA kit (IBL international) previously validated in dogs (Ljungvall et al., 2013).

2.3. Statistical methods

Descriptive statistics (XLStat software, Addinsoft) was used for clinical (gender, age, and weight), biochemical, and hematologic results; data were reported as median and range. Serum CXCL8, CCL2, and 5-HT concentrations were compared between breeds using a global linear model (SAS software, SAS Institute Inc.) integrating the effects of age and gender as covariables; data were expressed as least square mean ± SE. Proportions were compared using the Chi^2 test with the threshold 5%, data were expressed as percentage (XLStat software, Addinsoft). *P*-values ≤ 0.05 were considered as significant.

Table 1
Characteristics of the study population.

Breed	*N*	Sex, male/female	Age, yr (median, range)	Weight, kg (median, range)
WHWT	18	8/10	9.2 (2.9–16.9)	8.7 (7.4–12.0)
ST	14	3/11	5.1 (0.9–9.5)	10.0 (8.5–13.6)
JRT	16	2/14	7.2 (1.0–11.8)	6.9 (5.9–12.6)
M	15	4/11	6.2 (0.9–12.6)	5.3 (4.0–9.0)
KCS	14	5/9	5.8 (0.5–10.3)	8.3 (6.8–12.0)
LR	12	5/7	5.7 (1.6–12.2)	36.4 (23.0–42.0)
MBS	14	10/4	5.5 (1.5–7.8)	31.2 (23.0–35.0)

WHWT, West Highland white terrier. ST, Scottish terrier. JRT, Jack Russell terrier. M, Maltese. KCS, King Charles spaniel. LR, Labrador retriever. MBS, Malinois Belgian Shepherd.

3. Results and discussion

3.1. Physical, biochemical, and hematologic examination

Physical examination was unremarkable in all dogs included in this study. Dogs did not have any signs or findings indicating disease. Biochemical and hematologic data are summarized in Table 2. Most measured parameters were within laboratory reference ranges or only discreetly increased, except for alkaline phosphatase (ALP) and platelet count. ALP was above the upper limit of reference range in most dogs from WHWT (73%) and ST (75%) breeds; these percentages were high in comparison with those obtained in M (8%), KCS (7%), LR (13%), and MBS (0%) ($P < 0.0001$). These observations are in agreement with previously published data indicating an increased ALP activity in dogs from the WHWT and ST breeds (Gallagher et al., 2006; Nestor et al., 2006; Heikkilä et al., 2011), and possibly attributed in ST to benign subclinical hyperadrenocorticism (Zimmerman et al., 2010). Whether such phenomenon also exists in the WHWT breed has not been investigated. Platelet count was below the lower limit of reference range in most dogs from KCS (54%) breed compared with other breeds: WHWT (0%), ST (7%), JRT (0%), M (0%), LR (29%), and MBS (23%) ($P = 0.002$ vs. WHWT, JRT, and M). In thrombocytopenic KCS, macrothrombocytes were observed on blood smear. This finding can be related to the existence of an autosomal mutation in beta1-tubulin gene in KCS leading to asymptomatic macrothrombocytopenia in this breed (Davis et al., 2008). On the opposite, platelet count was most frequently above the upper limit of reference range in WHWT dogs (39%) compared with other breeds: ST (21%), JRT (17%), M (8%), KCS (15%), LR (0%), and MBS (8%) ($P = 0.147$). Thrombocytosis in apparently healthy WHWT, although not significant in the present study, has already been reported previously (Heikkilä et al., 2011). In human IPF patients, increased platelet reactivity has recently been demonstrated and may participate to the fibroproliferative process observed in the disease by the release of pro-fibrotic mediators such as TGF-β1 (Fernandez and Eickelberg, 2012; Crooks et al., 2014). In dogs, although the reactivity of platelets has not been investigated, a similar hypothesis might explain why breeds with high basal platelet numbers would be more prone to develop lung fibrosis.

3.2. CXCL8 and CCL2 concentrations

Serum CXCL8 and CCL2 concentrations (pg/mL) are presented in Figs. 1 and 2, respectively. Significantly higher serum CXCL8 concentrations were observed in healthy WHWT in comparison with all other groups of healthy dogs ($P \leq 0.05$). Significant differences were also

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Table 2
Biochemical and hematologic data.

Breed	WHWT	ST	JRT	M	KCS	LR	MBS	References
TP, (g/L)	62 (52–74)	67 (54–87)	59 (50–73)	61 (52–70)	65 (60–75)	67 (65–72)	68 (56–71)	60–80
Creat, (μmol/L)	79.3 (65.0–115.7)	81.7 (60.8–93.0)	89.3 (74.1–106.4)	90.2 (78.9–101.9)	73.1 (55.7–97.5)	112.5 (57.2–144.4)	80.4 (51.3–122.3)	<133
ALT, (IU/L)	37 (23–156)	47 (26–158)	71 (47–133)	34 (23–156)	33 (19–288)	58 (39–119)	50 (23–73)	5–62
ALP, (IU/L)	191 (40–654)	176 (32–816)	64 (24–235)	40 (9–136)	63 (29–125)	56 (23–269)	54 (21–117)	12–121
Ht, (%)	51 (38–59)	58 (46–67)	50 (43–59)	48 (42–54)	42 (34–47)	49 (35–56)	52 (46–58)	37–55
WBC, ($\times 10^6$ μ/L)	7820 (4130–11800)	7925 (4590–22200)	9160 (6830–11900)	7340 (4770–14600)	9040 (6020–15160)	8070 (4480–18100)	9000 (5970–13400)	6000–15000
Plt, ($\times 10^3$ μ/L)	446 (254–754)	383 (136–543)	414 (248–554)	437 (248–535)	180 (54–625)	225 (72.5–340)	247 (92–560)	200–500

WHWT, West Highland white terrier. ST, Scottish terrier. JRT, Jack Russell terrier. M, Maltese. KCS, King Charles spaniel. LR, Labrador retriever. MBS, Malinois Belgian Shepherd. TP, total protein. Creat, creatinine. ALT, alanine-amino transferase. ALP, alkaline phosphatase. Ht, hematocrit. WBC, white blood cell count. Plt, platelet count. Data are expressed as median and range.

noticed between M and both KCS and LR ($P \leq 0.008$). Serum CCL2 concentrations were significantly higher in healthy WHWT and M in comparison with KCS and MBS ($P \leq 0.05$). A significant difference for serum CCL2 concentrations was also observed between LR and MBS ($P = 0.04$). Effects of age and gender on serum CCL2 and CXCL8 concentrations were not significant.

In humans, both CXCL8 and CCL2 concentrations were found to be increased in blood (Ziegenhagen et al., 1998a; Suga et al., 1999; Fujiwara et al., 2012) and bronchoalveolar lavage fluid (BALF) (Capelli et al., 2005; Antoniou et al., 2006; Baran et al., 2007) of IPF patients compared with healthy volunteers and correlated with lung function (Capelli et al., 2005; Emad and Emad, 2007; Martina et al., 2009; Vasakova et al., 2009), disease progression (Ziegenhagen et al., 1998b; Totani et al., 2002), and outcome (Shinoda et al, 2009; Richards et al., 2012). Furthermore, several studies suggested an involvement of the chemokine CCL2 in the pathogenesis of IPF, notably through its action on resident pulmonary fibroblast and circulating fibrocytes, promoting the generation of abundant extracellular matrix in the lungs (Gharaee-Kermani et al., 1996; Phillips et al., 2004; Moore et al., 2005; Inomata et al., 2014). Such strong evidence involving CXCL8 in the pathogenesis of the disease are lacking, although this chemokine is thought to act as a pro-fibrotic factor in IPF via the promotion of exacerbated

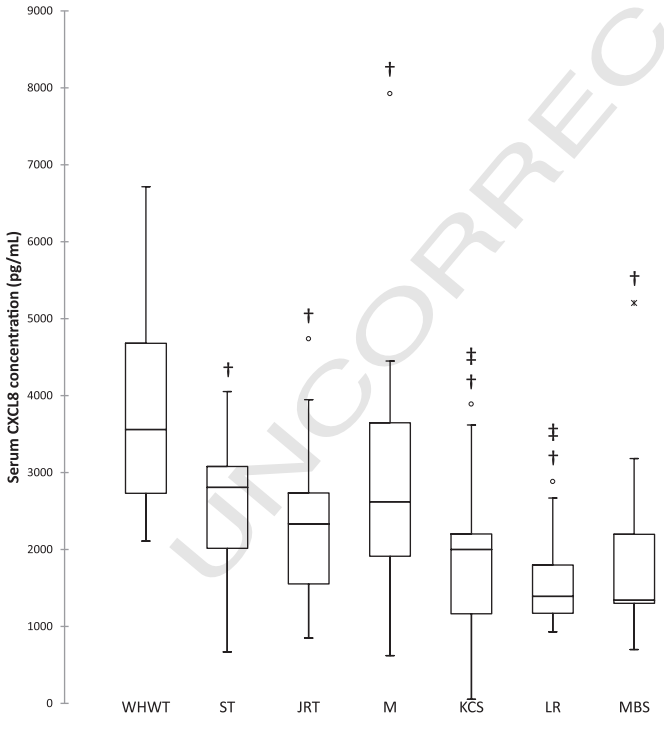

Fig. 1. Box plot of serum CXCL8 concentrations (pg/mL) obtained from healthy West Highland white terriers (WHWT, n = 18), Scottish terriers (ST, n = 14), Jack Russell terriers (JRT, n = 16), Maltese (M, n = 15), King Charles spaniels (KCS, n = 14), Labrador retrievers (LR, n = 12), and Malinois Belgian Shepherds (MBS, n = 14). The box represents the interquartile range, with the median indicated by the horizontal line. The whiskers extend from the minimum to the maximum values, excluding outliers that are presented by an open circle or extreme outliers that are presented by asterisks. †Statistically different from WHWT ($P \leq 0.05$). ‡Statistically different from M ($P \leq 0.008$).

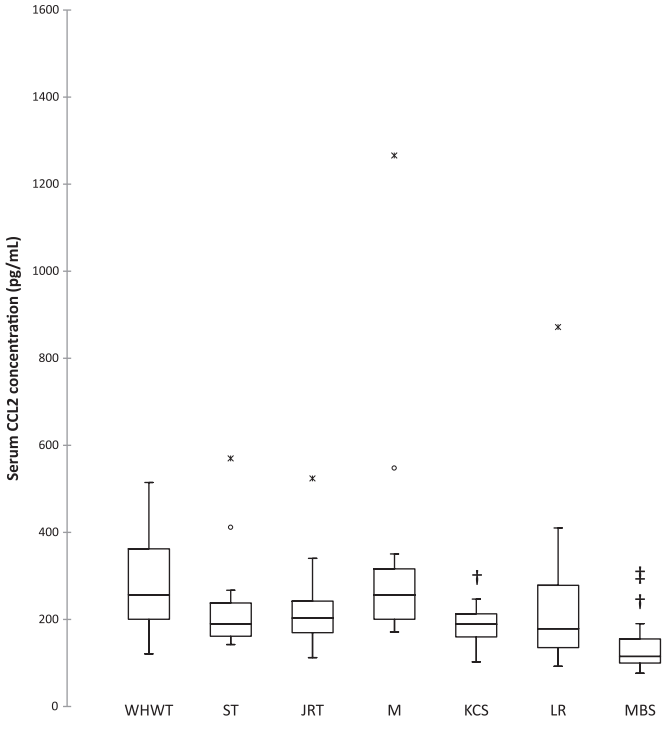

Fig. 2. Box plot of serum CCL2 concentrations (pg/mL) obtained from healthy West Highland white terriers (WHWT, n = 18), Scottish terriers (ST, n = 14), Jack Russell terriers (JRT, n = 16), Maltese (M, n = 15), King Charles spaniels (KCS, n = 14), Labrador retrievers (LR, n = 12), and Malinois Belgian Shepherds (MBS, n = 14). The box represents the interquartile range, with the median indicated by the horizontal line. The whiskers extend from the minimum to the maximum values, excluding outliers that are presented by an open circle or extreme outliers that are presented by asterisks. †Statistically different from WHWT and M ($P \leq 0.01$). ‡Statistically different from L ($P = 0.04$).

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angiogenesis (Strieter et al., 2002; Rosenkilde and Schwartz, 2004; Antoniou et al., 2006; Martina et al., 2009; Cui et al., 2010).

In dogs, mRNA expression of CXCL8 and CCL2 was found to be increased in CIPF lungs compared with controls (Krafft et al., 2013). Higher CXCL8 concentrations were found in BALF, but not in serum, of WHWT with CIPF in comparison with healthy WHWT, and both BALF and serum CCL2 concentrations were shown to be increased in WHWT with CIPF compared with healthy WHWT (Krafft et al., 2013; personal communications).

In the present study, finding various serum CXCL8 and CCL2 concentrations in healthy dogs from different breeds suggests that such concentrations might be genetically determined in dogs. Although not proven, a cause–effect relationship between the development of CIPF and the high circulating CXCL8 and CCL2 concentrations observed in healthy WHWT might exist. A similar hypothesis was already proposed for serum TGF-β1 concentrations which were also found to be increased in healthy WHWT compared with breeds less predisposed to CIPF (Krafft et al., 2014). However, not all dogs from the WHWT breed develop the disease at an advanced age. Therefore, the high serum TGF-β1, CXCL8, or even CCL2 concentrations found in apparently healthy WHWT might serve as one of the multiple predisposing factors for CIPF development by triggering an inappropriate lung response after an injury, leading subsequently to pulmonary fibrosis. In humans, whether healthy people with increased CXCL8, CCL2, or TGF-β1 blood concentrations are specifically at risk for development of fibrosis has not been studied. Nevertheless, some studies highlighted the fact that IPF patients with a high TGF-β1 producing genotype are incline to have a worse prognosis and a more rapid deterioration in lung function (Arkwright et al., 2000; Alhamad et al., 2013). A single nucleotide polymorphism (rs4073T > A) was also recently found in the promoter of the CXCL8 gene and was significantly associated with higher BALF CXCL8 concentrations and an increased risk of development of IPF in humans (Ahn et al., 2011). Existence of such polymorphism in CIPF dogs has not yet been investigated.

Increased serum CCL2 concentrations observed in healthy M in the present study does not seem to predispose this breed to a fibrotic disease. In human medicine, the chemokine CCL2, while involved in the pathogenesis of IPF, is also involved in a variety of other diseases, ranging from immune-mediated and vascular diseases to cancer (Locati et al, 2005). Whether the high serum CCL2 concentrations observed in healthy M might predispose this breed to specific pathological conditions is unknown.

3.3. Serum 5-HT concentrations

Serum 5-HT concentrations (ng/mL) obtained in healthy KCS, ST, M, and MBS were significantly higher in comparison with those obtained in healthy WHWT, JRT, and LR ($P \leq 0.05$). Data are presented in Fig. 3. Effects of age and gender were not significant. These results do not indicate relevant interbreed differences regarding CIPF predisposition and are not in favor of any influence of basal 5-HT concentrations on CIPF development in WHWT. In humans, altered regulation of the serotonin pathway is thought to be associated with the development of pulmonary fibrosis since an increased expression of 5-HT receptors was found in IPF lungs (Fabre and Crestani, 2010; Konigshoff et al., 2010) and anti-serotonin therapy was shown to attenuate induced pulmonary fibrosis in mice (Konigshoff et al., 2010; Skurikhin et al., 2012). In dogs, 5-HT has essentially been studied in the pathogenesis of the DMVD, another fibrotic disease (Oyama and Levy, 2010; Ljungvall et al., 2013; Cremer et al., 2014; Mangklabruks and Surachetpong, 2014). Increased 5-HT blood concentrations were found in healthy KCS in comparison with healthy dogs from other breeds predisposed or not to DMVD (Arndt et al., 2009). In the present study, although KCS displayed the highest serum 5-HT concentration, the difference with other breeds was only significant in comparison with WHWT, JRT, and LR.

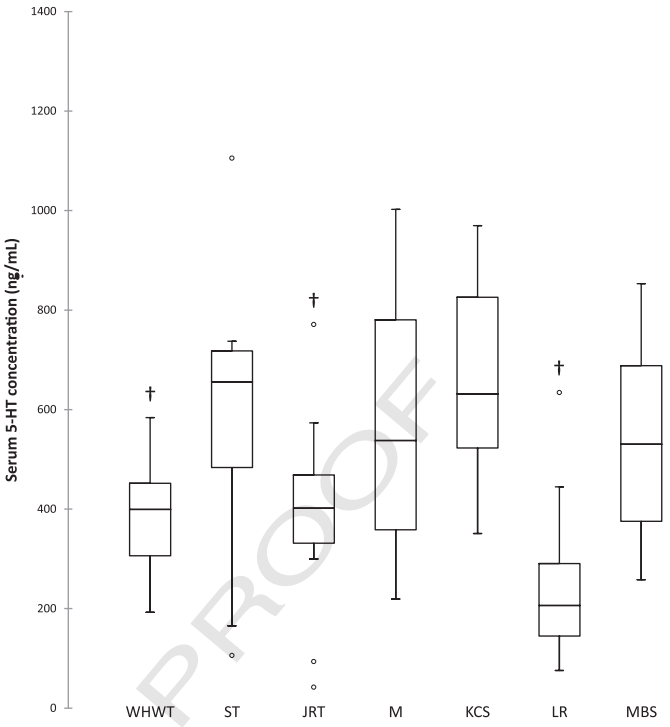

Fig. 3. Box plot of serum 5-HT concentrations (ng/mL) obtained from healthy West Highland white terriers (WHWT, n = 18), Scottish terriers (ST, n = 14), Jack Russell terriers (JRT, n = 16), Maltese (M, n = 15), King Charles spaniels (KCS, n = 14), Labrador retrievers (LR, n = 12), and Malinois Belgian Shepherds (MBS, n = 14). The box represents the interquartile range, with the median indicated by the horizontal line. The whiskers extend from the minimum to the maximum values, excluding outliers that are presented by an open circle or extreme outliers that are presented by asterisks. †Statistically different from ST, M, KCS, and MBS ($P \leq 0.05$).

3.4. Serum VEGF concentrations

The majority of samples tested for serum VEGF concentrations, 92 out of 103 (89.3%), were below the ELISA kit detection limit (39.1 pg/mL). By consequence, a quantitative comparison between breeds was not possible. Results above the kit detection limit were found in 3 KCS (21.4%), 3 JRT (18.8%), 3 LR (25.0%), 1 WHWT (5.6%), and 1 ST (7.1%). Frequency of positive results was not different between breed groups ($P = 0.147$). In humans, VEGF was found significantly decreased in BALF of IPF patients compared with healthy volunteers (Koyama et al., 2002) and serum concentrations were found to correlate with the disease progression (Ando et al., 2010). Moreover, nintedanib, a tyrosine kinase receptor antagonist which inhibits a number of key receptors including the VEGF receptor, was proven to slow down the progression of the disease and to improve the quality of life in patients with IPF (Woodcock et al., 2013). These observations made in human IPF enhance the interest toward the VEGF molecule in CIPF, although results of the present study were not conclusive due to the assay limitation.

3.5. Limitations

Limitations of the present study were that only half of the healthy WHWT underwent a thoracic HRCT and that thoracic X-rays were not available for the healthy other dogs included in this study. Moreover, although CIPF HRCT findings were described in detail (Johnson et al., 2005; Heikkilä et al., 2011), the sensitivity of this imaging technique for detection of early lung lesions has not been established. Therefore, some included WHWT, even the ones that underwent a thoracic HRCT, might already have subclinical CIPF lesions at the time of

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sampling, which could have interfered with the results of the present study. This is unlikely, in view of absence of development of respiratory clinical signs in time intervals ranging from 6 months to 6 years after blood sampling. However, the mild interstitial fibrosis noticed on lung histopathology from three included WHWT (2.6–4.6 years after blood sampling), without evidence of CIPF clinical signs, highlights the potential existence of a subclinical CIPF state in dogs from the WHWT breed. Another limitation of the present study could be that some dogs included, no matter from which breed they are belonging, may have suffered from subclinical inflammatory or neoplastic diseases at the time of sampling, that could have influenced serum concentrations.

4. Conclusion

The present study demonstrated increased serum CXCL8 concentrations in healthy dogs from the WHWT breed in comparison with other breeds less or not predisposed to CIPF. Serum CCL2 concentrations were increased in healthy WHWT, but also in M, a non CIPF-predisposed breed. No relevant interbreed differences were observed for 5-HT with regard to CIPF predisposition. Breed-related differences in VEGF blood concentrations could not be investigated since most of the results obtained were below the kit detection limit. Increased serum CXCL8 concentrations, and possibly CCL2 concentrations, found in healthy WHWT might be related to the breed predisposition of the WHWT for CIPF and possibly serve as predisposing factor for disease development. Further investigations are warranted to explore how those chemokines systemically and locally participate to pulmonary fibrosis mechanisms.

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