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Contribution to the understanding of the pathogenesis and the therapeutic management of canine idiopathic eosinophilic bronchopneumopathy

Contribution à la compréhension de la pathogénie et à la prise en charge thérapeutique de la bronchopneumopathie éosinophilique idiopathique du chien

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List of Abbreviations

ABPA	Allergic bronchopulmonary aspergillosis
ANCA	Antineutrophil cytoplasmic antibodies
A. fumigatus	*Aspergillus fumigatus*
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
Bb	*Bordetella bronchiseptica*
BBF	Bronchial brushing fluid
CCR-3	CC-chemokine receptor-3
CIRD	Canine infectious respiratory disease
Ct	Cycle threshold
CT	Computed tomodensitometry
EBP	Eosinophilic bronchopneumopathy
ECM	Extracellular matrix
ECP	Eosinophils cationic protein
EDN	Eosinophil-derived neurotoxin
EP	Eosinophilic pneumonias
EPO	Eosinophil peroxidase
FeNO	Fractional exhaled nitric oxide
GM-CSF	Granulocyte macrophage colony stimulating factor
ICS	Inhaled corticosteroid
ICEP	Idiopathic chronic eosinophilic penumonia
Ig	Immunoglobulins
IgCAM	Immunoglobulin-like cell adhesion molecules
IL	Interleukine
INF	Interferon
IST	Inhaled steroid therapy
IU	International unit
MBP	Major basic protein
M. canis	*Mycoplasma canis*
M. cynos	*Mycoplasma cynos*
MCP	Monocyte chemoattractant protein

NAEB	Non asthmatic eosinophilic bronchitis
PAA	Pituitary-adrenal axis
PGE2	Prostaglandin E2
pMDI	Pressurized metered dose inhaler
qPCR	Quantitative PCR
TGF	Transforming-growth factor
TNCC	Total nucleated cells count

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Summary – Résumé

Summary

Idiopathic eosinophilic bronchopneumopathy (EBP) is a chronic disease characterized by eosinophilic infiltration of the lung and bronchial mucosa in young adult dogs of medium-large breeds such as Siberian Huskies (Corcoran et al. 1991, Clercx et al. 2000, Clercx & Peeters 2007). Definitive diagnosis of idiopathic EBP requires combination of compatible clinical signs, radiographical features, bronchoscopy findings, cytologic evidence of bronchial or bronchoalveolar eosinophilic infiltration, and exclusion of potential other causes of eosinophilic airway inflammation such as cardio-pulmonary parasites. The aetiology of this chronic inflammatory condition is still unclear. An underlying type I hypersensitivity reaction is highly suspected while the inciting antigens remain mostly unidentified (Clercx et al. 2002, Peeters et al. 2005). The treatment usually consists in oral steroid therapy (Clercx & Peeters 2007). Because of potential side effects or contraindicative comorbidities, the use of inhaled steroid therapy (IST) is common in practice (Clercx & Peeters 2007, Casamian-Sorrosal et al. 2020).

As idiopathic EBP is a diagnosis by exclusion, specific investigation of cardio-pulmonary parasites is needed once eosinophilic airway inflammation is demonstrated. *Angiostrongylus vasorum* is one of the major parasites that are able to cause an eosinophilic airway inflammation. It is important to discriminate between EBP and angiostrongylosis, especially since long-term management and prognosis differ. Over the last 10 years, several studies high-lightened the presence of *A. vasorum* in all countries of western Europe including neighbouring countries of Belgium (Bourque et al. 2008, Yamakawa et al. 2009, Taubert et al. 2009, Barutzki & Schaper 2009, Van Doorn et al. 2009, Helm et al. 2010, Gredal et al. 2011, Conboy 2011, Gallagher et al. 2012, Traversa et al. 2013). The aims of Study 1 and Study 2 were to investigate *a posteriori* the possibility of previously undiagnosed angiostrongylosis among a series of coughing and healthy dogs using qPCR on collected and stored broncho-alveolar lavage specimens and to compare the usefulness of qPCR on lavage with non-invasive tests for the diagnosis of angiostrongylosis. Based on results of Study 1, pulmonary angiostrongylosis was negligible in Belgium until 2013 and previous misdiagnosis of idiopathic EBP is unlikely. Study 2 confirmed that qPCR on BALF is the most sensitive technique to definitely rule out angiostrongylosis in dogs with eosinophilic inflammation on BALF, as non-invasive diagnostic tools (faecal analysis and rapid serological device) have imperfect sensitivities.

Precedent clinical studies unsuccessfully investigated some potential infectious triggers of the development of EBP, including non specific bacteria or fungi (Clercx et al. 2000, Clercx et al. 2002, Johnson et al. 2019a). Although human asthma and canine EBP differ because of the lack of bronchial hyper-responsiveness in dogs with EBP, the role of bacterial genera, that are known to be implicated in induction or exacerbation in humans, has never been investigated in dogs with EBP. In human medicine, infections with specific bacteria such as *Mycoplasma pneumoniae* and *Bordetella pertussis* have been associated with asthma for decades (Hansbro et al. 2004, Harju et al. 2006, Blanchard & Raherison

2010, Atkinson 2013). Although *Mycoplasma cynos* was recently identified as an emerging and possibly lethal pathogen in dogs with canine infectious respiratory disease (CIRD) (Rycroft et al. 2007, Zeugswetter et al. 2007, Priesnall et al. 2014), the role of *M. canis* and *M. cynos* as primary respiratory pathogens still remains unclear (Chandler & Lappin 2002, Chalker et al. 2004, Chan et al. 2013). On the other hand, *Bordetella bronchiseptica* (*Bb*) is recognized as one of the primary causative pathogen agents of CIRD. It can also cause chronic cough without any systemic signs or alveolar foci on radiographs; therefore, chronic infection might remain undiagnosed without the use of sensitive methods such as qPCR on BALF. The diagnostic utility of qPCR was firstly assessed in a clinical series of young dogs with typical *Bb* infection, in comparison with cytological examination and conventional culture of BALF (Study 3). Based on results of Study 3, qPCR was demonstrated as being the most sensitive method for *Bb* confirmation as compared to conventional bacterial culture, especially in dogs previously-treated with antibiotics. Secondly, the presence and the bacterial load of *M. canis, M. cynos* and *Bb* were evaluated in dogs newly-diagnosed with EBP in comparison with dogs having chronic bronchitis and healthy dogs, using specific qPCR on bronchoalveolar lavage samples (Study 4). Results failed to support a potential role of *M. canis* and *M. cynos* in the pathogenesis of EBP. Nevertheless, in EBP dogs, *Bb* was detected more frequently in dogs with more severe clinical signs and moderate or high loads were only observed in dogs with EBP.

Considering other potential infectious triggers, the investigation of *Aspergillus* spp. in idiopathic EBP has been limited to fungal culture (Clercx et al. 2002, Johnson et al. 2019a). In humans, sensitization to *Aspergillus fumigatus* can occur in asthmatics experiencing life-threatening dyspneic episodes (Shah & Panjabi 2016, Page et al. 2015) and the investigation can combine dosage of specific serum immunoglobulins G and E, fungal culture and PCR. Results of qPCR on BALF and specific serum immunoglobulins E were not different between dogs with EBP, dogs with chronic bronchitis and healthy dogs but higher concentrations of serum immunoglobulins G for *A. fumigatus* at several dilutions were found in dogs with EBP compared to dogs with chronic bronchitis and healthy dogs (Study 5).

Lastly, the well-described treatment of canine EBP consists in long-term oral administration of steroids such as prednisolone (Clercx et al. 2000) and/or IST (Bexfield et al. 2006). Despite a rapid positive clinical response, discontinuation of the oral treatment is frequently followed by clinical recurrence (Corcoran et al. 1991, Clercx et al. 2000, Casamian-Sorrosal et al. 2020). Chronic oral steroid therapy may however lead to iatrogenic hyperadrenocorticism and its use may also be contraindicated wih some comorbidities. So far, despite its regular use in practice in dogs, publications related to long-term clinical response and potential side effects of IST as single therapy in dogs with idiopathic EBP were scarce (Bexfield et al. 2006). Study 6 demonstrated that long-term fluticasone monotherapy fails to control cough in part of dogs with EBP, in which oral treatment is ultimately required. Furthermore, inhibition of the pituitary-adrenal-axis was also confirmed in two dogs treated with IST for 48 months and one of them developed progressive clinical signs of iatrogenic hyperadrenocorticism.

Résumé

La bronchopneumopathie éosinophilique idiopathique (EBP) est une affection respiratoire chronique caractérisée par une infiltration éosinophilique du parenchyme pulmonaire et de la muqueuse bronchique chez des chiens jeunes adultes et de moyenne à grande race (Corcoran et al. 1991, Clercx et al. 2000, Clercx & Peeters 2007). Le diagnostic définitif s'établit sur la combinaison de signes cliniques évocateurs, de lésions radiographiques et endoscopiques suggestives, la mise en évidence d'une prépondérance d'éosinophiles lors de l'analyse du lavage bronchoalvéolaire ou d'une biopsie bronchique et après exclusion de toute autre cause d'inflammation éosinophilique des voies respiratoires inférieures telle que les parasites cardio-pulmonaires. La cause exacte de cette affection reste encore mal comprise ; une réaction d'hypersensibilité de type I est fortement suspectée bien que les antigènes responsables n'aient pas encore été clairement identifiés (Clercx et al. 2002, Peeters et al. 2005). La prise en charge habituelle consiste en une corticothérapie orale prolongée (Clercx & Peeters 2007) ; en raison des effets secondaires, l'utilisation d'une thérapie inhalée (IST) est très courante en pratique.

La confirmation d'une EBP repose sur un diagnostic d'exclusion ; une fois confirmée la présence d'une inflammation éosinophilique des voies respiratoires inférieures, la recherche spécifique de parasites cardio-pulmonaires est incontournable. *Angiostrongylus vasorum* est l'un des parasites les plus prévalents. Sur les dix dernières années, de nombreuses études ont en effet démontré la prévalence croissante d'*A.vasorum* dans l'Europe de l'Ouest, incluant entre autres les pays frontaliers avec la Belgique (Bourque et al. 2008, Yamakawa et al. 2009, Taubert et al. 2009, Barutzki & Schaper 2009, Van Doorn et al. 2009, Helm et al. 2010, Gredal et al. 2011, Conboy 2011, Gallagher et al. 2012, Traversa et al. 2013). Il était donc nécessaire de chercher si un diagnostic erroné d'EBP idiopathique avait pu être établi chez des chiens présentant en réalité une angiostrongylose. Les objectifs des études 1 et 2 étaient donc de rechercher *a posteriori* la possibilité d'une angiostrongylose non détectée chez une population de chiens tousseurs et de chiens sains en exploitant la PCR quantitative sur le lavage bronchoalvéolaire et de comparer l'utilité diagnostique de la PCR avec celle d'examens non invasifs tels que l'analyse fécale ou encore l'analyse sérologique rapide. D'après l'étude 1, la présence de l'angiostrongylose sous sa forme respiratoire était négligeable en Belgique jusqu'en 2013 et un faux diagnostic d'EBP idiopathique n'a pas été établi au sein de la population de chiens recrutés. L'étude 2 a par ailleurs confirmé que la PCR quantitative sur le lavage bronchoalvéolaire est l'examen jouissant de la plus haute sensibilité pour définitivement exclure une angiostrongylose chez des chiens pour lesquels une éosinophilie du lavage a été mise en évidence.

Jusqu'à présent, les publications n'ont pas permis d'identifier d'agents pathogènes spécifiques comme facteurs incitateurs ou exacerbateurs d'une EBP idiopathique chez le chien (Clercx et al. 2000, Clercx et al. 2002, Johnson et al. 2019a). Bien que l'asthme de l'Homme et l'EBP du chien diffèrent par l'absence d'une hyper-réactivité bronchique lors d'EBP canine, le rôle d'espèces bactériennes spécifiques, connues chez l'homme pour initier ou exacerber un asthme, comme les genres *Mycoplasma*

ou *Bordetella* (Hansbro et al. 2004, Harju et al. 2006, Blanchard & Raherison 2010, Atkinson 2013), n'a jusqu'à présent pas été étudié chez le chien lors d'EBP idiopathique. Le rôle exact de *M. canis* et *M. cynos* comme agent pathogène primaire reste débattu chez le chien (Chandler & Lappin 2002, Chalker et al. 2004, Chan et al. 2013). D'autre part, *Bordetella bronchiseptica* (*Bb*) peut être responsable d'une toux chronique sans repercussion systémique ni lesions radiographiques alvéolaires ; ainsi, une infection chronique peut être complexe à confirmer sans l'utilisation d'outils sensibles tels que la PCR sur le lavage bronchoalvéolaire. L'utilité diagnostique de cette analyse a d'abord été évaluée au sein d'une population de jeunes chiens infectés par *Bb* en comparaison à la culture bactériologique et à l'examen cytologique du lavage (Etude 3). La PCR quantitative s'est distinguée par sa sensibilité supérieure, en particulier chez les chiens ayant reçu une précédente antibiothérapie. L'étude 4 a par la suite comparé la présence et la charge bactérienne de *M. canis, M. cynos* et *Bb* dans le lavage bronchoalvéolaire provenant de chiens avec une EBP idiopathique, de chiens avec une bronchite chronique et de chiens sains. Les résultats de l'étude 4 conduisent à réfuter un rôle potentiel de *M. canis* et *M. cynos*. En revanche, chez les chiens avec une EBP, *Bb* a été retrouvée plus fréquemment chez les chiens ayant des signes cliniques marqués et parmi tous les chiens testés, une charge bactérienne modérée à forte n'a été retrouvée que chez les chiens ayant une EBP.

Jusqu'à présent, l'investigation d'*Aspergillus* spp. dans l'EBP du chien s'est limitée à la réalisation d'une culture fongique sur le lavage collecté chez quelques chiens (Clercx et al. 2002, Johnson et al. 2019a). Chez l'Homme, la sensibilisation à *Aspergillus fumigatus* peut apparaitre chez certains patients asthmatiques (Shah & Panjabi 2016, Page et al. 2015) et l'investigation repose sur le dosage d'immunoglobulines sériques spécifiques de type E et G et une culture fongique ou une analyse PCR sur les sécrétions respiratoires. L'étude 5 a de nouveau regroupé des chiens avec une EBP, des chiens avec une bronchite chronique et des chiens sains. L'analyse PCR sur le lavage et le dosage des immunoglobulines E n'ont pas montré de résultats différents entre les 3 groupes de chiens. En revanche, les concentrations en immunoglobulines spécifiques de type G étaient plus élevées chez les chiens avec une EBP que chez les chiens des deux autres groupes et ce, pour plusieurs dilutions successives.

Enfin, le traitement le plus décrit lors d'EBP chez le chien consiste en l'administration prolongée de prednisolone (Clercx et al. 2000) et/ou l'utilisation de l'IST (Bexfield et al. 2006). Malgré une résolution clinique très rapide, l'interruption du traitement oral est régulièrement suivie d'une récurrence clinique (Clercx et al. 2000). Une corticothérapie orale prolongée peut néanmoins conduire à l'apparition d'un syndrome de Cushing iatrogène. Malgré son utilisation très courante en pratique, la documentation de la réponse clinique obtenue avec l'IST seule chez des chiens présentant une EBP est très limitée dans la littérature (Bexfield et al. 2006). L'étude 6 démontre que l'IST en monothérapie à long terme n'apporte pas de résolution clinique chez certains chiens avec EBP, chez lesquels l'utilisation d'une corticothérapie orale peut finalement être requise. Par ailleurs, une inhibition de l'axe hypophyso-surrénalien a été confirmée chez 2 chiens recevant l'IST depuis 2 ans ; l'un de ces deux chiens a par ailleurs développé progressivement des signes cliniques évocateur d'un syndrome de Cushing iatrogène.

General Preamble

The present work will focus on a chronic and poorly-understood respiratory disease characterized by eosinophilic infiltration of the lung and bronchial mucosa in young adult dogs called "canine idiopathic eosinophilic bronchopneumopathy" (EBP).

The first occurrence in literature was in 1989 and the term of "pulmonary eosinophilia" was historically used. The first two case series were published in 1991 and 2000 (Corcoran et al. 1991, Clercx et al. 2000). Since then, publications focused on immunologic investigation (Clercx et al. 2002, Peeters et al. 2005, Peeters et al. 2006) and on evaluation of markers of lower airway and lung destruction (Rajamaki et al. 2002a, Rajamaki et al. 2002b, Määttä et al. 2021). A search in the pubmed database shows that only less than thirty publications have been listed about this disease. Canine idiopathic EBP is a distinct and specific disease in dogs which does not share much resemblance with eosinophilic airway diseases described in cats, horses or humans. To date, interest for canine EBP still remains intact as evidenced by three relevant clinical and radiographical publications over the last 2 years (Johnson et al. 2019a, Casamian-Sorrosal et al. 2020, Lo et al. 2021).

However, some aspects concerning pathogenesis and treatment of canine EBP remain unelucidated. While a type I hypersensitivity reaction regulated by Th2 lymphocytes has been hypothesized (Peeters et al. 2005, Peeters et al. 2006), the aetiology of this chronic inflammatory condition is still unclear and potential inciting antigens remain unidentified. Implication of specific fastidious bacteria and saprophyte fungi have thus to be explored using, among others, newly-available and highly-sensitive techniques such as quantitative polymerase chain reaction (qPCR) assays. Secondly, the treatment of canine idiopathic EBP usually consists in prolonged oral steroid therapy, leading to rapid and dramatic clinical improvement. Because of potential side effects or contraindicative comorbidities, use of inhaled steroid therapy (IST) is more relevant but long-term efficacy in such young dogs and potential clinical and biological side effect have not been addressed.

In the introduction, after a brief review about biology of eosinophils, we will synthetize the current knowledge about canine idiopathic EBP. We also will present some eosinophilic airway and lung diseases in humans to potentially find points of similarity with the canine idiopathic EBP.

Introduction

1.   The Eosinophil: structure, production, biology

Eosinophils, neutrophils and basophils are subpopulations of granulocytes. The eosinophil was named by P. Ehrlich in 1879 based on its affinity for anionic dyes, such as eosin. Eosinophils are present in all vertebrates, suggesting important physiologic functions which, however, remain largely unclear. Eosinophils are now known as powerful effector cells. This cell defends against helminthic parasites and is also important in host defense, innate, acquired and adaptive immunity, tissue damage and remodeling, and developmental biology. As examples, eosinophils play an important role in allergic and inflammatory processes and are implicated in host resistance against viral, bacterial, helminthic and fungal pathogens (Young & Meadows 2010, Sastre et al. 2018, Simon et al. 2020, Jackson et al. 2022).

1.1   Structure and morphology

1.1.1. Granules

Eosinophils are highly specialized granulocytic effector cells with a very active metabolism. They produce and store diverse biologically active molecules, including cytotoxic, cytostimulatory proteins, lipid mediators, chemotactic peptides and cytokines (Figure 1) (McBrien & Menzies-Gow 2017, Jackson et al. 2022).
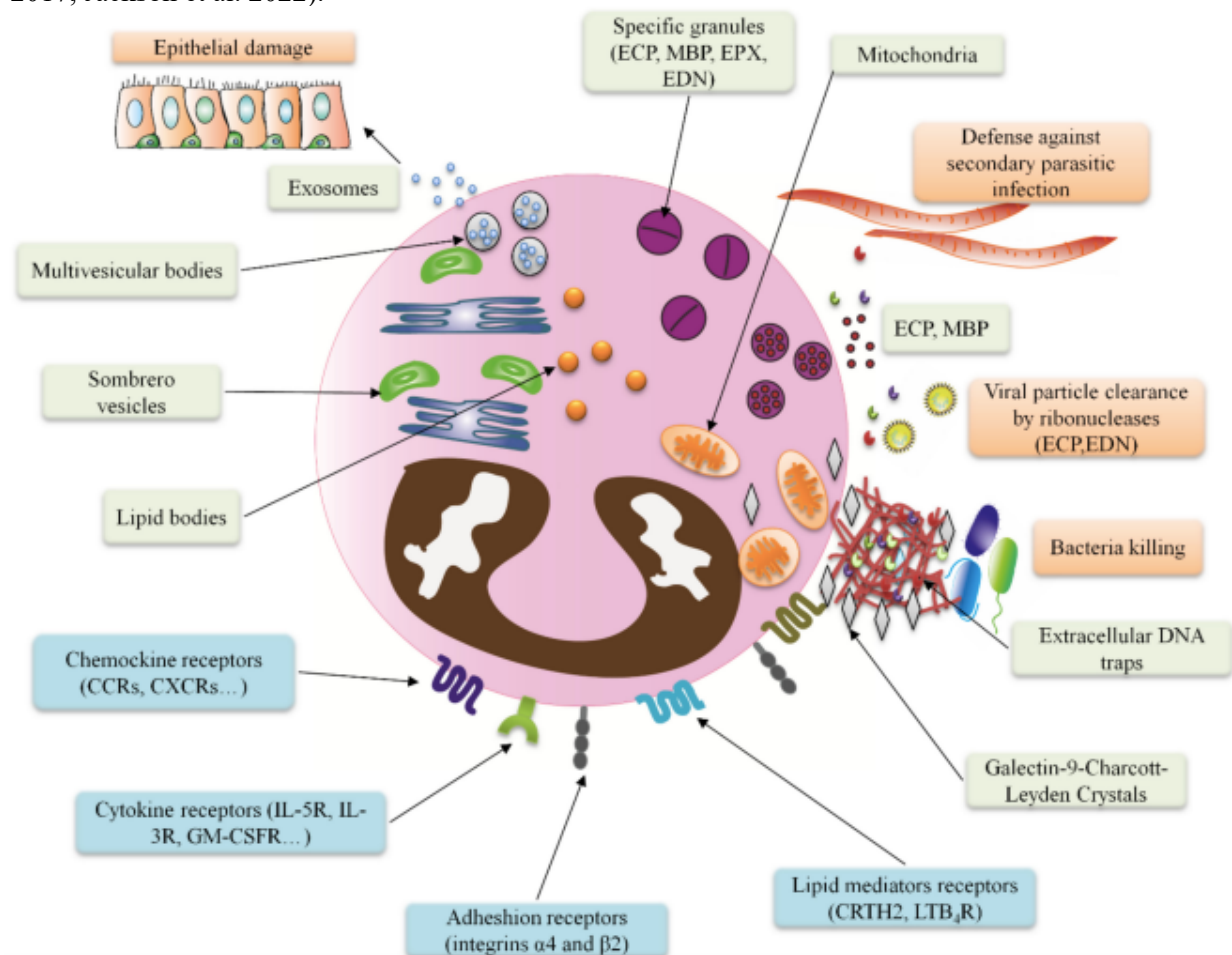
Figure 1: Human Eosinophil ultrastructure (Rodrigo-Muñoz et al. 2021).   - 29 -

Specific granules contain potent cytotoxic proteins, including major basic protein (MBP), eosinophil peroxidase (EPO), eosinophils cationic protein (ECP), eosinophil-derived neurotoxin (EDN), catalase, peroxisomal lipid B oxidation enzymes, b-glucuronidase, cathepsin D and serine/pyruvate aminotransferase. MBP can harm helminths, microbes and mammalian cells by disrupting cell membranes or altering enzymatic activity. EPO produces proinflammatory oxidants and also plays a cytotoxic role against both parasites and mammalian cells. EDN has cytotoxic, neurotoxic and anti-viral activity. ECP has marked toxicity for a wide variety of helminths, bacteria, viruses and host tissues. Primary granules include lysophospholipases and small dense granules contain acid phosphatase (McBrien & Menzies-Gow 2017). Lipid bodies store arachidonic acid used in the generation of lipid mediators such as leukotrienes, thromboxane A2 and prostaglandins. Eosinophils also synthesize cytokines, proteoglycans, vitamin B-binding proteins, and numerous enzymes, including collagenase, histaminase, phospholipase D, and non-specific esterases. At least 35 cytokines, chemokines and growth factors are produced by eosinophils. Eosinophils are the chief producers of cytokines such as TGF-β (McBrien & Menzies-Gow 2017). Besides, Charcot-Leyden crystal protein (carbohydrate-binding family of galectin-10) is another type of eosinophil content, characterized for forming extracellular hexagonal bipyramidal crystals, which exhibit lysophospholipase activity and have been identified as a hallmark of eosinophil involvement in allergic inflammation (Rodrigo-Muñoz et al. 2021). If Charcot-Leyden crystals (CLC) are common in eosinophilic diseases in man, there is lack of reports of CLCs in nonhuman species, and only one single report in canine, suggesting that suggest that CLCs are human-specific (Choi et al. 2017).

1.1.2   Receptors

The varied roles of the eosinophil are reflected in its wide repertoire of surface molecules and receptors, which integrate eosinophils with both the innate and adaptive immune systems.

The receptor for IL-5 is thought to be the most important cytokine receptor expressed by eosinophils. Eosinophils also express receptors for multiple other cytokines and growth factors, including for IL-3, IL-4, IL-13, IL-33, thymic stromal lymphopoietin, GM-CSF, IFNγ and TGF-β. CC-chemokine receptor-3 (CCR3) is an important G protein-coupled receptor expressed on eosinophil cell membranes. CCR3 binds to all three subtypes of eotaxin. CCR3 also binds to several other chemokines including monocyte chemoattractant protein-3 (MCP-3) and MCP-4. Eosinophils also possess cell surface receptors for lipid mediators such as leukotrienes, prostaglandins, and platelet-activating factor. Fc receptors to imunoglobulins (Ig) A, Ig D, Ig E, Ig G, and Ig M are also expressed on the surface of eosinophils, facilitating interaction with the adaptive immune system.

Lastly, adhesion receptors allow eosinophils to adhere to the extracellular matrix (ECM) and to other cells. They also allow the eosinophil to sense its surroundings and respond accordingly. Adhesion

receptors are divided into four main groups: integrins, cadherins, selectins, and immunoglobulin-like cell adhesion molecules (Ig-CAM) (Young & Meadows 2010, Sastre et al. 2018, Simon et al. 2020).

1.2   Regulation, production and release

Eosinophils are constantly replenished from a pool of pluripotent and lineage-committed hematopoietic progenitor cells in the bone marrow. Committed eosinophil progenitors originate from granulocyte/monocyte progenitors (CD34^{+} hematopoietic stem cells) (Hogan et al. 2008). Differentiation and maturation occur as follows:

Myeloblast → Promyelocyte → Eosinophil myelocyte → Eosinophil metamyelocyte → Eosinophil.

The main cytokines driving eosinophil proliferation, differentiation, and survival are IL-3, GM-CSF, and IL-5 (Figure 2a) (Akuthota & Weller 2012a).
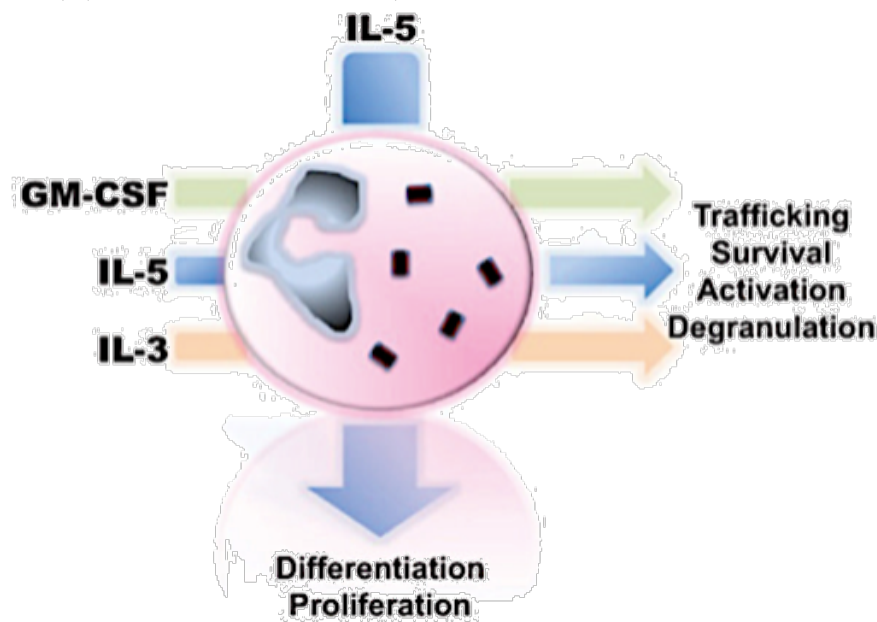
Figure 2a: The effect of major stimulatory cytokines on eosinophils. IL-5 promotes eosinophils differentiation and proliferation. IL-5, GM-CSF, and IL-3 all have important effects on trafficking, survival, degranulation, and activation of eosinophils (Akuthota & Weller 2012a).

IL 3 is primarily produced by activated T-lymphocytes; however, other cell types such as bone marrow stromal cells, natural killer (NK) cells, eosinophils, and mast cells also can produce IL-3. Granulocyte-macrophage CSF (GM-CSF) is produced by a variety of cells including macrophages, T and B cells, bone marrow stromal cells, osteoblasts, fibroblasts, endothelial cells, neutrophils, eosinophils, and mast cells. IL-3 and GM-CSF play a role in the early steps of proliferation and commitment of the eosinophil progenitor pool (Akuthota & Weller 2012a).

IL-5 is an essential cytokine in eosinophil development as it is required for proliferation, terminal differentiation, functional maturation and survival of eosinophils (Figure 2a) (Akuthota & Weller 2012a). Th2 T-helper cells are the primary source of IL-5. However, IL-5 can be produced by other cells such as mast cells, eosinophils, NK cells and innate lymphoïd cells (ILC2) (Figure 2b) (Takatsu et al. 2008). In addition to its role in proliferation and differentiation of eosinophils, IL-5 prevents apoptosis, stimulates release of eosinophils from the bone marrow, promotes adhesion of eosinophils to endothelial cells, and activates eosinophil effector function (Figure 2b). Interestingly, IL-5 knockout mice have normal basal numbers of eosinophils in their bone marrow but fail to develop tissue or blood eosinophilia when stimulated by parasite infection or inhalant allergen (Foster et al. 1996). This suggests that IL-5 is necessary for an expanded eosinophilic response to inflammatory stimuli.

Bone marrow production time from myeloblast to mature eosinophils takes 2–6 days, depending on the species. Secondary or specific granules begin to form at the late promyelocyte stage and granule contents continue to mature through the segmented stage.
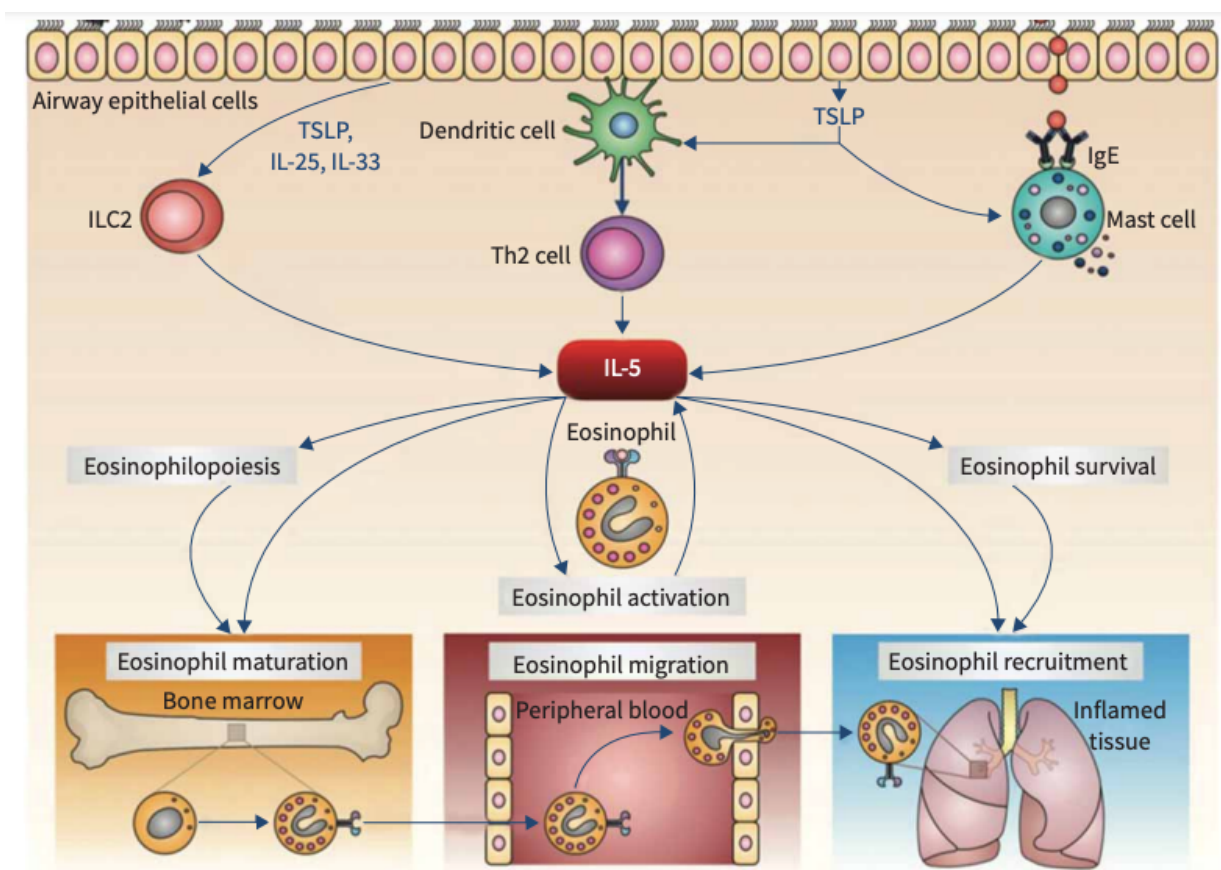
Figure 2b: The role of interleukin (IL)-5 in eosinophil biology. T helper type 2 (Th2) lymphocytes, group 2 innate lymphoid cells (ILC2), and mast cells release IL-5 in response to a variety of triggers. IL-5 promotes eosinophil maturation, migration out of the bone marrow, and recruitment to peripheral sites. TSLP: thymic stromal lymphopoietin (Jackson et al. 2022)

1.3 Recruitment to tissues

Recruitment to tissues is mediated by chemokines, notably those of the eotaxin family, IL-5 and IL-13. IL-5 acts synergistically with eotaxins, a variety of C-C motif chemokine ligands (CCLs), which are selective chemotactic factors that mediate the migration and recruitment of these cells into the body tissues and their activation. Eotaxin (CCL11), eotaxin-2 (CCL24), and eotaxin-3 (CCL26), among others, bind to CCchemokine receptors-3 (CCR3) on eosinophil cell membranes and can induce chemotaxis in allergic inflammation (Figure 3) (George & Brightling 2016). In healthy individuals, the half-life of eosinophils in circulation varies from less than 1 hour in the dog (Young & Meadows 2010) to 18–24 hours in people. Eosinophils migrate into tissues where they live for about 2 days unless anti-apoptotic factors, such as IL-5, prolong their survival for up to several weeks (Figure 3) (Young & Meadows 2010, Simon et al. 2020).
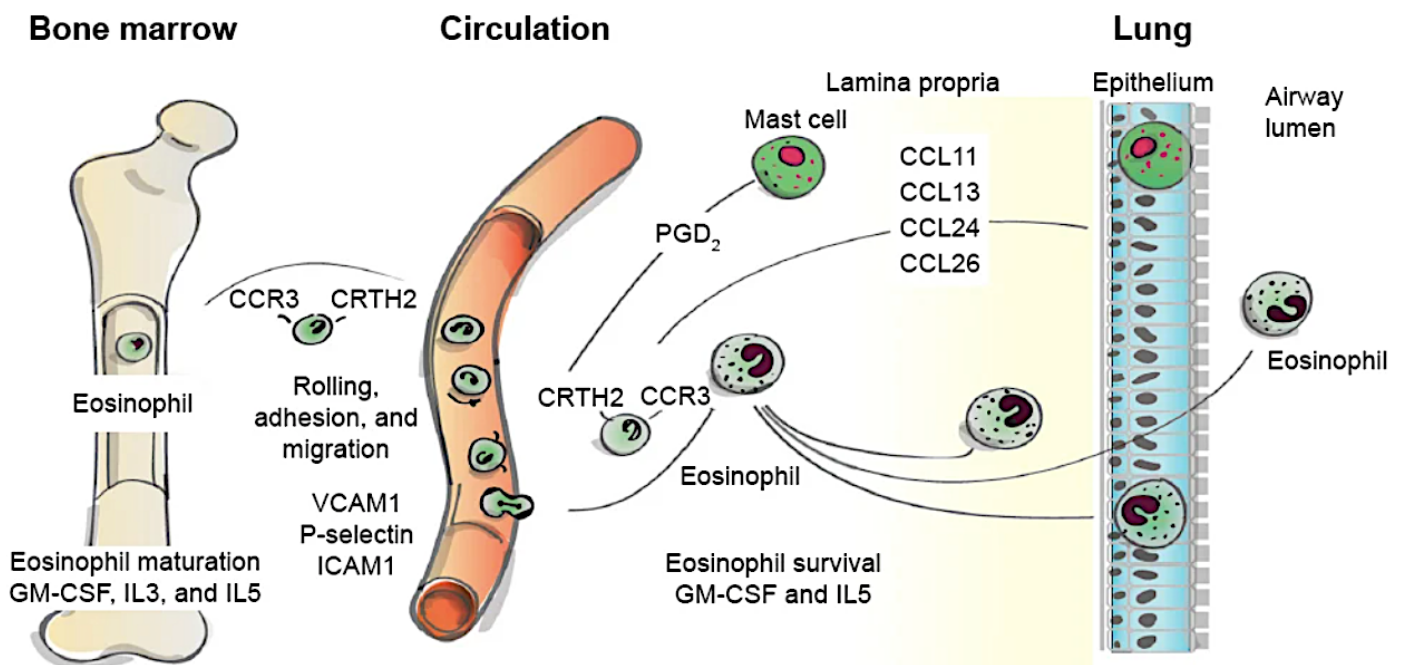
Figure 3: Eosinophil trafficking from the bone marrow to airways (George & Brightling 2016).

1.4 Involvement in the immune system

Eosinophils are multifunctional cells. They produce and release functional cytokines and chemokines and are able to modulate functions of various immune cells (Figure 4).

For instance, eosinophils can produce IL-4, IL-13 and IL-25 which trigger Th2 differentiation and B cell activation and maintain alternatively activated macrophages. In addition, the recruitment of dendritic cells and Th2 cells is supported by producing CC chemokine ligand 17 (CCL17), and CCL22. They can also produce and release large amounts of IL-8, GM-CSF, and IL-10, suggesting that they

might be able to attract neutrophils, auto-stimulate themselves in an autocrine/paracrine manner, and suppress immune responses.Eosinophils express MHCII and costimulatory molecules, process antigens, and promote proliferation and cytokine production on T cells in an antigen-specific manner. So, eosinophils participate in T cell-mediated immunity through recognition and presentation of microbial, viral, and parasitic antigens. Eosinophils can induce histamine release by mast cells and can indirectly activate neutrophils, basophils and macrophages in adipose tissue.
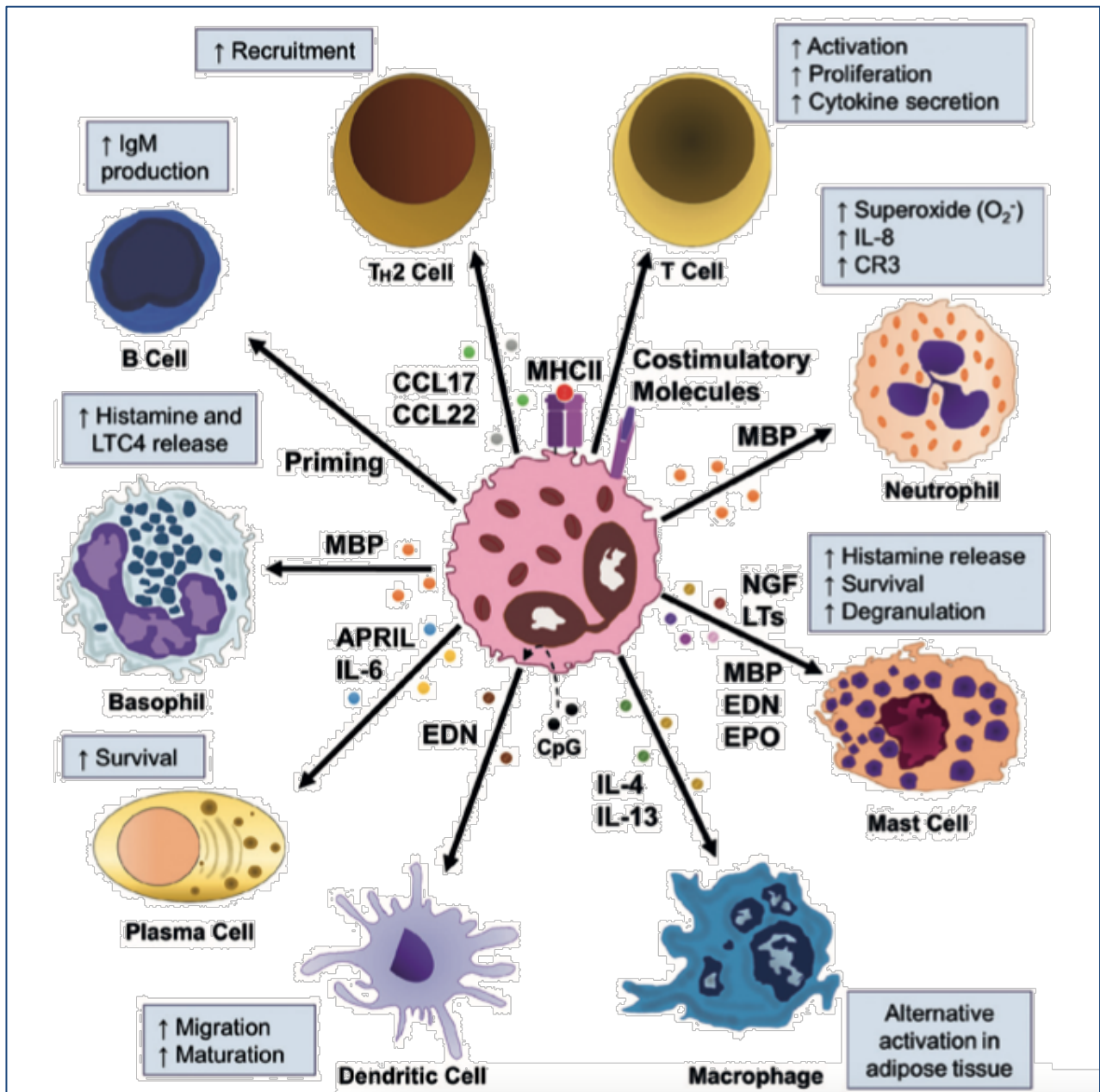
Figure 4: Eosinophils may influence other leukocytes both directly (e.g., IL-6-induced B cell activation) and indirectly. APRIL, a proliferation-inducing ligand; CCL, CC-chemokine ligand; CR3, cell-surface integrin receptor 3; EDN, eosinophil-derived neurotoxin; EPO, eosinophil peroxidase; IL, interleukin; LTs, leukotrienes; MBP, major basic protein; MHCII, major histocompatibility complex class II; NGF, nerve growth factor (Sastre et al. 2018).

1.5   Tissue damages

Eosinophil granule products, reactive oxygen species, and lipid mediators cause damage to tissues (Figure 5). Major basic protein in particular is toxic to epitheliums and triggers degranulation of mast cells and basophils. Moreover, eosinophils can produce and secrete IL-6, tumor necrosis factor alpha (TNF-α), and interferon-gamma (IFN-γ), which are proinflammatory cytokines that can cause tissue damage. TGF-β from eosinophils may play an important role in in fibrotic processes and tissue remodeling in chronic inflammatory diseases via fibroblast proliferation and increased production of collagen and glycosaminoglycans. Prostaglandins, leukotrienes and IL-13 secreted by eosinophils mediate vascular permeability, secretion of mucus, and constriction of smooth muscle. In addition, cytokines involved in recruitment also prolong survival of eosinophils by delaying apoptosis. Moreover, epithelial cell-derived alarmins, IL-25 (also known as IL-17E), IL-33, and TSPL (thymic stromal lymphopoietin) promote eosinophilopoiesis by increasing IL-5 production by ILC2 cells (Figure 5).
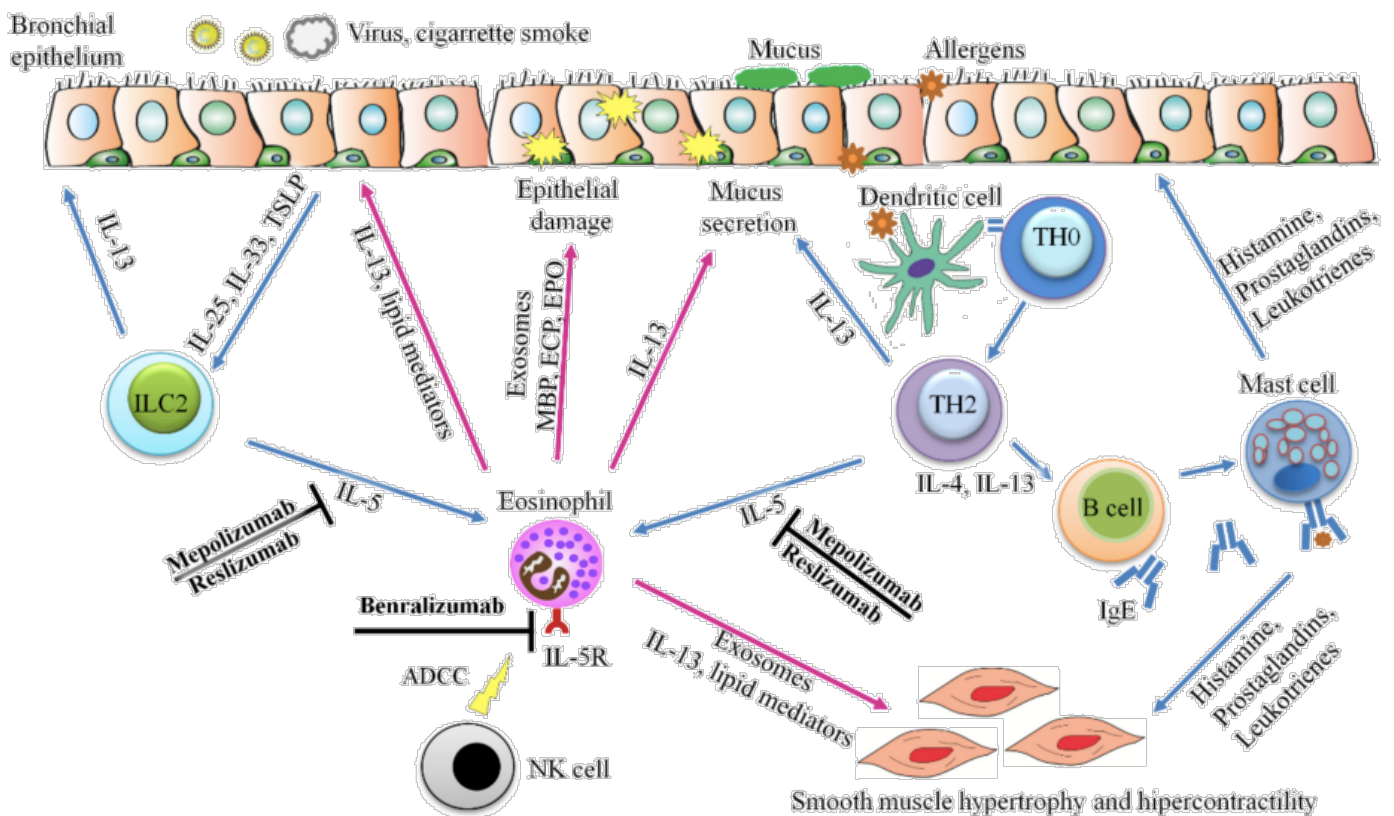
Figure 5: Role of eosinophils in asthma and biological drugs for its control. Type 2 innate lymphoid cells (ILC2s) are activated by alarmins (IL-33, IL25 and TSLP) and releasing Il-5. Allergens are recognized by dendritic cells and presented to T naïve helper cells (Th0), which are polarized to Th2 cells, secreting Il-4 and Il-13. Th2 cells are also capable of releasing IL-5, which activates eosinophils. Eosinophils release IL-13 and lipid mediators (which induce mucus secretion) and discharge exosomes, toxic proteins (ECP, MBP and EPO) and other mediators as ROS and NO capable of inducing epithelial damage. Exosomes, IL-13 and other lipid mediators are also inductors of smooth muscle hypertrophy and contraction (Rodrigo-Munoz et al. 2021).

2.   Idiopathic eosinophilic bronchopneumopathy in dogs: an update

2.1 Historic description of canine idiopathic eosinophilic bronchopneumopathy

Several terms, including pulmonary eosinophilia, pulmonary infiltrates with eosinophilia (PIE), pulmonary eosinophilia and eosinophilic pneumonia have been used in connection with dogs and humans to describe eosinophilic infiltration of the airways or pulmonary parenchyma sometimes associated with peripheral blood eosinophilia. To date, in canine medicine, no clear method of classification and nomenclature exists; nowadays, the most commonly used term in dogs is idiopathic eosinophilic bronchopneumopathy (EBP), taking into account that bronchial infiltration and/or parenchymal involvement are present in these cases. EBP can develop in response to parasitic, fungal or neoplastic diseases, systemic eosinophilic disease or as an immunologic response to an unknown trigger. However, in most instances, no underlying etiology is identified and most cases are considered idiopathic. Idiopathic EBP is a poorly understood inflammatory airway disease that results in substantial morbidity in dogs. It is suspected that hypersensitivity plays a role in these cases but the exact mechanism has not been fully elucidated (Bauer 1989, Corcoran et al. 1991, Clercx et al. 2000, Clercx et al. 2002, Clercx & Peeters 2007, Peeters et al. 2005, Peeters et al. 2006). In human classification, eosinophilic bronchitis and eosinophilic pneumonia are considered as separate processes. However, in dogs, EBP has been described as a heterogenous group of disorders with a spectrum of clinical presentations and bronchoscopic appearances and disease primarily in the airways or parenchyma or in both segments of the lungs (Corcoran et al. 1991, Clercx et al. 2000, Johnson et al. 2019a). Moreover, several reports have defined a variant characterized by eosinophilic granuloma (or "eosinophilic granulomatosis"), often resembling to neoplastic process and associated with a poorer prognosis (Katajavuori et al. 2013, Fina et al. 2014, Johnson et al. 2019a).

2.2 Clinical characterization and diagnosis of canine idiopathic EBP

2.2.1   Epidemiology

Dogs affected with idiopathic EBP are usually young adult dogs: age at diagnosis varies between 3 months and 13 years with a mean age between 3 and 4 years (Corcoran et al. 1991, Clercx et al. 2000, Rajamaki et al. 2002a, Johnson et al. 2019a, Casamian-Sorrosal et al. 2020, Lo et al. 2021).

Mean body weight varies from 16 to 25 kg with a majority of dogs weighing more than 20 kg (Clercx et al. 2000, Johnson et al. 2019a, Casamian-Sorrosal et al. 2020, Lo et al. 2021).

The most frequently represented breeds include Siberian Husky, Labrador and German Shepherd (Clercx et al. 2000, Johnson et al. 2018, Johnson et al. 2019a). Rottweiler, Beauceron, Australian Shepherd, Whippet and Belgian Shepherd are also frequently affected (Johnson et al. 2018, Johnson et al. 2019a, Casamian-Sorrosal et al. 2020, Lo et al. 2021).

Some studies report a gender bias with female dogs being apparently more frequently affected than male dogs (Clercx et al. 2000, Clercx et al. 2002, Casamian-Sorrosal et al. 2020) while other recent large studies describe similar proportions of female and male dogs (Jonhson et al. 2019a, Lo et al. 2021).

2.2.2   Clinical presentation

The time to diagnosis varied from 3 weeks to 6 years (Clercx et al. 2000, Rajamaki et al. 2002a, Jonhson et al. 2019a, Casamian-Sorrosal et al. 2020). Medical history reveals continuous chronic cough to be the most common symptom (92%-100%) (Clercx et al. 2000, Clercx et al. 2002, Jonhson et al. 2019a, Casamian-Sorrosal et al. 2020, Lo et al. 2021). Cough is frequent or persistent in 40 and 50% of dogs, respectively (Clercx et al. 2000). Cough is generally harsh and sonorous and is frequently followed by gagging and retching. In early cases, gagging and retching may be the only clinical sign and they might be confused with a disorder of the digestive system. Other frequent clinical complaints include exercise intolerance or dyspnea (from 20 to 90%) and nasal discharge or nasopharyngeal signs (30-50%) (Clercx et al. 2000, Rajamaki et al. 2002a, Johnson et al. 2018, Johnson et al. 2019a, Casamian-Sorrosal et al. 2020, Lo et al. 2021). The discharge varies in aspect and is usually mucopurulent, of yellow or green color (Clercx et al. 2000). On physical examination, thoracic auscultation can be normal but increased lung sounds, wheezes, or crackles are often found (Clercx et al. 2000).

2.2.3   Blood work

Blood eosinophilia is observed in 20 to 60% of dogs with a mean eosinophil count between 3360 and 6700 cells/µl (Corcoran et al. 1991, Clercx et al. 2000, Rajamaki et al. 2002a, Casamian-Sorrosal et al. 2020). Prevalence of blood eosinophilia could be underestimated by corticosteroid therapy prior diagnosis. Thus, in both naïve or previously treated dogs, absence of peripheral eosinophilia does not exclude a diagnosis of EBP. Basophilia can be associated to eosinophilia (Corcoran et al. 1991, Rajamaki et al. 2002a). A previous study documented positive correlation of the number and percentage of BALF eosinophils with the number of eosinophils in blood (Rajamaki et al. 2002a). Circulating eosinophilia is less often present in dogs with bronchial radiographic pattern and minimal bronchoscopic

abnormalities compared to dogs with other radiographic and bronchoscopic findings (i.e interstitial or alveolar pattern, thick mucoid exudate and bronchiectasis on bronchoscopy) (Johnson et al. 2019a).

2.2.4   Diagnostic imaging

On thoracic radiographs, almost all dogs have diffuse radiographic pulmonary lesions with moderate to severe interstitial and bronchial densities being almost always noted (Figures 6a and 6b) (Clercx et al. 2000, Johnson et al. 2019a, Lo et al 2021). Abnormal lung changes are most frequently seen in the caudodorsal lung fields and 30% of dogs have changes in lung opacity in only the caudodorsal lung fields (Lo et al. 2021). Among 35 dogs with EBP having radiographic abnormalities, the severity grades were classified as mild (37%), moderate (26%) or severe (37%) (Lo et al. 2021). Other typical lesions include peribronchial cuffing and bronchiectasis (Figures 6a and 6b) and/or an alveolar pattern (Figure 6c) (Clercx et al. 2000). Bronchial plugging with intraluminal soft tissue opacities and nodular or mass lesions may also be seen and these modifications are referred as eosinophilic granulomatosis (Figures 6d and 6e) (Johnson et al. 2019a). Lastly, thoracic radiographs can be also normal in up to 20% of dogs (Casamian-Sorrosal et al. 2020, Lo et al 2021).
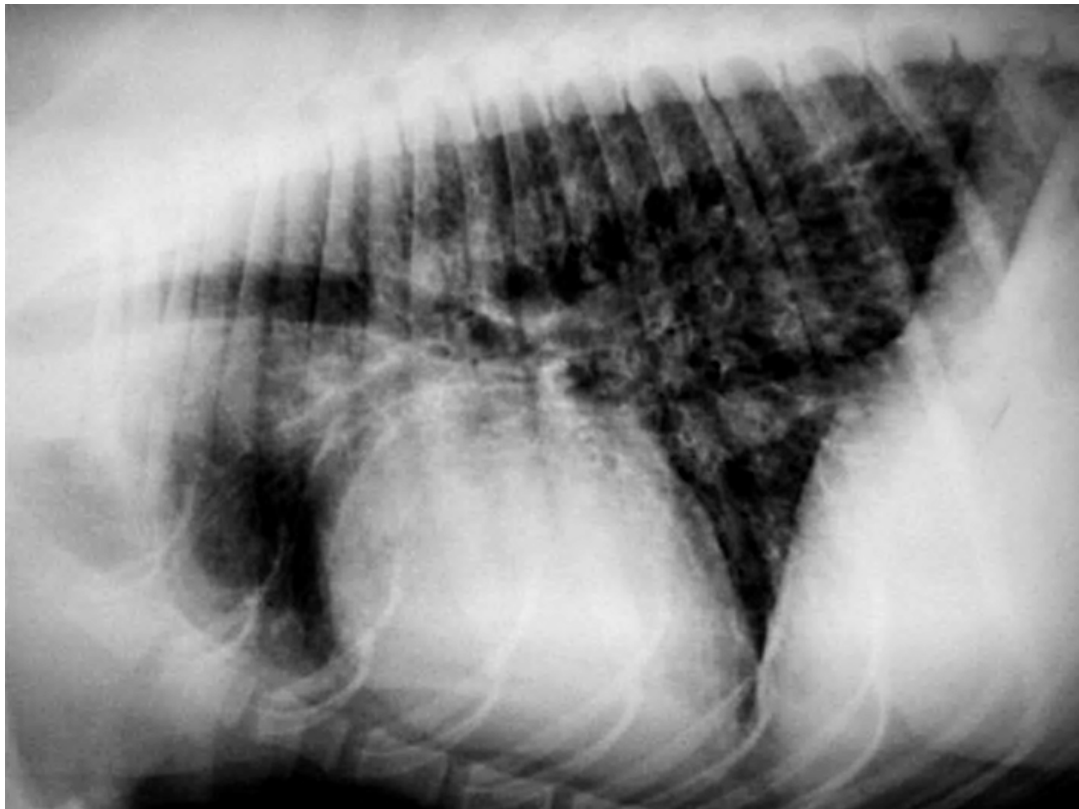
Figure 6a: Left lateral thoracic radiograph of a 3-year-old Husky with EBP, showing severe bronchointerstitial pattern with peribronchial infiltration (Clercx et al. 2000).
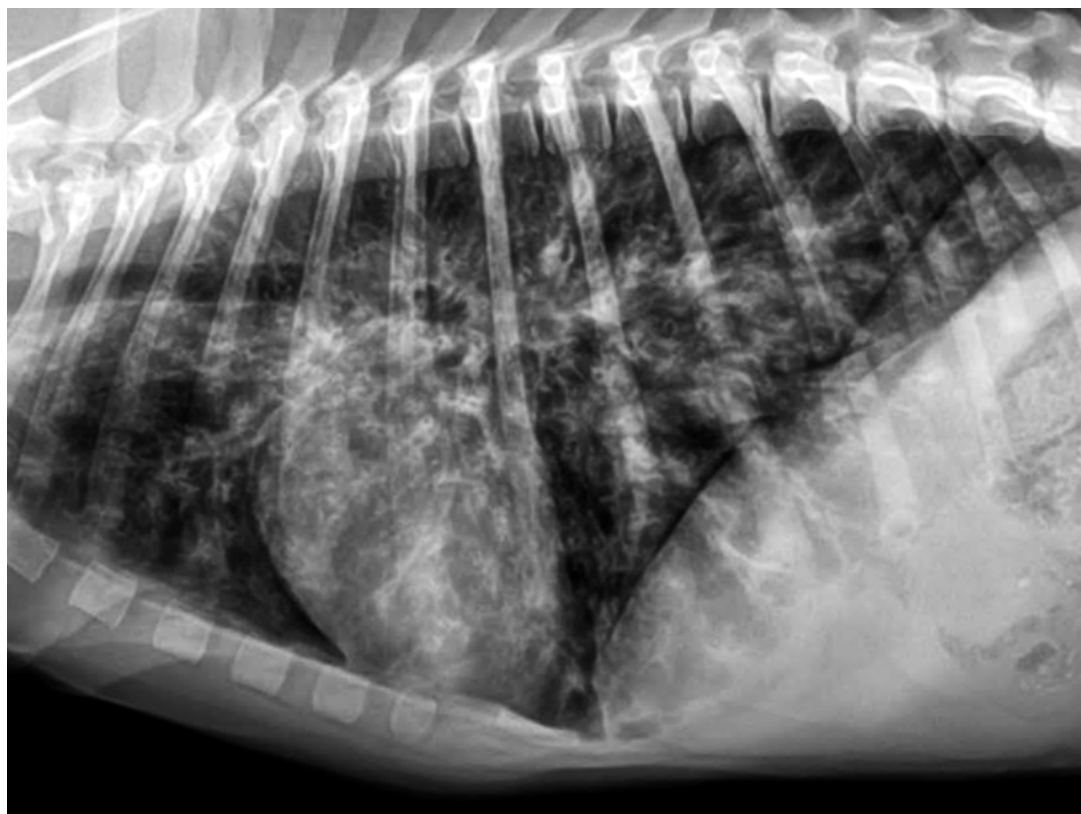
Figure 6b: Right lateral projection of the thorax of a dog with EBP that demonstrates peribronchial cuffing, marked patchy unstructured interstitial pattern and bronchiectasis (Reinero 2019).

Figure 7c: Right lateral projection of the thorax of a dog with EBP: a severe bronchointerstitial pattern, some peribronchial cuffing and alveolar infiltration (Clercx et al. 2000).
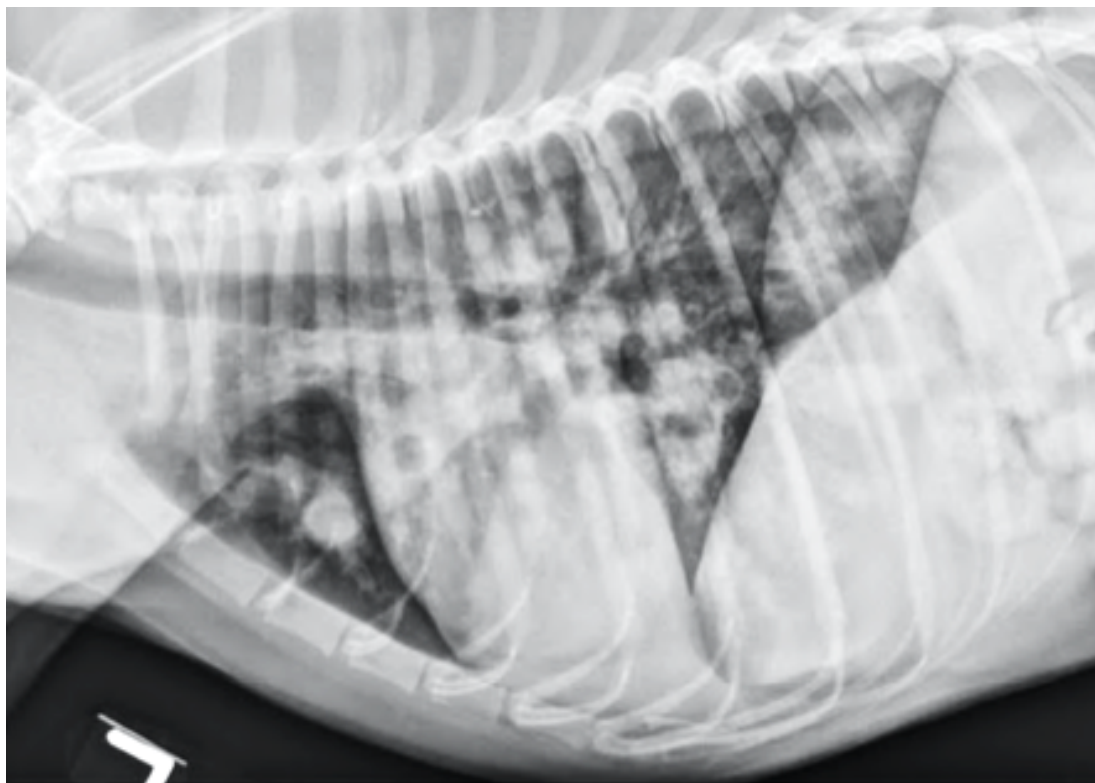
Figure 6d: Severe bronchial pattern with bronchiectasis and gross enlargement of the right cranial and right caudal lobar bronchi that are filled with soft tissue opacity (Johnson et al. 2019a)

Figure 6e: Severe bronchial pattern with bronchiectasis and, in particular, gross enlargement of the right cranial and right caudal lobar bronchi that are filled with soft tissue opacity (Johnson et al. 2019a)

A recent study documents the tomodensitometric findings in 15 dogs with confirmed EBP (Mesquita et al. 2015). Pulmonary parenchymal abnormalities were found in 14/15 (93%) dogs and was the most common abnormality found (Figures 7a to 7d). Main other abnormalities included: bronchial wall thickening (Figures 7a to 7f), lung consolidation (Figures 7b and 7c), plugging of the bronchial lumen by mucus/debris (Figures 7d and 7e) and focal bronchiectasis (Figure 7f).
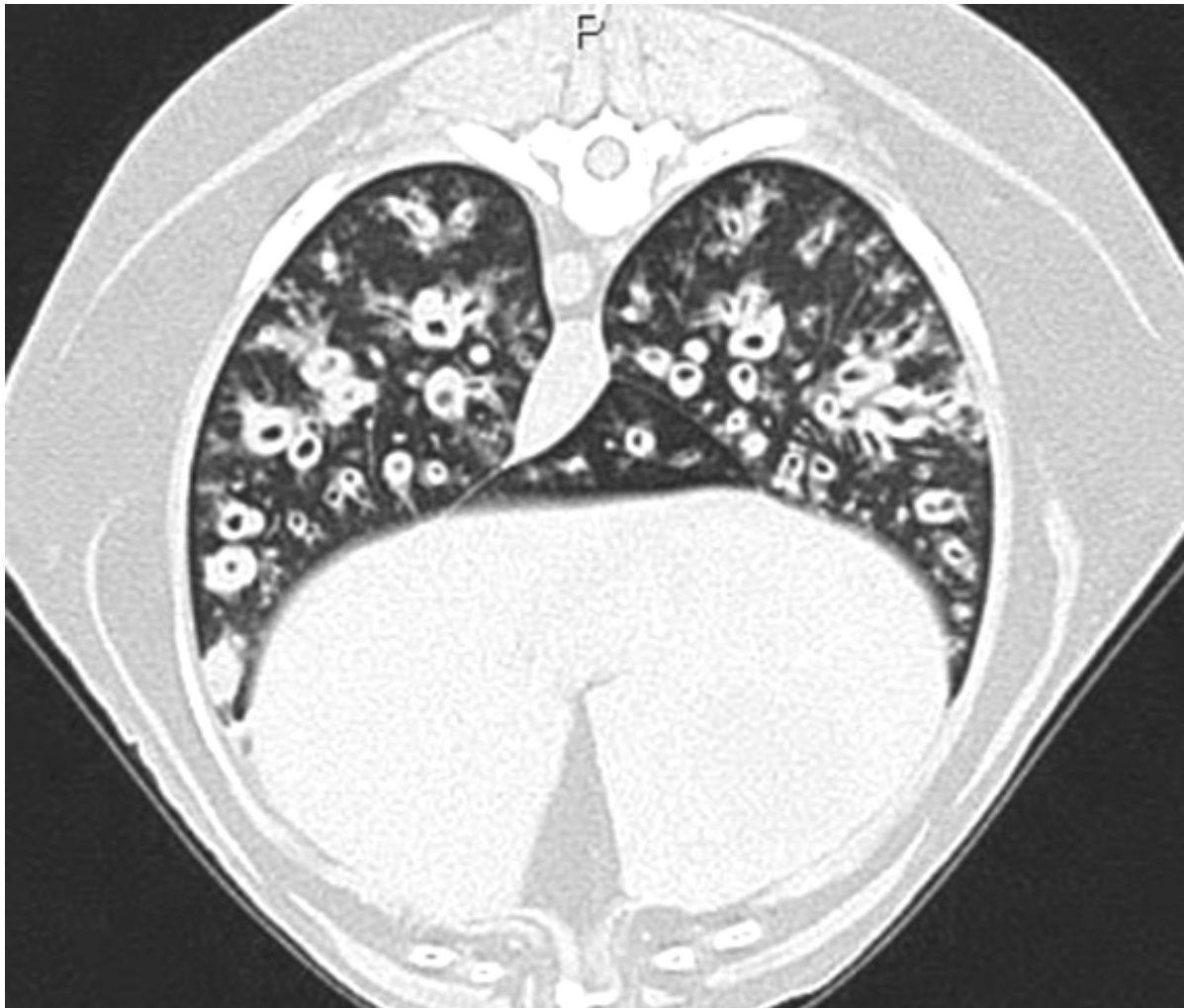
Figure 7a: Noncontrast transverse CT image highlighting the bronchocentric nature of the lesions with increases in peribronchovascular opacification that in cross-section appears as "donuts" (Reinero 2019).
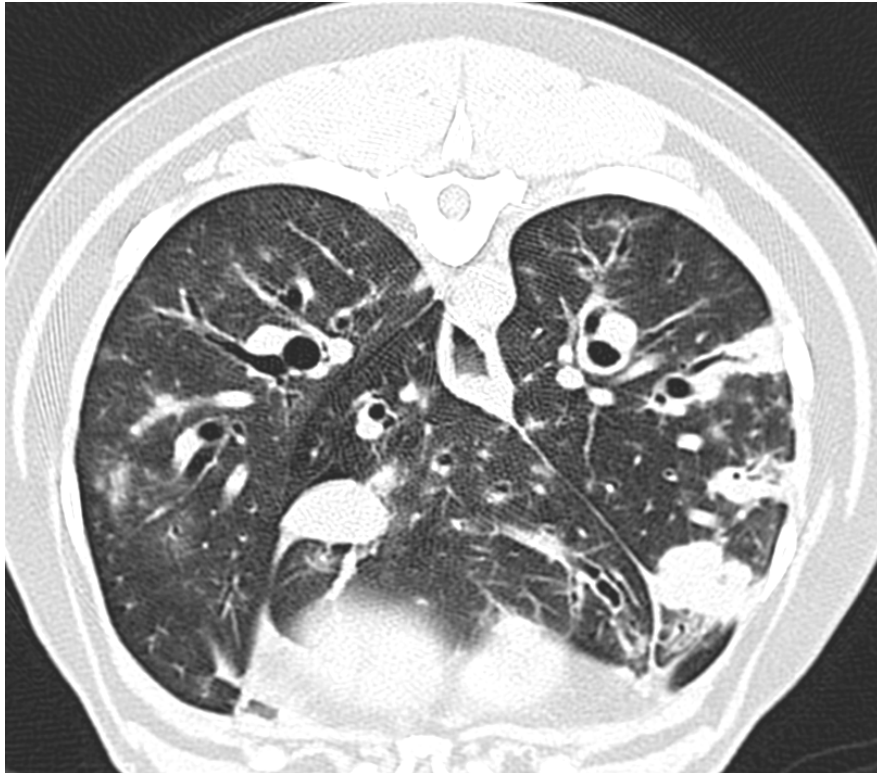
Figure 7b: Noncontrast transverse CT image (lung window), of a dog with eosinophilic bronchopneumopathy. Note the multifocal areas of peribronchial consolidation, generalized slight ground glass pattern and bronchial wall thickening (Mesquita et al. 2015).

Figure 7c: Noncontrast transverse CT image (lung window) of a dog with eosinophilic bronchopneumopathy. A rounded soft tissue attenuating nodule is present in right cranial lung lobe (arrow). Note also the multifocal ground glass pattern and bronchial wall thickening (Mesquita et al. 2015).
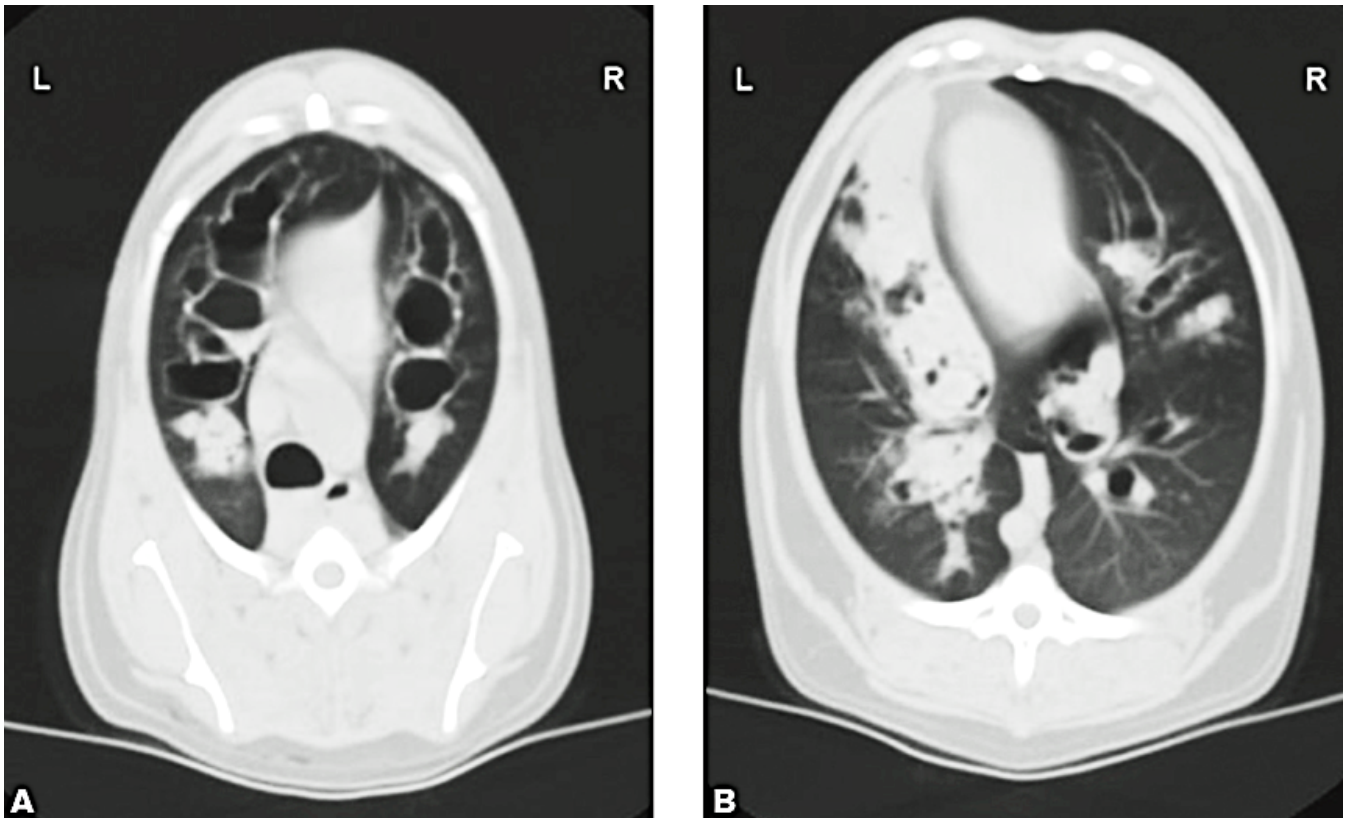
Figure 7d: Transverse CT images of the thorax. Severe, diffuse cylindrical bronchiectasis is present in all lung fields. Soft tissue- attenuating material is present in the dilated bronchi of the ventral lung fields. A — Transverse image of the cranial thorax. Severe bronchiectasis is evident in all lung lobes. B — Transverse image of the caudal lung fields. Multiple dilated bronchi are filled with soft tissue- attenuating material, most pronounced in the left hemithorax adjacent to the heart (Meler et al. 2010).

Figure 7e: Noncontrast CT images (sagittal plane multiplanar reconstruction (A) and transverse (B), lung window) in of a dog with eosinophilic bronchopneumopathy. Note the plugging of the bronchial lumen by mucus/debris of the right caudal (A) and right cranial main bronchus (B) (Mesquita et al. 2015).

Figure 7f: Minimum intensity projection CT image (lung window) of a dog with cylindrical bronchiectasis affecting the right caudal and the accessory lung lobes (Mesquita et al. 2015).

Computed tomodensitometry characteristics of confirmed idiopathic eosinophilic granulomatosis have been also recently described (Fina et al. 2014). Masses and nodules of variable size were reported with lesions most commonly located in the caudal lung lobes (Figure 7g) and a honeycomb-like enhancement pattern was often observed after intravenous administration of iodinated contrast medium (Figure 7h).

Figure 7g: Contrast enhanced CT image of a large eosinophilic granuloma in the right caudal lung lobe in a dog. Note the well-defined margins and the alveolar infiltration at the lateral aspect of the mass (Fina et al. 2014).
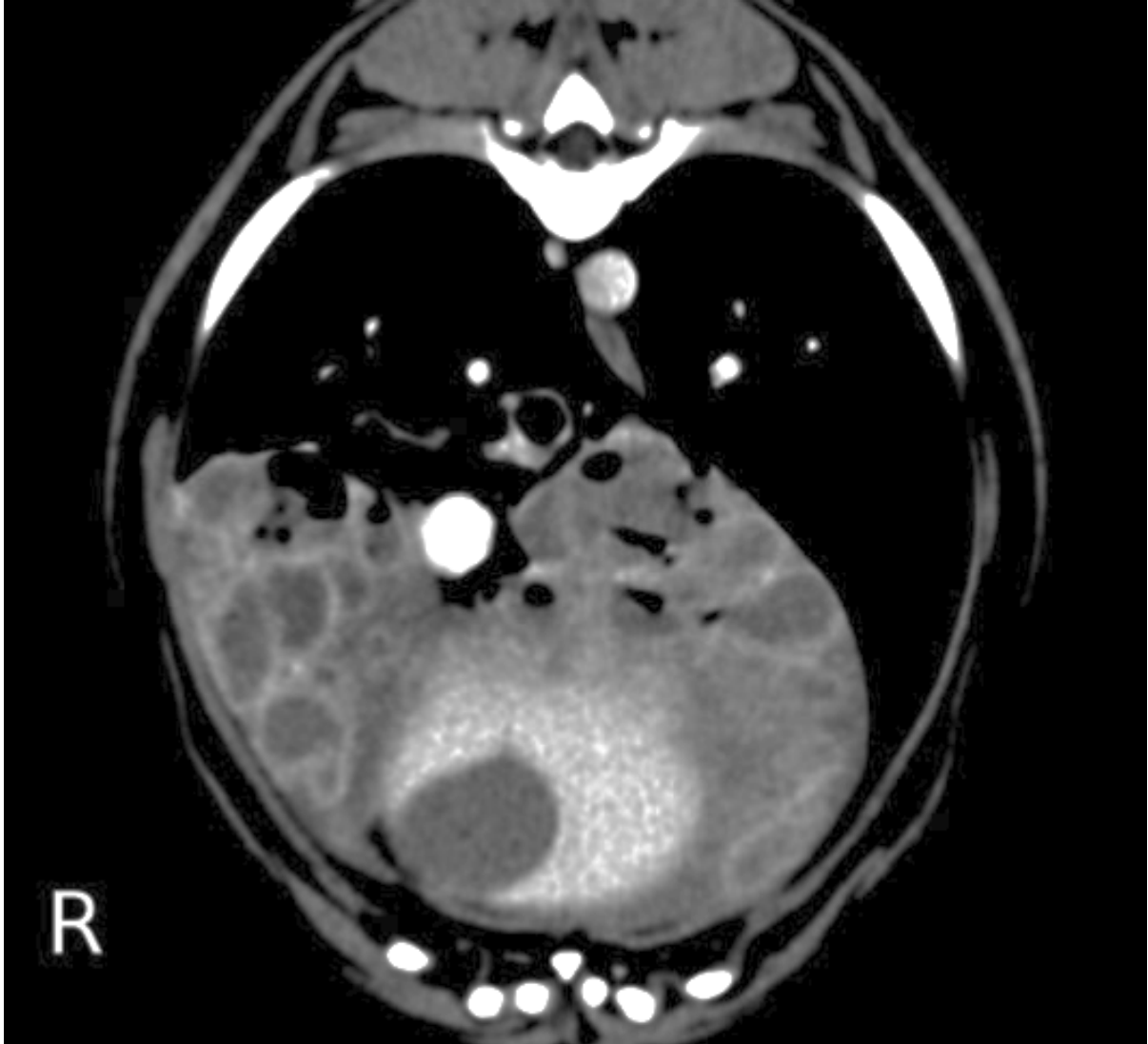
Figure 7h: Contrast enhanced CT image of eosinophilic granuloma. Large masses were present within the right caudal and accessory lung lobe. Enhancement pattern within the large masses consists in several contrast enhancing rings delineating central hypoattenuating areas, giving the granulomas a honeycomb-like appearance (Fina et al. 2014).

2.2.5   *Bronchoscopy and bronchoalveolar lavage fluid*

The most common bronchoscopic findings are dramatic airway hyperemia (70%), presence of moderate to abundant thick yellow-green mucus or mucopurulent material (Figures 8a and 8b) (60-70%), moderate to severe thickening of the mucosa with irregularity or polypoid proliferation (Figure 8c) (52%), exaggerated concentric airway collapse during expiration (Figure 8d) (28-35%) and bronchiectasis (12%). Inspissated intraluminal material was observed in 10% of dogs (Figure 8e) (Clercx et al. 2000, Johnson et al. 2019a). Presence of yellow- green mucus or mucopurulent material, mucus plugging and polypoid mucosal changes are considered particularly specific for EBP (Clercx et al. 2000, Clercx & Peeters 2007, Johnson et al. 2019a).

In case of nasal discharge, rhinoscopy can be performed. The nasal mucosa can be congested and edematous with polypoid proliferations in severe cases and mucus or mucopurulent material is observed (Clercx et al. 2000, Clercx & Peeters 2007).

Figure 8a: Endoscopic finding in a dog with EBP showing moderate to abundant thick yellow-green mucus or mucopurulent material (University of Liege, Internal Medicine)
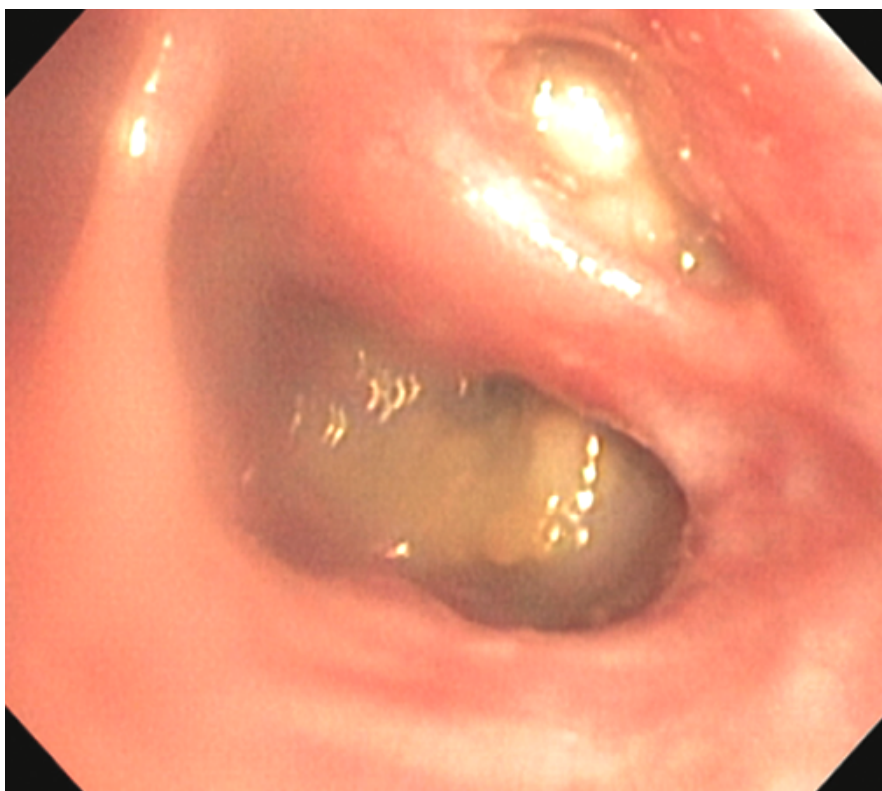
Figure 8b: Endoscopic image of the right caudal and accessory lung lobes. Partially to fully obstructive yellow-green mucopurulent material is evident in both lobar bronchi. Mild diffuse mucosal hyperemia is also evident (Meler et al. 2010).
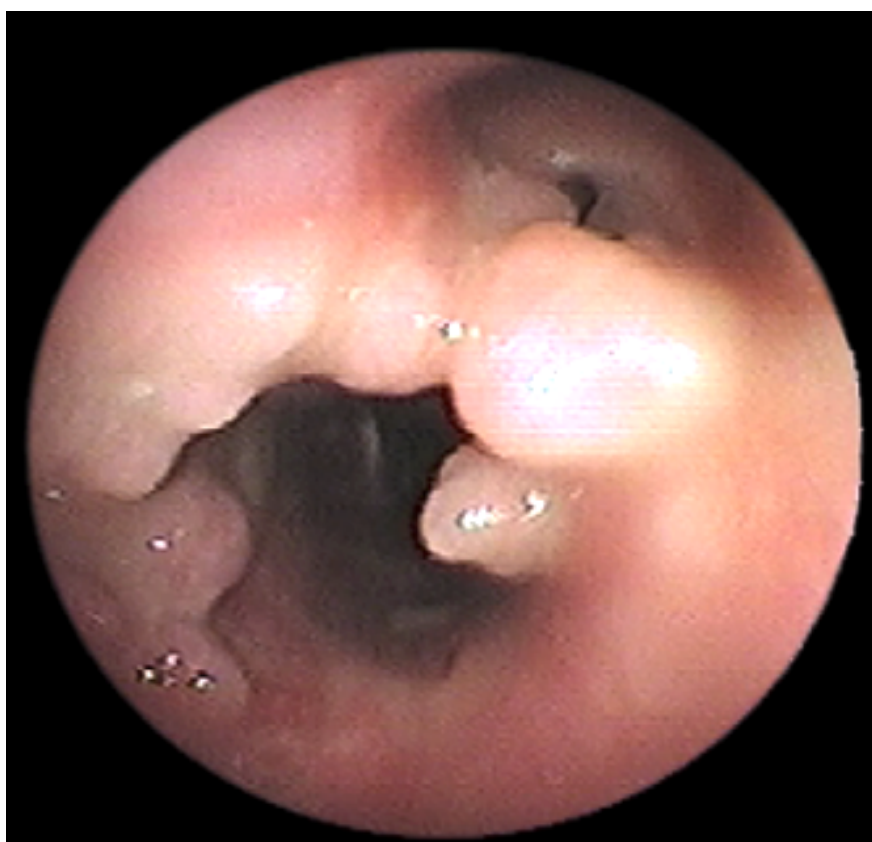
Figure 8c: Endoscopic finding in a dog with EBP showing moderate to severe thickening of the mucosa with irregularity or polypoid proliferation (University of Liege, Internal Medicine)
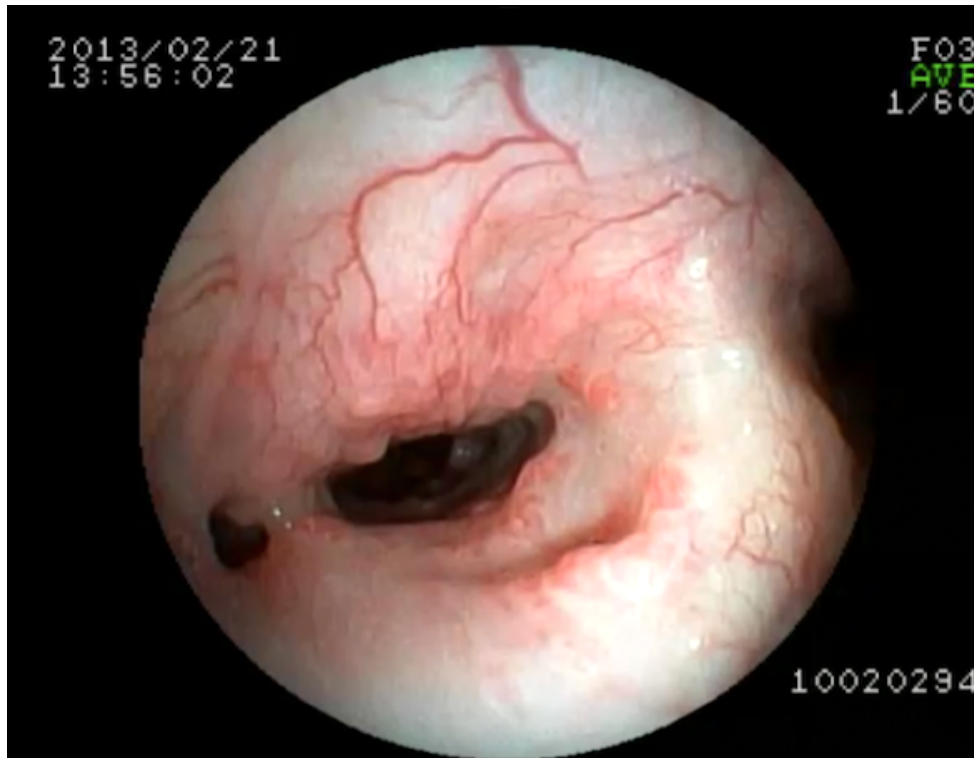
Figure 8d: Endoscopic finding in a dog with EBP showing exaggerated concentric airway collapse during expiration (University of Liege, Internal Medicine)
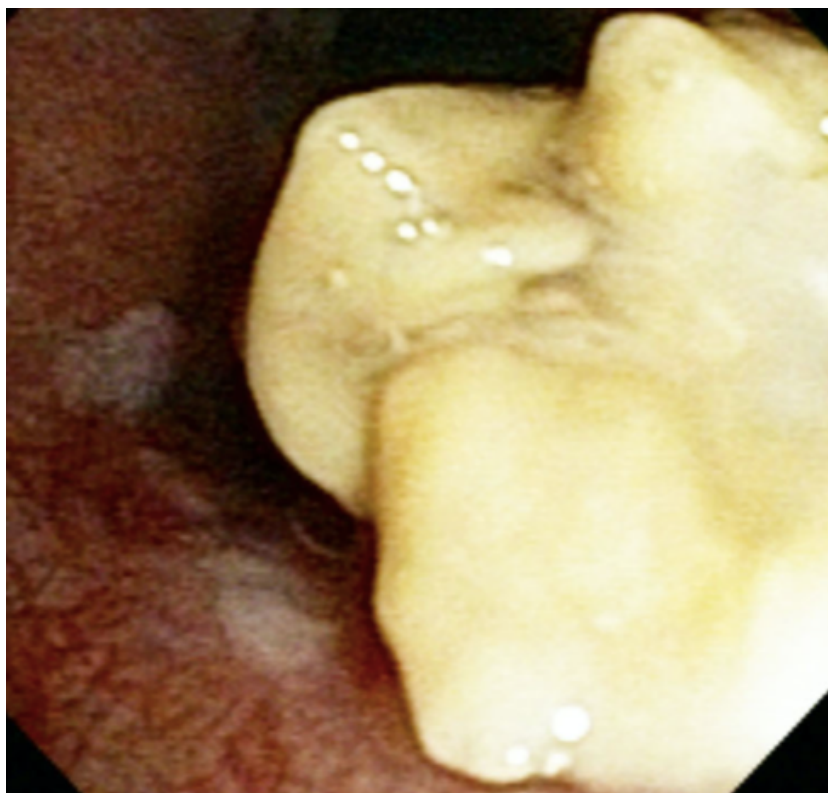
Figure 8e: Endoscopic finding in a dog with EBP showing inspissated intraluminal material consolidated in a large yellow mass lesion, partially obstructing a bronchus (Johnson et al. 2019a)

Airway sampling is necessary to confirm a diagnosis of EBP through cytologic assessment and exclusion of infection. Bronchoalveolar lavage (BAL) is considered to be a safe procedure in dogs, although in a case report, a dog with EBP developed respiratory distress after BAL, presumably because of eosinophil degranulation and potential bronchoconstriction (Cooper et al. 2005). Therefore, careful monitoring of cardiac and respiratory parameters is always recommended.

Aliquots of BALF must be cytocentrifuged immediately to obtain good-quality cytologic samples. EBP is characterized by an increase in the total number of cells in BALF and an increase in the percentage of eosinophils. Less than 9% eosinophils are generally found in the BALF from healthy dogs (Hawkins et al. 1990). Siberian Huskies seem predisposed to a high number of eosinophils in BALF in the absence of obvious clinical signs of airway inflammation (Clercx, unpublished data, 2000). In dogs with EBP, BALF total nucleated cells count range from 200 to 33 800 cells/microL (Johnson et al. 2019a) and median BALF percent of eosinophils range from 50 to 90% (Clercx et al. 2000, Rajamaki et al. 2002a, Johnson et al. 2019a) (Figure 9).

Some dogs (<10%) may also have concurrent lymphocytic airway inflammation with lymphocyte differential count >20% (Johnson et al. 2019a). Increased lymphocytes may be expected as cytokines produced by lymphocytes could play a role in triggering IgE production and attracting eosinophils in Th2 hypersensitivity-type disease. Previous studies reported a dramatic increase in the BALF lymphocyte CD4:CD8 ratio because of a large increase in CD4 cells (Peeters et al. 2005).
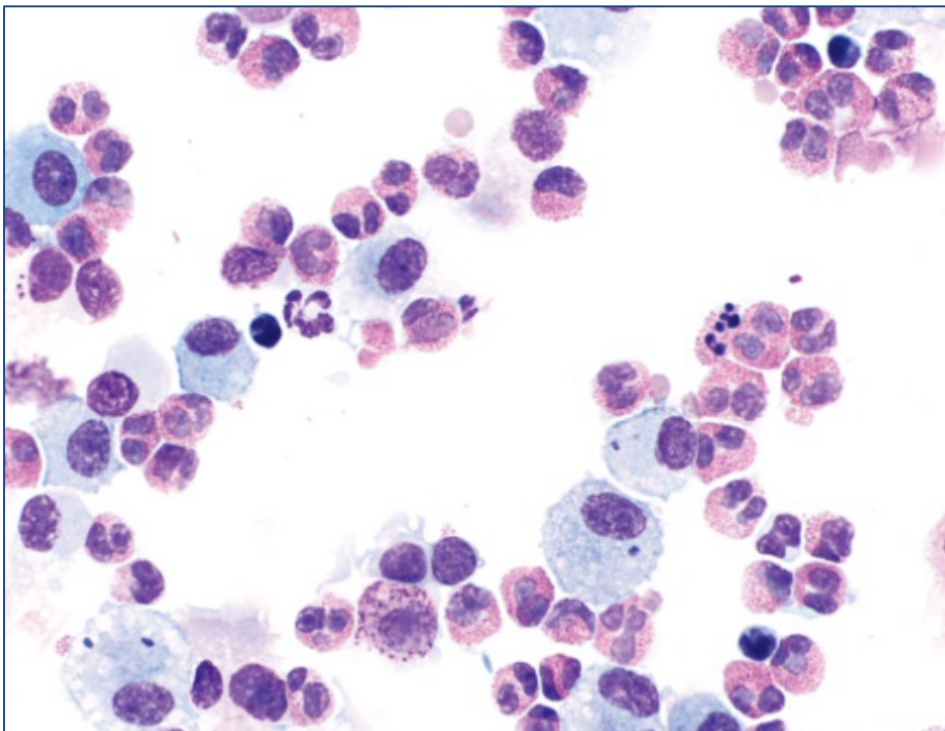
Figure 9: Cytologic preparation (BALF cytospin) showing 47% of eosinophils (total cell count was 7000 cells/mL) (May-Grünwald-Giemsa Stain, University of Liege, Internal Medicine).

2.2.6   Histopathological features of bronchial biopsy

Perendoscopic mucosal bronchial biopsies may be performed for histopathologic examination. Eosinophils are often clustered immediately beneath the respiratory epithelium or actively migrating through epithelium to the luminal surface (Figure 10). Scattered mast cells are also present in the superficial lamina. In case of severe inflammation, microhemorrhage, hemosiderin-laden macrophages, collagenolysis and fibrosis are sometimes noted (Clercx et al. 2000). Cytologic grade based on BALF analysis and histopathologic grade do not seem to be correlated (Clercx et al. 2000).

In case of nasal discharge, biopsies of nasal mucosa can also reveal eosinophilic infiltration (Clercx et al. 2000).
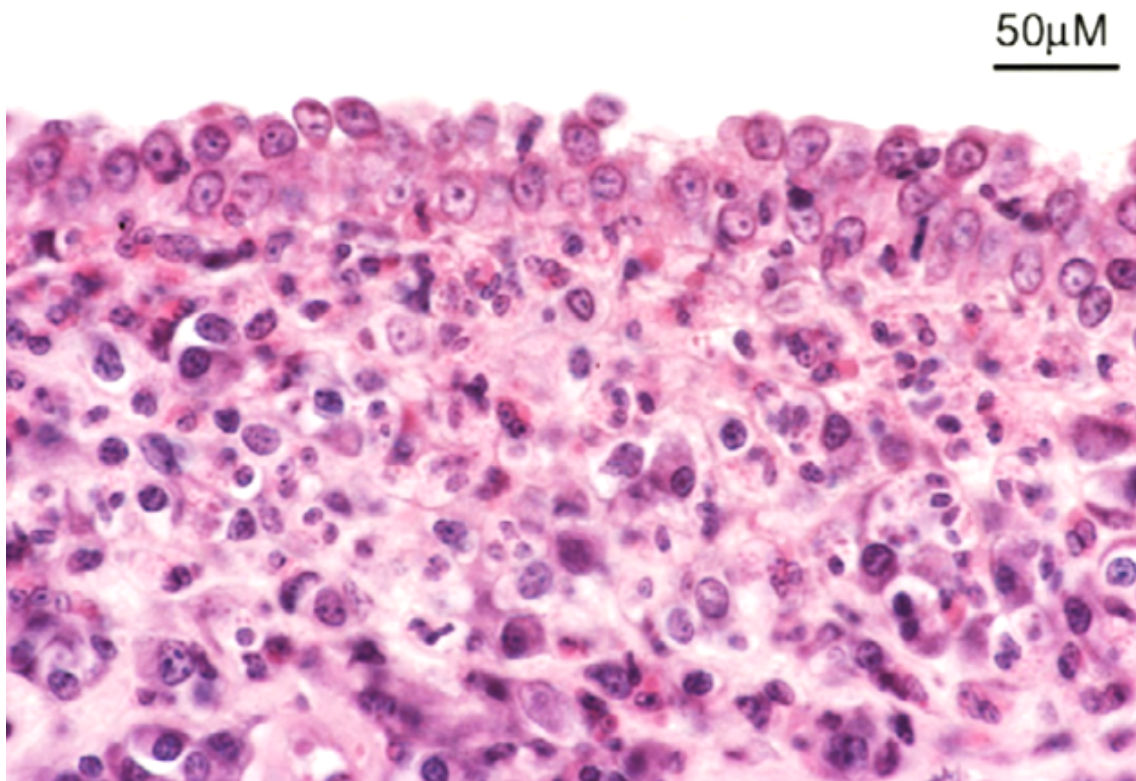
Figure 10: Histological examination of a bronchial biopsy in a dog with EBP showing eosinophilic infiltration (Hematoxylin Eosin Safran Stain) (Clercx et al. 2000)

2.2.7   Exclusion of other causes of eosinophilic airway inflammation

Canine idiopathic EBP may be suspected based on signalment, clinical signs and previous history of a positive response to corticosteroids. Definitive diagnosis relies on radiographic and bronchoscopic findings, blood eosinophilia, tissue eosinophilic infiltration demonstrated by cytological examination of BALF or bronchial cytobrushing or histopathologic examination of bronchial biopsies, and specific exclusion of potential known causes of eosinophilic infiltration of the lower airways.

Most known causes of airway eosinophilia in dogs include fungi, molds, bacteria, and parasites, including canine heartworm disease (infection by *Dirofilaria immitis*). As occult heartworm disease can occur, it is strongly advised to run a heartworm antigen test and to perform echocardiography in endemic areas or in dogs that have traveled to or lived in endemic areas.

Helminth parasites are implicated in eosinophilic bronchopulmonary reactions through primary infection (among others *Angiostrongylus vasorum, Oslerus osleri, Filaroides hirthi, Crenosema vulpis, Capillaria aerophila* or *Paragonimus kellicotti*) or by migration through lung tissue during parasitic cycle (*Strongyloides stercoralis, Toxocara canis, Ancylostoma caninum…*). Zinc sulfate centrifugation-flotation and Baermann sedimentation of feces are advised, because these tests detect eggs or larvae for most pulmonary parasites. Since fecal excretion occurs intermittently, a negative fecal examination by either method is not conclusive; it is therefore advised to treat against potential parasites using appropriate antihelminthic drugs (eg, fenbendazole).

Prevalence of infection by *Crenosoma vulpis* is not negligible in dogs and disease can be easily confused with canine idiopathic EBP because of similities in radiographic or tomodensitometric lesions or bronchoscopic findings (Unterer et al. 2002, Caron et al. 2014, Mortier et al. 2018, Vekšins et al. 2021, Pohly et al. 2022). Fecal analysis is hightly sensitive and cytological examination of BALF could also identify helminth larvae (Unterer et al. 2002). Lastly, qPCR for *Crenosoma vulpis* is also currently available in some countries and may be performed on BALF samples (Pohly et al. 2022).

On the contrary, a recent unpublished study evaluated prevalence of *Capillaria aerophila* infection in coughing, client-owned, domestic dogs in Belgium by qPCR on BALF samples. Results were negative for all dogs suggesting that *C. aerophila* infection is not prevalent in Belgium in dogs with chronic cough (Roels et al. 2022).

Moreover, some tumors, such as lymphoma and mast cell tumor, have been anecdotally-associated with eosinophilic pulmonary infiltrate in dogs (Bauer 1989). While several drugs have been associated with eosinophilic pneumonia in human beings, drug-induced eosinophilic pneumonia has not been reported in dogs.

2.2.8 *Arterial blood gas analysis*

Arterial blood gas analysis is a valuable test providing insights into the severity of pulmonary dysfunction in dogs with parenchymal disease. Mild decreased values in PaO2 and increased values in the alveolar-arterial oxygen gradient (A-aDO2) have been described in dogs with EBP as compared with healthy animals (Rajamaki et al. 2002a).

2.2.9   Allergy testing

In a previous study, an intradermal skin test using a panel of 48 standardized allergens, including house dust mites, *Dermatophagoides pteronyssinus, Dermatophagoides farina, Tyrophagus*, human dander, mixed feathers, molds, pollens of grasses, trees, weeds, and mixed insects, was positive in 4 out of 12 dogs with untreated EBP (Clercx et al. 2002). Positive reactions against *Tyrophagus* spp. and *D farinae* were observed in one dog; other positive reactions were noted against human dander in 2 dogs and against mixed feathers in another. In another study, 3 dogs with EBP were tested with various antigens and all 3 were negative (Corcoran et al. 1991). The causal relation between positive intradermal skin testing and documentation of aeroallergens responsible for EBP is difficult to establish. A positive intradermal skin test does not necessarily indicate that the allergen identified is responsible for the pulmonary response.

2.3 Therapeutic management

The treatment of choice for canine idiopathic EBP is oral corticosteroid therapy that leads to a dramatic clinical improvement in all dogs (Clercx et al. 2000). Steroids inhibit mast cells, eosinophils, lymphocytes, neutrophils and macrophages involved in the generation and exacerbation of allergic airway inflammation. Release of cytokines and mediators such as histamine, eicosanoids or leukotrienes, is inhibited as well. In the first large case series, an initial dose of prednisolone of 1 mg/kg administered orally twice daily during the first week was usually prescribed (Clercx et al. 2000). This dose was then given on alternate days during the second week, and further reduced to 1 mg/kg on alternate days during the third week. If clinical signs were well controlled, the dose was gradually decreased until maintenance dosage was established; the maintenance dose of prednisolone ranged between 0.125 mg/kg and 0.5 mg/kg every other day or even every 3 or 4 days (Clercx et al. 2000). Among the 36 dogs for which a dose of prednisolone at 1 mg/kg/d was initially started, only 5% of dogs subsequently required an increase to an immunosuppressive dose (2 mg/kg/d) to control their clinical signs. Overall, 25% of dogs receiving dual therapy (oral and inhaled steroids) had their oral therapy discontinued (Casamian-Sorrosal et al. 2020).

The response to steroid therapy is typically excellent with cough, dyspnea and exercise intolerance improving within 1 to 3 days after initiation of treatment. However, full resolution of clinical signs can take several months. Nasal discharge is sometimes more refractory to steroid therapy (Clercx et al. 2000). During treatment, radiographic and bronchoscopic scores decrease with often persisting chronic lesions (Clercx et al. 2002) (Figure 11). Peripheral blood eosinophil counts generally return to normal in dogs that initially had eosinophilia 1 month after the start of the treatment (Clercx et al. 2000).
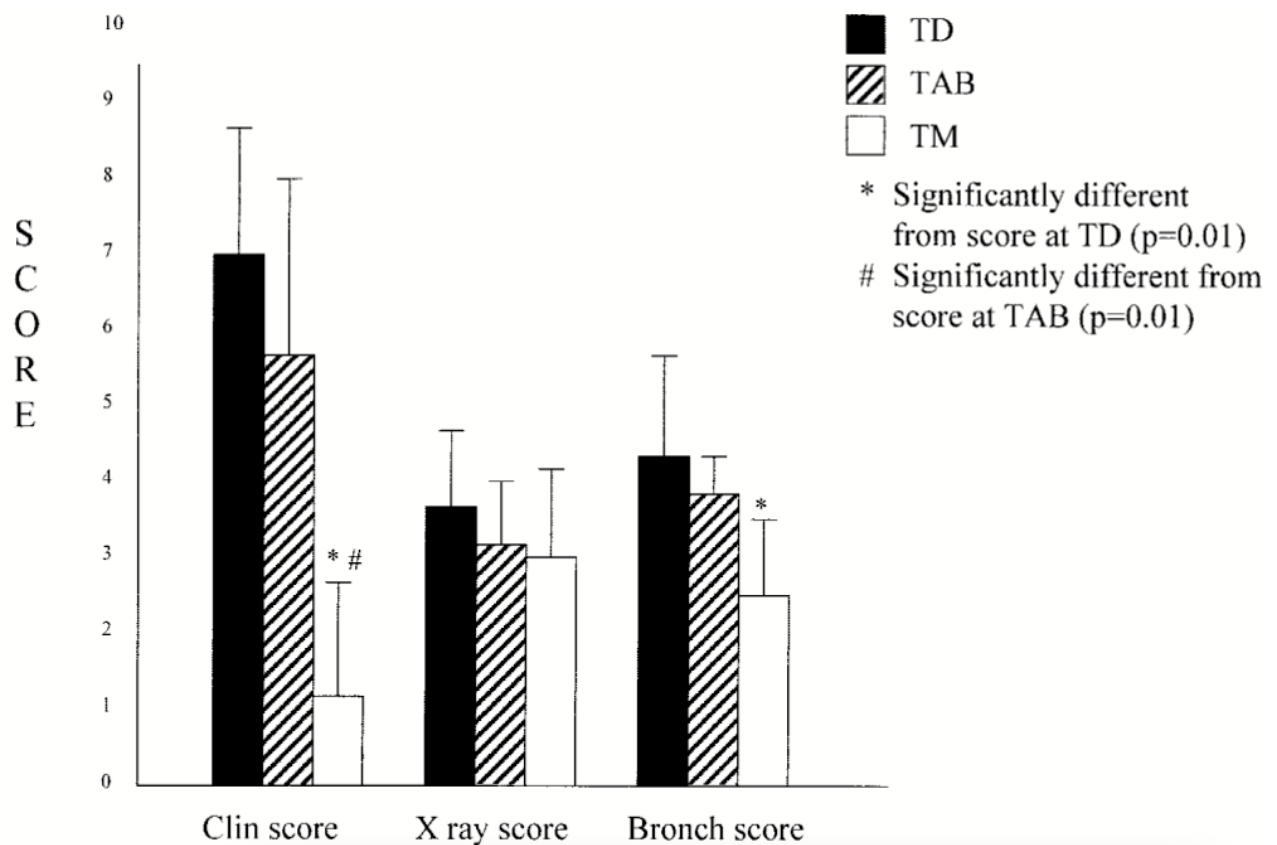
Figure 11: Clinical, radiographic, and bronchoscopic scores at the time of diagnosis, after antibiotic treatment, and at glucocorticoid maintenance dosage (Clercx et al. 2002).

Eosinophilic granulomatosis is usually considered as poorly-responsive to medication; a more aggressive treatment is previously-described with combination of steroids with other immunosuppressive drugs (cyclosporine) (Johnson et al. 2019a) or cytotoxic agent (azathioprine) (Katajavuori et al. 2013). In rare cases, lobectomy may be needed (Johnson et al. 2019a).

2.4 Long term outcome

Some dogs seem to be cured by then previously-described steroid protocol. However, relapse of clinical signs can occur within weeks or months after drug discontinuation (Clercx et al. 2000, Casamian-Sorrosal et al. 2020). In a study including dogs treated with steroids for 8 weeks, 6 of 20 dogs relapsed and needed immediate reinstitution of therapy, suggesting that a longer period of tapering medication might be required in some dogs (Rajamaki et al. 2002a). In another publication, 8 of 11 dogs relapsed after prednisolone cessation and relapses occur either immediately or within the first 3 months (Clercx et al. 2000). In the recent study by Casamian-Sorrosal, relapse was reported in 26% of dogs for which treatment was discontinued and occurs in the first 6 months after the end of the first treatment (Casamian-Sorrosal et al. 2020).

The delay between onset of clinical signs and diagnosis and age at the time of diagnosis do not seem to impact the clinical response to steroids. However, a poorer clinical response was reported in dogs previously treated with high doses of glucocorticoids that are abruptly discontinued or in those treated with irregular parenteral administration of depository steroid injections (Clercx et al. 2000). Casamian and al. studied factors potentially implicated in short-term and long-term clinical remission; univariate analysis did not demonstrate any statistical association between presence of bronchiectasis, bronchoscopy score or degree of BALF eosinophilia and percentages of dogs experiencing clinical remission in both short and long-term (Casamian-Sorrosal et al. 2020).

Despite a gradual decrease in dosage of prednisolone, some dogs still require relatively high doses of treatment to control cough, and so, weight gain, polyuria or polydipsia, and panting become undesirable side effects. Moreover, in some dogs, presence of concurrent diabetes mellitus or obesity contraindicates the use of steroids. In such cases, inhaled steroids could be beneficial. Inhaled medications offer the advantage of high drug concentrations within the airways while limiting systemic absorption and side effects. To date, no study has investigated the long-term response of dogs with EBP to inhaled steroids.

Long-term outcome of dogs with eosinophilic granulomatosis is currently not largely described in literature. Follow-up description are limited to some case reports (Katajavuori et al. 2013, Johnson et al. 2019a). Lobectomy combined to long-term oral cyclosporine treatment was favorably described in one dog followed for > 55 months (Johnson et al. 2019a). Oral prednisolone combined to azathioprine was also associated with an excellent outcome in a dog treated during 7 months (Katajavuori et al. 2013).

Although the role of aero-allergens in EBP is largely unclear, hyposensitization therapy directed against allergens identified by skin testing has rarely resulted in clinical improvement (B.C. McKiernan, unpublished data, 2004).

2.5 Understanding the pathogenesis of canine EBP

2.5.1   A Th-2-mediated response

While the potential triggering antigen(s) is (are) still unidentified, some of the pathogenesis has been elucidated. A primary immune-mediated hypersensitivity reaction to aeroallergens is highly suspected in canine idiopathic EBP. Indeed, predominance of CD4$^+$ T cells and a selective decrease in CD8$^+$ T cells were found in blood, BALF and bronchial mucosa (Figure 12) at the time of diagnosis (Clercx et al. 2002). Moreover, counts of CD4$^+$ T cells decreased with treatment (Clercx et al. 2002).
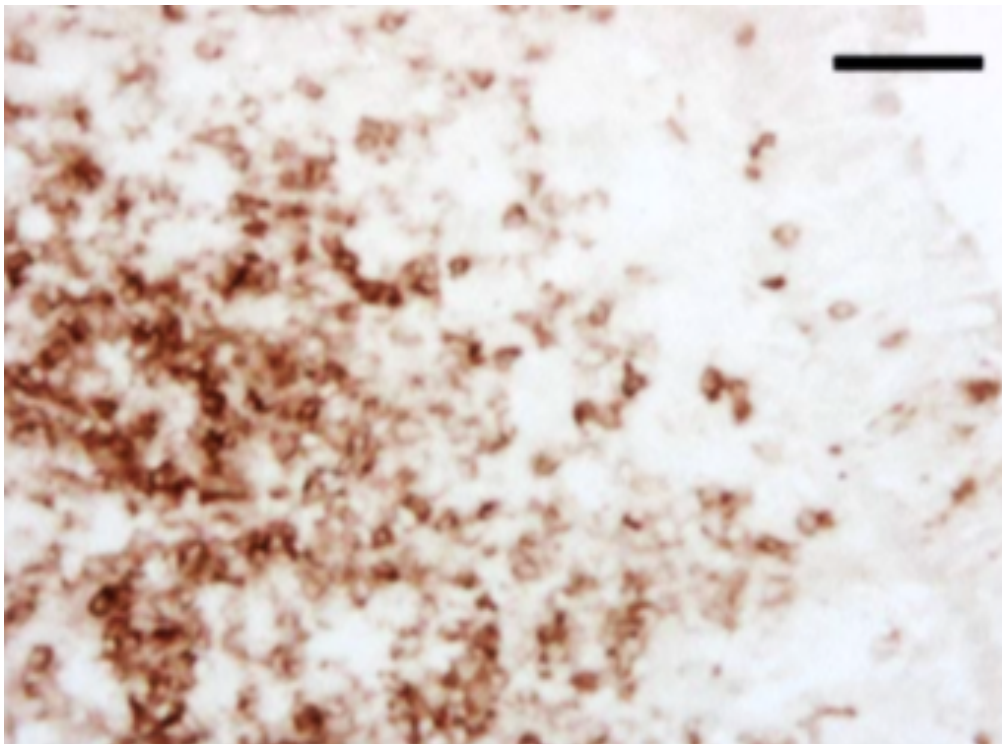
Figure 12: Bronchial mucosa from a dog with EBP, showing many CD4+TCR-αβ+ lymphocytes in the lamina propria (Peeters et al. 2005).

These results suggested that the influx of eosinophils into airways and lungs would be mediated, at least in part, by type I hypersensitivity mechanisms regulated by Th2 lymphocytes. In humans with asthma or idiopathic chronic eosinophilic pneumonia, the ratio of CD4$^+$ T cells to CD8$^+$ T cells was also reported to increase with activated Th2 cells accumulating at site of inflammation.

Despite these first observations, when real-time RT-PCR assays were used for the quantification of mRNA encoding for a panel of Th2-cytokines (IL-4, IL-5 and IL-13), CC chemokines and CC chemokine receptor 3 (CCR3) in perendoscopic bronchial biopsies from eight dogs with EBP, no significant difference in the expression of any cytokine and CCR3 was found compared to control dogs (Peeters et al. 2006) (Figure 13a). Nevertheless, in bronchial biopsies from dogs with EBP, expression

of transcripts for eotaxin-2 and eotaxin-3 and monocyte chemoattractant protein (MCP)-3, strong chemo-attractants for eosinophils, was significantly greater than in samples from control dogs (Peeters et al. 2006) (Figure 13b).
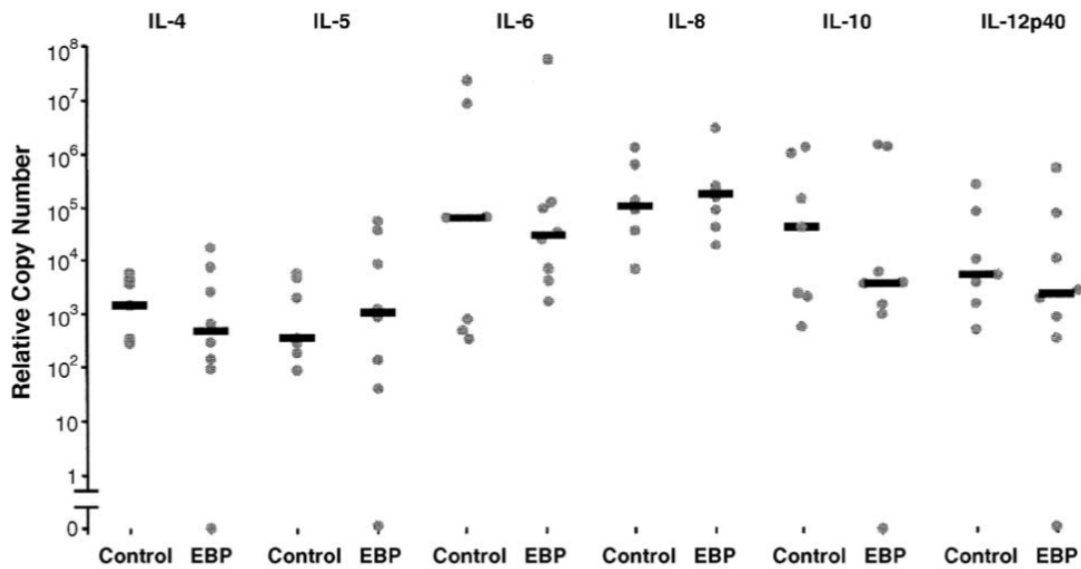
Figure 13a: Plots of the relative copy number assessed via quantitative RT-PCR assays for IL-4,IL-5,IL-6,IL-8,IL-10,IL-12p40 in control dogs and dogs having EBP. The horizontal line corresponds to the median value of that group (Peeters et al. 2006).
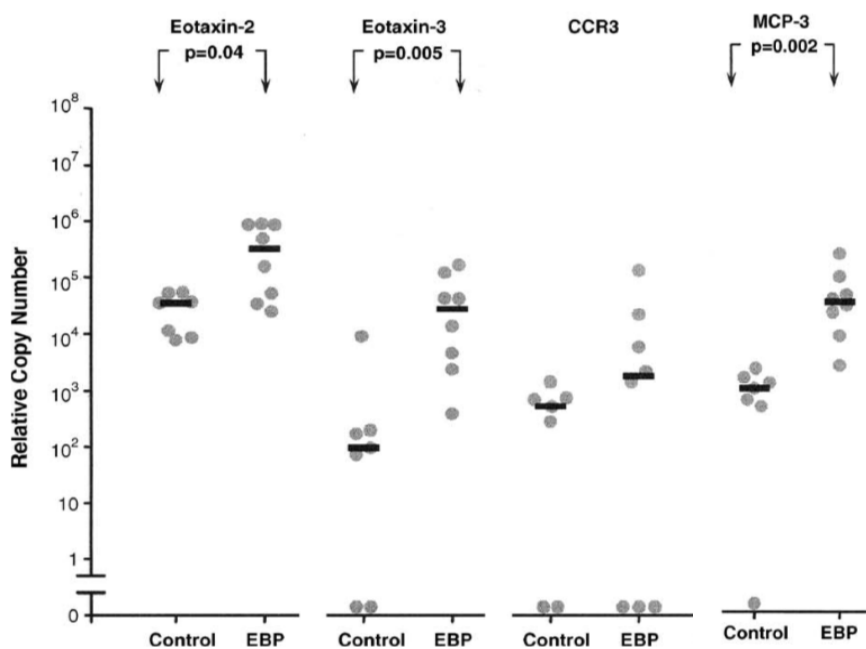
Figure 13b: Plots of the relative copy number assessed via quantitative RT-PCR assays for assays for MCP-3, eotaxin-2,-3 and CCR3 in control dogs and dogs having EBP (Peeters et al. 2006).

2.5.2   Increased collagenase activity

Canine EBP has been also associated with increased lower airways and lung destruction and remodelling with upregulation of collagenolysis and proteolysis (Ramajaki et al. 2002a, Ramajaki et al. 2002b, Heikkilä et al. 2013, Määttä et al. 2021). Indeed, collagenase activity of matrix metalloproteinases (MMPs) was reported to be increased in cell-free and native BALF from dogs with EBP as compared with BALF from healthy dogs (Ramajaki et al. 2002a) (Figures 14a and 14b). A significant positive correlation was noted between the percentage of degraded collagen I and the counts of eosinophils, macrophages, lymphocytes, and mast cells (Rajamaki et al. 2002a). A recent study confirmed a high relative proMMP-9 activity in BALF collected from dogs with EBP (Määttä et al. 2021) (Figure 14c).
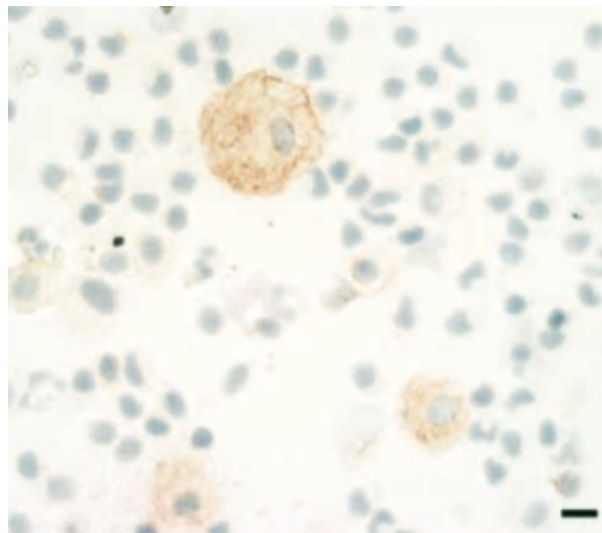
Figure 14a : Collagenolytic matrix metalloproteinase 13 (MMP-13) immunopositive macrophages
(Rajamaki et al. 2002a)
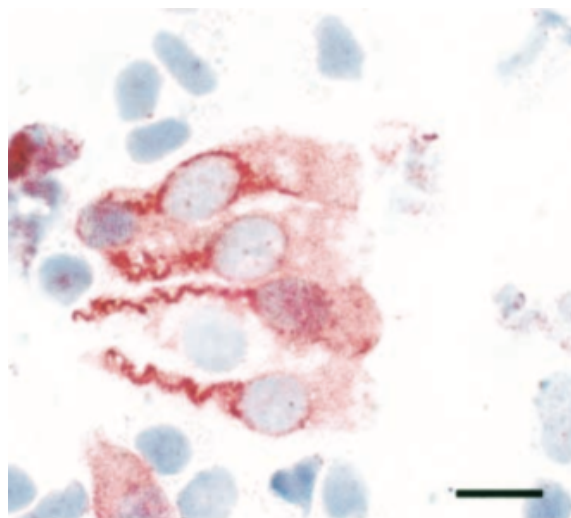
Figure 14b : Collagenolytic matrix metalloproteinase-8 (MMP-8) immunopositive epithelial cells
(Rajamaki et al. 2002a)
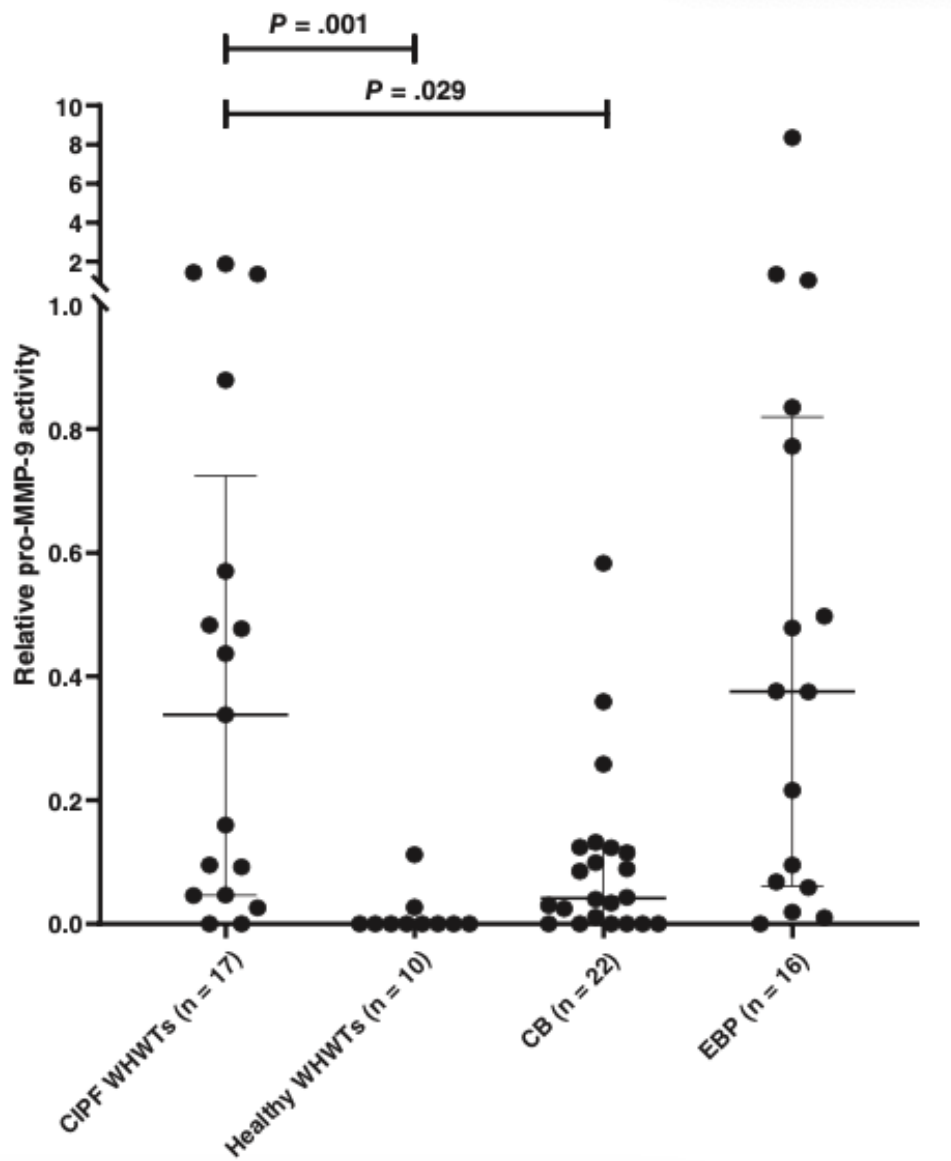
Figure 14c: Scatter plot (median and interquartile range) of bronchoalveolar lavage fluid pro-MMP-9 activities in West Highland white terriers with CIPF compared with other groups. CB, chronic bronchitis; CIPF, canine idiopathic pulmonary fibrosis; EBP, eosinophilic bronchopneumopathy; WHWT, West Highland white terrier (Määttä et al. 2021)

2.5.3   Increased fibroblast activity

Procollagen type III amino terminal propeptide (PIIINP) is a marker of fibroblast activity and enhanced collagen type III turnover. PIIINP was thus shown to be a marker of organ fibrosis. High PIIINP concentrations have been found on BALF collected from dogs with EBP (Schuller et al. 2006, Heikkila et al. 2013) (Figure 15). Elevation of PIIINP in BALF is probably due to secondary fibrotic changes caused by chronic and intense eosinophilic inflammation.
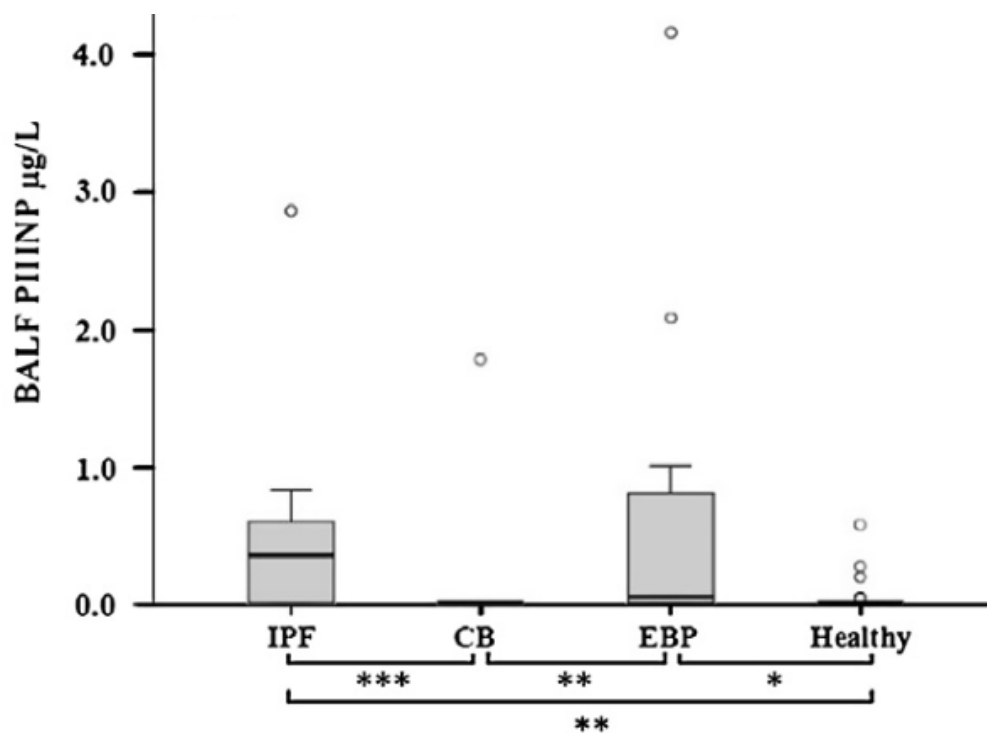
Figure 15: Bronchoalveolar lavage fluid (BALF) procollagen type III amino terminal propeptide (PIIINP) concentrations in dogs with idiopathic pulmonary fibrosis (IPF), chronic bronchitis (CB) and eosinophilic bronchopneumopathy (EBP) and in healthy dogs.

Statistically significant differences: * : P < 0.05, ** : P < 0.01, *** : P < 0.001

(Heikkilä et al. 2013)

2.6 Uninvestigated fields

2.6.1   Etiologic infectious agents

Precedent clinical studies unsuccessfully investigated some potential infectious triggers of the development of EBP, including helminths, non-specific bacteria or fungi (Clercx et al. 2000, Clercx et al. 2002, Johnson et al. 2019a) with fecal analysis and BALF analysis.

Specific knowledge of the prevalence of cardiopulmonary parasites in a specific geographic area is helpful to guide the diagnostic approach. *Angiostrongylus vasorum* is one of the major parasites that are able to cause an eosinophilic airway inflammation in dogs. As the sensitivity of all available diagnostic methods is unfortunately imperfect, confusion with canine idiopathic EBP could clearly be done. Over the last 10 years, several studies highlighted the presence of *A. vasorum* in all countries of western Europe including neighbouring countries of Belgium (Bourque et al. 2008, Yamakawa et al. 2009, Taubert et al. 2009, Barutzki & Schaper 2009, Van Doorn et al. 2009, Helm et al. 2010, Gredal et al. 2011, Conboy 2011, Gallagher et al. 2012, Traversa et al. 2013). Definitive diagnosis of *A. vasorum* may be challenging in some dogs (Bolt et al. 1994, Chapman et al. 2004, Bourque et al. 2008, Koch & Willesen 2009, Helm et al. 2010, Gredal et al. 2011, Elsheika et al. 2014). Actually, sensitivity of faecal Baermann analysis is considered as suboptimal because of irregular shedding of L1 larvae (Bolt et al. 1994, Elsheika et al. 2014, Oliveira-Junior et al. 2006, Schnyder et al. 2015). Furthermore, pre-patent lung pathology can occur before evidence of faecal excretion (Barcante et al. 2008). Alternative methods including serological or molecular assays have been successively developed to improve diagnosis. An in-house rapid ELISA assay (Angio Detect™ Test©, Idexx Laboratories) has been documented as a valid alternative when positive Baermann analysis was considered as gold standard (Schnyder et al. 2014). On the other hand, qPCR could also represents very useful diagnostic tool (Jefferies et al. 2011). The respective clinical usefulness of different diagnostic methods was not investigated in clinical setting. In order to be able to definitively exclude angiostrongylosis before making the diagnosis of idiopathic EBP, determination of the most sensitive test could have a particular importance. Thus, evaluation of comparative results of Baermann analysis, in-house rapid ELISA assay (AngioDetectTM Test©), serology for specific circulating adult *A. vasorum* antigens and specific antibodies by ELISA assays and qPCR on bronchoalveolar lavage is required.

Although human asthma and canine EBP differ because of the lack of bronchial hyper-responsiveness in dogs with EBP, the role of bacterial genera, that are known to be implicated in induction or exacerbation in humans, has never been investigated in dogs with EBP. In human medicine, infections with specific bacteria such as *M. pneumoniae* and *B. pertussis* have been associated with asthma for decades (Hansbro et al. 2004, Harju et al. 2006, Blanchard & Raherison 2010, Atkinson 2013). The proportion of asthmatics who are seropositive for *M. pneumoniae* is positively associated

with clinical severity (Iramain et al. 2016, Yin et al. 2017). *B. pertussis* has been discussed as a triggering and exacerbating factor in chronic inflammatory bronchial diseases with *B. pertussis* toxin favouring eosinophilic airway inflammation (Ennis et al. 2004, Harju et al. 2006, Wakashin et al. 2008, Kavanagh et al. 2013, Nicolai et al. 2013, Yin et al. 2017). In dogs, *M. pneumoniae* and *B. pertussis* infection have not been reported. Among mycoplasmal genera, *M. canis, M. cynos* are classically referred to the respiratory tract of dogs. Nevertheless, while *M. cynos* was recently identified as an emerging and possibly lethal pathogen in dogs with canine infectious respiratory disease complex (CIRD-C) (Rycroft et al. 2007, Zeugswetter et al. 2007, Mannering et al. 2009, Priesnall et al. 2014), the role of *M. canis* and *M. cynos* as primary respiratory pathogens still remains unclear (Chan et al. 2013, Chandler & Lappin 2002, Chalker et al. 2004). On the other hand, *Bordetella bronchiseptica* (*Bb*) is recognized as one of the primary causative pathogen agents of CIRD-C. *Bb* can exist in canine respiratory tract as either commensal or pathogen (Schultz et al. 2014, Lavan et al. 2015). Implication of *M. canis, M. cynos* and *Bb* in canine EBP has never been investigated.

Furthermore, other non-bacterial infectious agents have been identified to trigger or exacerbate some inflammatory bronchial conditions in humans. In this regard, implication of *Aspergillus* has been largely documented (Shah & Panjabi 2016, Page et al. 2015). *Aspergillus* bronchitis and allergic bronchopulmonary aspergillosis are sufficiently common to be considered as a public health issue. Sensitization to *Aspergillus* can occur in asthmatic people, complicating 1-4% of adult cases (Shah & Panjabi 2016, Page et al. 2015). Clinical presentations of both *Aspergillus* bronchitis and allergic bronchopulmonary aspergillosis are reported to considerably overlap with other well-recognized conditions, and it is likely that the great majority of cases remain undiagnosed. The diagnosis of *Aspergillus* bronchitis requires raised *Aspergillus*-specific IgG or culture or persistent positive PCR from BALF. On the other hand, allergic bronchopulmonary aspergillosis is diagnosed based on elevated level of total Ig E and evidence of sensitivity from either skin testing or raised *Aspergillus*-specific IgE antibodies. Until now, in dogs with EBP, no study has investigated the presence of *A.fumigatus* in BALF by qPCR nor the presence of specific serum or BALF antibodies.

2.6.2   *Interest of inhaled steroid therapy as single treatment*

Current literature on management of EBP is scarce but suggests that oral steroid therapy provides a rapid positive clinical response; however, discontinuation of the treatment is followed by recurrence of clinical signs within weeks or months after drug cessation in 30 to 70% of cases (Corcoran et al. 1991; Clercx et al. 2000); these dogs frustratingly require long-term oral therapy to maintain clinical remission. Chronic oral steroid therapy may unfortunately lead to iatrogenic hyperadrenocorticism and its use may also be contraindicated in dogs with concurrent diseases such as diabetes mellitus, obesity or cardiac disease. Therefore, alternative treatment with inhaled steroid therapy (IST) has been increasingly used in the past years, with the suggested advantages of providing

both high drug concentrations within the airways and fewer side effects because of reduced systemic absorption. So far, despite its widespread and regular use by clinicians in practice in dogs with several respiratory conditions, published clinical response to IST as single therapy in dogs with idiopathic EBP is limited to one case treated with fluticasone (Bexfield et al. 2006). Moreover, clinicians commonly consider IST as a practical therapy in clinical practice but the daily compliance of the owners has not been precisely assessed.

2.6.3   Long term clinical and biological tolerance of inhaled steroid therapy

Although clinical and endocrine effects of a 3- to 4-week period of IST have been previously assessed in healthy dogs (Cohn et al. 2008, Melamies et al. 2012), long-term side effects of IST in dogs suffering from chronic diseases such as idiopathic EBP have not been evaluated. Investigation of possible biological inhibition of the pituitary–adrenal axis (PAA) is required in long-term treated dogs.

2.7 Diagnostic methods used in Studies 1 to 5: AngioDetect ND and qPCR

In experimental studies, in-clinic rapid serological device (AngioDetect ND) was positive 14 weeks after inoculation with a specificity of 100%; in naturally-infected dogs with positive Baermann faecal analysis, sensitivity was 85% (Schnyder et al. 2014). For Studies 1 and 2, quantitative PCR for *Angiostrongylus vasorum* was used on BALF. First description of PCR analysis was done by Jefferies in 2009. The PCR assay was capable of detecting a a single first stage larva (L1) in 200 microliters of canine EDTA blood and a single L1 in 200 mg of canine faeces. The assay exhibited a high level of specificity to *A. vasorum* and it did not amplify DNA from a range of other canine parasitic nematodes. According, Jefferies et al (2011), sensitivity on blood or feces were 60%; thus, blood and feces PCR offers no great advantage over Baermann test in terms of sensitivity in naturally infected dogs. Before Studies 1 and 2, investigation of qPCR on BALF in a series of dogs with angiostrongylosis was lacking.

In Studies 3 and 4, commercially-available PCR for *Bb, M.canis* and *M.cynos* were used. First description of PCR for *Bb* was done in 2009 on nasal swabs from vaccinated dogs. Since then, PCR was used on nasal swab to assess prevalence of *Bb* on asymptomatic dogs (Lavan et al. 2015) or dogs with acute cough (Schulz et al. 2014) or on bronchoalveolar lavage in dogs with bronchopneumonia (Viitanen et al. 2015). Chalker et al. (2004) described canine *Mycoplasma* species PCR primers and reaction conditions. In comparison with bacterial culture, PCR was demonstrated to be much more sensitive (Schulz et al. 2015). In a previous study, BALF samples were PCR-positive in 37.9% of dogs with respiratory disease and 18.8% of asymptomatic dogs and a significant association was found between detection of *M. cynos* and presence of respiratory disease.

Lastly, in Study 5, a commercially-available qPCR for *Aspergillus fumigatus* was used. The oldest study that described this method in dogs (Peeters et al. 2008) reported all specific primers. To date, use of qPCR on BALF in dogs with chronic cough was never investigated.

3. Eosinophilic bronchial and lung diseases in humans

Eosinophilic bronchial and lung diseases are two heterogeneous groups of diseases that are respectively unified by the prominent infiltration of bronchi, the lung interstitium and the alveolar spaces by polymorphonuclear eosinophils. Several classification systems have been proposed but none is uniformly accepted (Weissler 2017). Some entities have predominantly airway involvement while others involve the alveolar space disproportionately (Weissler 2017). The clinical presentation and manifestations can include a broad spectrum ranging from quiescent disease to one that is life threatening (Gonlugur & Gonlugur 2008, Akuthota & Weller 2012b, Cottin 2016, Lai et al. 2017, Weissler 2017, Allen & Wert 2018, Suzuki & Suda 2019)

3.1 Classification of eosinophilic bronchial and lung diseases in clinical practice

- Eosinophilic airways diseases
  - Eosinophilic asthma
  - Non asthmatic eosinophilic bronchitis
  - Idiopathic hypereosinophilic constrictive bronchiolitis
- Eosinophilic pneumonias of unknown cause
  - Isolated
    - Idiopathic chronic eosinophilic pneumonia
    - Idiopathic acute eosinophilic pneumonia
  - Eosinophilic pneumonia in systemic syndromes
    - Eosinophilic granulomatosis with polyangiitis
    - Idiopathic hypereosinophilic syndrome
- Eosinophilic pneumonias of known cause
  - Allergic bronchopulmonary aspergillosis and related syndromes
  - Eosinophilic pneumonias of parasitic origin
  - Eosinophilic pneumonias of other infectious causes
  - Drug-induced eosinophilic pneumonias

As this classification regroups very distinct diseases, only entities presenting clinicopathological similarities with the canine idiopathic EBP will be presented and discussed in this manuscript. Thus, eosinophilic bronchitis, idiopathic chronic eosinophilic pneumonia and allergic bronchopulmonary aspergillosis (ABPA) will be developed.   As asthma is basically-defined based on reflex bronchoconstriction that does not occur in canine EBP, this entity will be not presented.

3.2 Non asthmatic eosinophilic bronchitis

Non asthmatic eosinophilic bronchitis (NAEB) is an important cause of chronic, irritable and dry cough. NAEB shares similar eosinophilic inflammation of airway and response to corticosteroid with asthma but in contrast, NAEB subjects have no airflow obstruction and airway hyperresponsiveness (Gonlugur & Gonlugur 2008, Lai et al. 2017).

3.2.1   Epidemiology and etiology

NAEB accounts for about 10-30% of cases with chronic cough (Lai et al. 2017). Only 30-40% of patients with NAEB are accompanied with allergy, including dust mites, flour, rubber latex, welding fumes, formaldehyde, pollen and the mushroom spores. There is no obvious relationship between eosinophilic bronchitis and smoking (Gonlugur & Gonlugur 2008, Lai et al. 2017).

3.2.2   Pathogenesis

The mechanism of the different phenotype between NAEB and asthma is not very clear up to date (Lai et al. 2017). Difference between inflammatory mediators and airway remodeling might explain difference in characteristics of NAEB and asthma (Figure 16).

	Asthma	NAEB
IFN-γ	+	++
TNF-α	±	±
VEGF	+	±
IL13	+ ~ ++	±
IL-8[a]	+	++
IL-8	+	++
IL-5	++	++
ECP	+ ~ +++	+ ~ +++
LTC4	+ ~ ++	± ~ +
LTD4/E4	+	+
PGE2	±	± ~ +++
PGD2	±	++
PGF2α	±	±
TXB2	±	±
Amphiregulin	+	−
Histamine	±	±
Histamine[a]	++	+
8-Isoprostane[a]	++	+
CXCL10[a]	−	+
TARC	+	−

Abbreviations: VEGF:Vascular endothelial growth factor; ECP:Eosinophilic cationic protein; LTC4/D4/E4:Leukotrienes C4/D4/E4; PGE2/D2/F2α:Prostaglandin E2/D2/F2; TXB2:Thromboxane B2; CXCL10:Interferon(IFN)-inducible protein-10; TARC:Thymus and activation-regulated chemokine.
[a] In the bronchoalveolar lavage fluid.

Figure 16: Levels of inflammatory mediator in sputum supernatants in non-asthmatic eosinophilic bronchitis and asthma (Lai et al. 2017).

3.2.3   Clinical features

NAEB may occur in any age of patients, but it is more common in middle-aged men. Irritable cough is often the sole symptom, with no or mild production of white mucoid sputum. Nocturnal cough is not so common in NAEB comparing with some cases of asthma (Lai et al. 2017). Most patients are sensitive to cooking-oil fumes, dust, odors, and cold air, which are triggers of coughing. From 30% to 40% patients are complicated with allergic rhinitis (Gonlugur & Gonlugur 2008, Lai et al. 2017).

3.2.4   Diagnosis

Diagnostic criteria are: (1) chronic cough, characterized by irritable dry cough or accompanying a little mucoid; (2) normal chest radiographs; (3) normal pulmonary function tests and peak expiratory flow rate variability; (4) eosinophil count >3% in sputum; (5) response to gluco-corticosteroids and (6) exclusion of other eosinophilic diseases (Gonlugur & Gonlugur 2008, Lai et al. 2017).

As a new noninvasive method, the fractional exhaled nitric oxide (FeNO) test has been widely used in clinic practice. FeNO is a biomarker associated with the eosinophilic inflammation of airway (Lai et al. 2017). A high level (>31.5 ppb) of FeNO indicates a greater likelihood of corticosteroid-responsive cough but there are many overlaps between NAEB and asthma.

3.2.5   Treatment and outcome

Patients with NAEB are responsive to inhaled corticosteroids (ICS). There is no data currently available about dose nor duration of ICS (Gonlugur & Gonlugur 2008, Lai et al. 2017). In patients with severe cough or being refractory to ICS, oral prednisolone for 3 to 5 days is suggested. After treatment, symptoms improve obviously or even disappear (Gonlugur & Gonlugur 2008, Lai et al. 2017). Relapse may occur in up to 60% of patients and sputum eosinophilia after 4-week treatment is a risk factor to recurrence. Occasionally, a few patients might need a long-term treatment with ICS even with oral corticosteroids to achieve control of symptoms or sputum eosinophilia (Lai et al. 2017).

3.3 Idiopathic chronic eosinophilic pneumonia (ICEP)

Although it is a rare disease, representing fewer than 3% of cases of various interstitial lung diseases, ICEP is the most common of the EP in nontropical areas where the prevalence of parasitic infection is low (Crowe et al. 2019).

3.3.1   Epidemiology

ICEP predominates in women (2:1 female/male ratio), and affects every age group with a mean age of 45 years at diagnosis, with no genetic predisposition (Sergew & Fernández Pérez 2016, Crowe et al. 2019). Some childhood cases have been reported (Cottin 2016, Giovannini-Chami et al. 2016, Allen & Wert 2018). Most patients with ICEP are nonsmokers. A close association between ICEP and allergic diseases has been noted, and more than half of the patients with ICEP have an allergic disease, such as bronchial asthma, atopic dermatitis, nasal polyposis, and allergic rhinitis (Allen & Wert 2018).

3.3.2   Pathophysiology

Specific causes of ICEP remain unknown. It has been hypothesized that some unidentified trigger leads to the overproduction and infiltration of eosinophils in the lung (Allen & Wert 2018).

3.3.3   Clinical manifestations

The onset of ICEP is an insidious and progressive disease, with several weeks or months between the onset of symptoms and the diagnosis (Cottin 2016, Allen & Wert 2018, Suzuki & Suda 2019, Crowe et al. 2019). Shortness of breath is usually moderate and is the prominent clinical manifestation, present in 60% to 90% of patients (Crowe et al. 2019). Cough (90%), rhinitis or sinusitis (20%), and rarely chest pain or hemoptysis (10% or less) may be present (Cottin 2016, Allen & Wert 2018, Suzuki & Suda 2019, Crowe et al. 2019). Wheezes or crackles are found in one-third of patients at auscultation. Approximately 75% of the patients with ICEP experience asthma at some time throughout the course of disease. Systemic symptoms are frequently associated, with fatigue, malaise, fever, night sweats and weight loss. Occasionally arthralgias, non- specific skin manifestations or pericardial effusion are observed (Cottin 2016, Allen & Wert 2018, Crowe et al. 2019).

3.3.4   Imaging

The imaging features of ICEP consist of bilateral alveolar infiltrates with ill-defined margins, with a typical peripheral predominance in approximately 25% of patients. On high-resolution computed tomography, typical features consist of bilateral confluent consolidations (Figures 17a to 17c) and ground-glass opacities (Figures 17b and 17c) (Allen & Wert 2018, Crowe et al. 2019). Fibrotic changes such as bronchiectasis and honeycombing are late findings (Sergew & Fernández Pérez 2016).
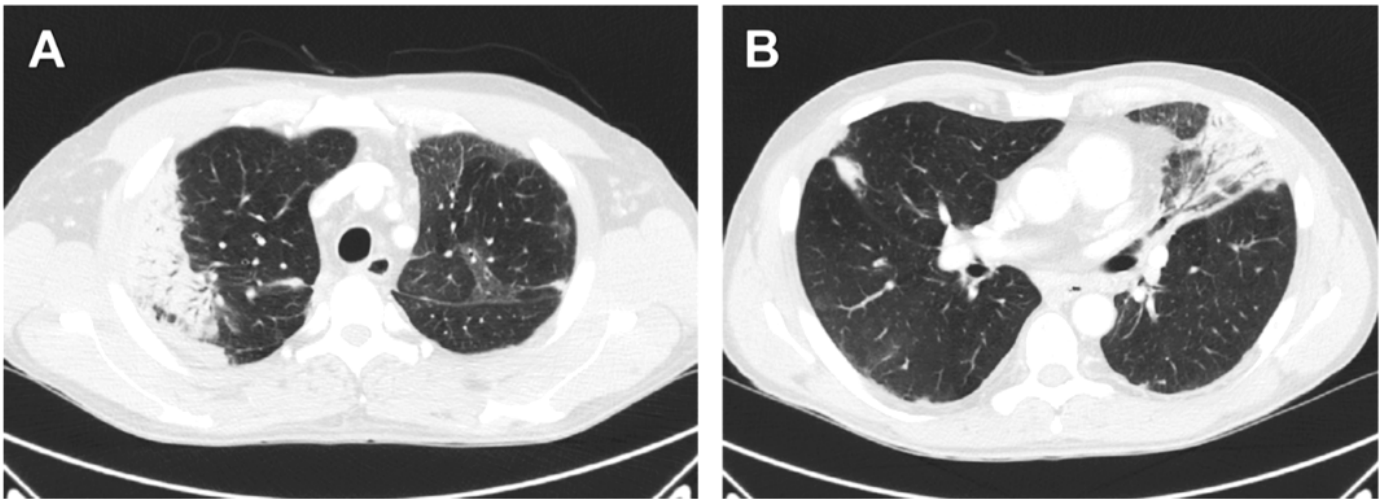
Figure 17a: Chest CT of a patient with idiopathic chronic eosinophilic pneumonia, demonstrating peripheral airspace consolidation predominating in the upper lobes. (A) Right upper lobe; (B) Lingula (Cottin 2016).
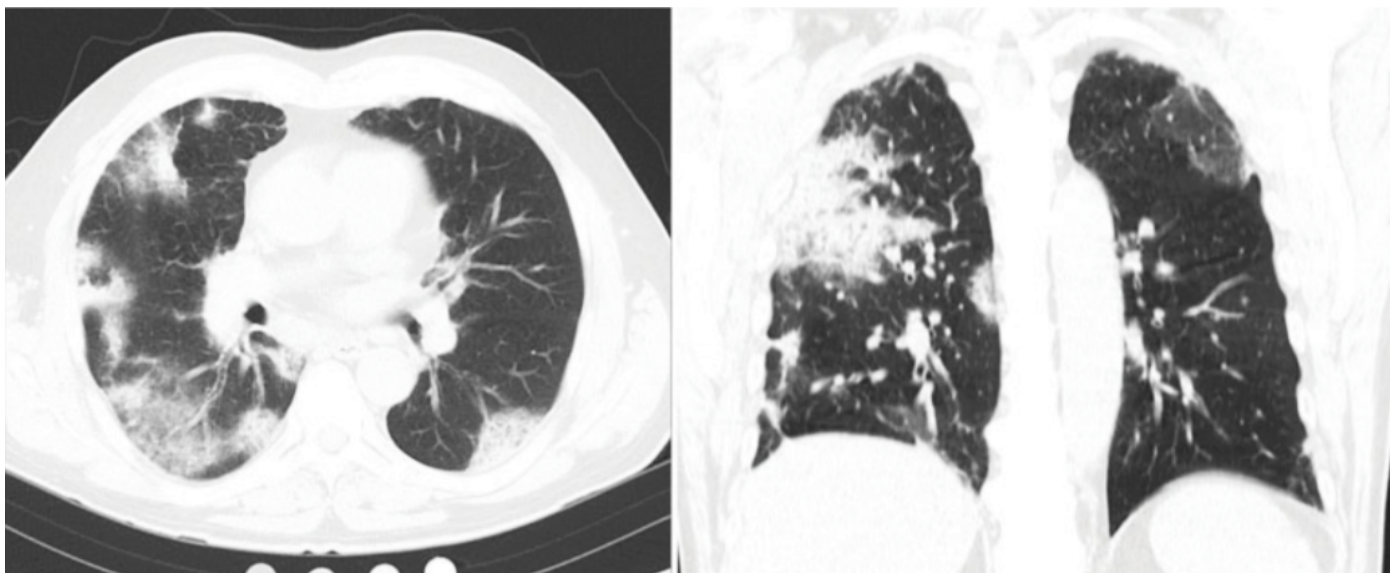
Figure 17b: Axial (left) and coronal (right) images from chest CT in a patient showing upper lung preponderant, right-greater-than-left, focal areas of consolidation, and ground-glass opacity, typical of idiopathic chronic eosinophilic pneumonia (Sergew & Fernández Pérez 2016).
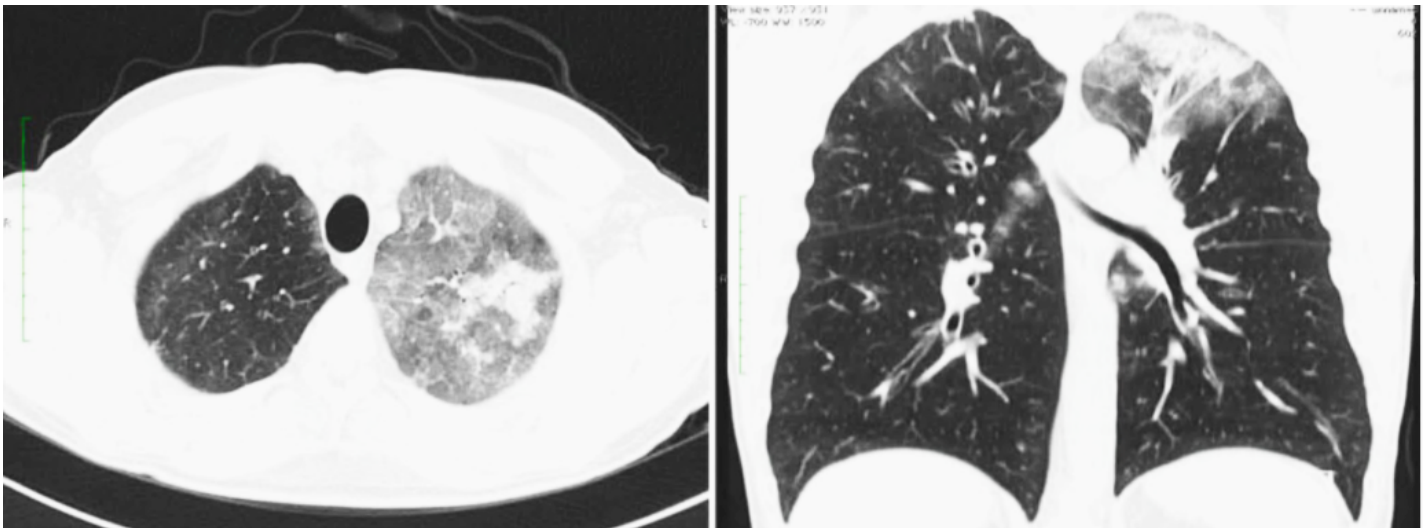
Figure 17c: Axial (left) and coronal (right) images from chest CT in a patient with idiopathic chronic eosinophilic pneumonia showing significant ground-glass opacity as well as consolidation in the left upper lobe (Sergew & Fernández Pérez 2016).

3.3.5   Laboratory findings

The vast majority of cases of ICEP (88-95%) are associated with peripheral blood eosinophilia (Allen & Wert 2018). The BAL eosinophil count is commonly greater than 40% (Allen & Wert 2018, Crowe et al. 2019). Increase of serum C-reactive protein and total immunoglobulin (Ig) E concentrations are common (Allen & Wert 2018).

3.3.6   Diagnostic evaluation

Working diagnostic criteria for ICEP are found in Figure 18 (Sergew & Fernández Pérez 2016, Allen & Wert 2018, Suzuki & Suda 2019, Crowe et al. 2019).

1. Diffuse pulmonary alveolar consolidation with air bronchogram and/or ground-glass opacities at chest imaging, especially with peripheral predominance.
2. Eosinophilia at bronchoalveolar lavage differential cell count >40% (or peripheral blood eosinophils > 1000 /mm3).
3. Respiratory symptoms present for at least 2 to 4 wk.
4. Absence of other known causes of eosinophilic lung disease (especially exposure to drug susceptible to induce pulmonary eosinophilia).

Figure 18: Diagnostic criteria for idiopathic chronic eosinophilic pneumonia (Allen & Wert 2018, Crowe et al. 2019)

Lung biopsy is usually not necessary to make a confident diagnosis (Allen & Wert 2018). Findings include interstitial and alveolar eosinophils and histiocytes (Sergew & Fernández Pérez 2016).

3.3.7   Differential diagnosis

Allergic bronchopulmonary aspergillosis (ABPA) is similar to ICEP in that there is a frequent pre-exisiting history in asthma, a peripheral blood eosinophilia and upper lobe radiographic opacities. However, in ABPA, serum IgE is typically greater than 1000IU/L. with serologic evidence of IgE antibodies to *Aspergillus* and a positive skin prick test to the *Aspergillus* antigen (cf infra).

3.3.8   Treatment and outcome

Treatment should be initiated as soon as a diagnosis of ICEP is made, as fewer than 10% of cases spontaneously improve or recover, and untreated ICEP can lead to irreversible fibrosis (Allen & Wert 2018). Systemic corticosteroids have been the most well-studied and effective therapy for ICEP and are the first-line treatment, with a goal of inducing remission as well as reducing the possibility of relapse (Crowe et al. 2019). Patients with ICEP often demonstrate a substantial and dramatic response once steroids are started with profound symptomatic relief within 48 hours in some cases (Allen & Wert 2018). If a patient has minimal to no improvement, an alternative diagnosis should be sought.

While the improvement in symptoms and infiltrates with corticosteroids is rapid, relapses occur in more than half the patients while decreasing or after stopping corticosteroids. After relapse, resumption of corticosteroid treatment leads to favorable response similar to that to the initial treatment. Up to 75% of patients required prolonged, and even lifelong, glucocorticoid therapy (Allen & Wert 2018, Crowe et al. 2019). Although inhaled steroids should not be used as initial or monotherapy, they may have some role in reducing the dose or duration of systemic steroid therapy (Allen &Wert 2018, Suzuki & Suda 2019, Crowe et al. 2019).

3.4 Allergic bronchopulmonary aspergillosis

Allergic bronchopulmonary aspergillosis (ABPA) is a rare lung disorder linked to Th2 CD 4[+]T-cell-mediated hypersensitivity to antigens of *Aspergillus fumigatus*, a ubiquitous thermophilic fungus that colonizes bronchial mucosa (Agarwal et al. 2013, Shah & Panjabi 2016, Agarwal et al. 2020). It occurs in non-immunocompromised patients, and manifest clinically as recurrent infiltrates, chronic bronchial obstruction, and the development of central bronchiectasis, complicating the course of patients with asthma and cystic fibrosis (Agarwal et al. 2013, Shah & Panjabi 2016).

3.4.1   Epidemiology

Aspergillus sensitization can be defined as the presence of immediate cutaneous hypersensitivity (or elevated serum IgE levels) against antigens of *A. fumigatus*. ABPA is an advanced stage of *Aspergillus* sensitization. It occurs almost exclusively in subjects with a prior history of chronic bronchial disease; most patients who develop ABPA have a history of atopy, asthma or cystic fibrosis (Agarwal et al. 2013, Shah & Panjabi 2016). Both the last conditions lead to greater bronchial adherence of the inhaled spores and intrabronchial development of filaments (hyphae). In adults, the prevalence in asthmatic patients is low, at around 2–8% and in patients with cystic fibrosis, ABPA is a common complication of the disease, occurring in approximately 10% of cases (Agarwal et al. 2013). Past research studies on ABPA have led to the conclusion that it is both underdiagnosed and much more prevalent than previously assumed. The underdiagnosing of ABPA is due to a lack of consensus regarding diagnosis.

3.4.2   Pathophysiology

Exposure to high concentrations of spores have been reported to cause ABPA but as not all asthmatics develop ABPA despite being exposed to the same milieu, environmental factors may not be the only factors in the pathogenesis of ABPA. Fungal conidia are immunologically inert. However, defective clearance of conidia in asthma or cystic fibrosis allows them to germinate into hyphae. Airway macrophages recognize fungi through pattern recognition receptors such as toll-like receptors and mannose-binding lectin (Agarwal et al. 2013). In ABPA, it is hypothesized that defects in innate and adaptive immunity cause persistence of *A. fumigatus* (Shah & Panjabi 2016).

The fungal antigens elicit mainly a type I (IgE-mediated) reaction that is responsible for the disease presentation (Shah & Panjabi 2016). Both type III (with the participation of IgG and IgA

antibodies) and type IV (exaggerated Th2 CD4$^+$ T-cell–mediated immune response) hypersensitivity responses with IL-4, IL-5, IL-9 and IL-13 cytokine secretion are also involved in the immunologic process (Agarwal et al. 2013, Patel et al. 2019, Agarwal et al. 2020). Excessive B-cell response, immunoglobulin production (total and *A.fumigatus* IgE), and high levels of circulating IL-4 play a key role.

Individual host genetic susceptibility appears more significant than environmental factors. Genetic risk factors include expression of HLA-DR2 and HLA-DR5 genotypes (Shah & Panjabi 2016). Surfactant protein-A2, toll- like receptor or interleukin receptor polymorphisms also play an important role in the development of ABPA (Shah & Panjabi 2016, Agarwal et al. 2020).

3.4.3   Clinical manifestations

Patients with ABPA experience chronic cough, dyspnea, breathlessness, expectoration of golden-brown or tan sputum plugs, haemoptysis, low-grade fever, and chronic rhinitis (Agarwal et al. 2013, Shah & Panjabi 2016). ABPA rarely progresses to chronic respiratory failure requiring oxygen supplementation (Shah & Panjabi 2016). Rhonchi, crepitations, and bronchial breathing may be heard depending on the degree of the lung disease present. Lastly, ABPA may be associated with allergic *Aspergillus* sinusitis (Agarwal et al. 2013, Shah & Panjabi 2016). ABPA is clinically and biologically divided into five clinical stages (Figure 19).

Stage	Clinical description	Radiographic findings	Total serum Ig E
I: Acute	Symptomatic (fever, cough, chest pain, hemoptysis, sputum)	Infiltrates	Elevated total serum IgE (> 1000 IU/ml); aspergillus-specific precipitins
II: Remission	Asymptomatic	Normal	Normal or elevated total serum IgE
III: Exacerbation	Symptomatic	New infiltrates appearing	Elevated total serum IgE (doubling from baseline); peripheral blood eosinophilia
IV: Corticosteroid-dependant asthma	Cyanosis, severe dyspnea	With or without infiltrates	Normal or elevated total serum IgE
V: End-stage (fibrotic lung disease)	Cyanosis, severe dyspnea	Bronchiectasis, fibrosis and chronic cavitation.	Normal or elevated total serum IgE

Figure 19: Clinical staging of Allergic Bronchopulmonary Aspergillosis (Agarwal et al. 2013, Shah & Panjabi 2016, Patel et al. 2019)

3.4.4   Imaging

High resolution CT of the thorax is currently the imaging modality of choice for ABPA (Patel et al. 2019, Agarwal et al. 2020). Bronchial abnormalities include central cylindrical bronchiectasis (Figures 20a and 20b), bronchial wall thickening, mucous plugging with "finger-in-glove" pattern, ground-glass or mosaic attenuation, cavitation, emphysematous bullae, and airspace consolidation. Pleural involvement may also be seen with pleural effusions or spontaneous pneumothorax. Central bronchiectasis and mucous impaction (Figure 20c) are pathognomonic for ABPA (Shah & Panjabi 2016, Patel et al. 2019, Agarwal et al. 2020).
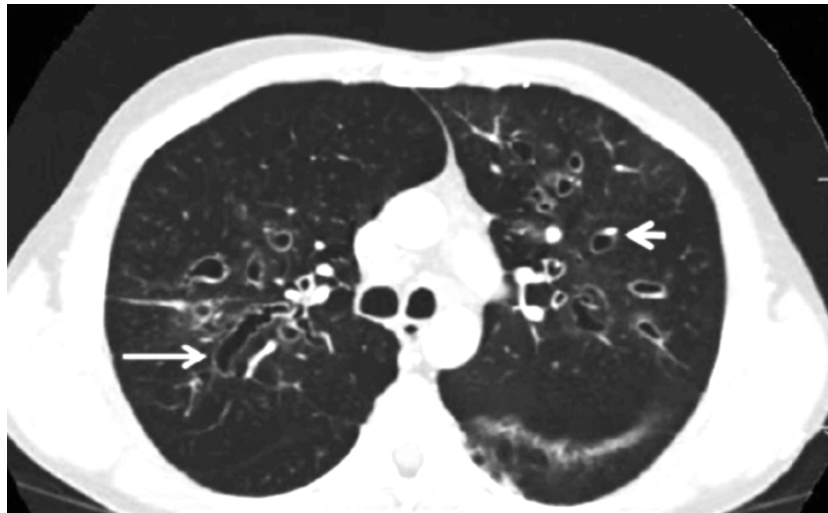
Figure 20a: 'Signet ring' (short, thick arrow) and 'string of pearls' (long, thin arrow) appearances, indicative of central bronchiectasis (Patel et al. 2019).
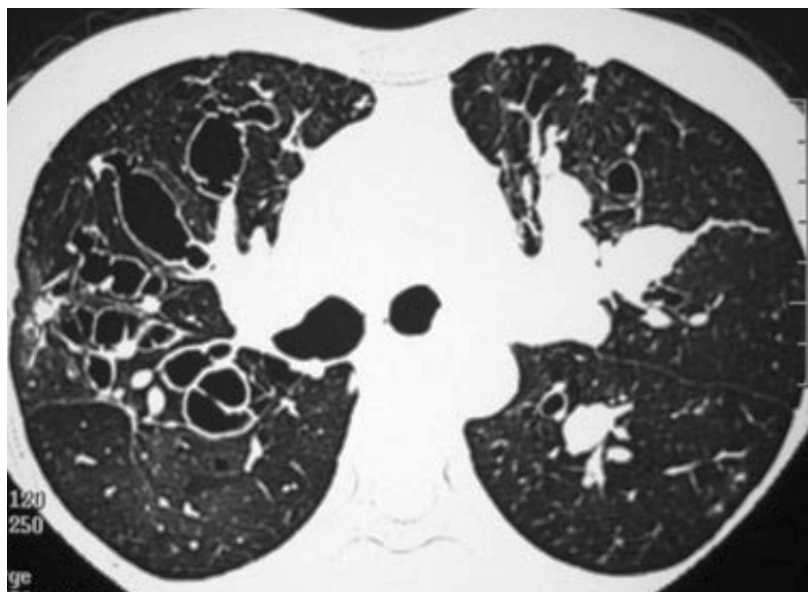
Figure 20b: Extensive bronchiectasis extending till the periphery (Agarwal et al. 2013)
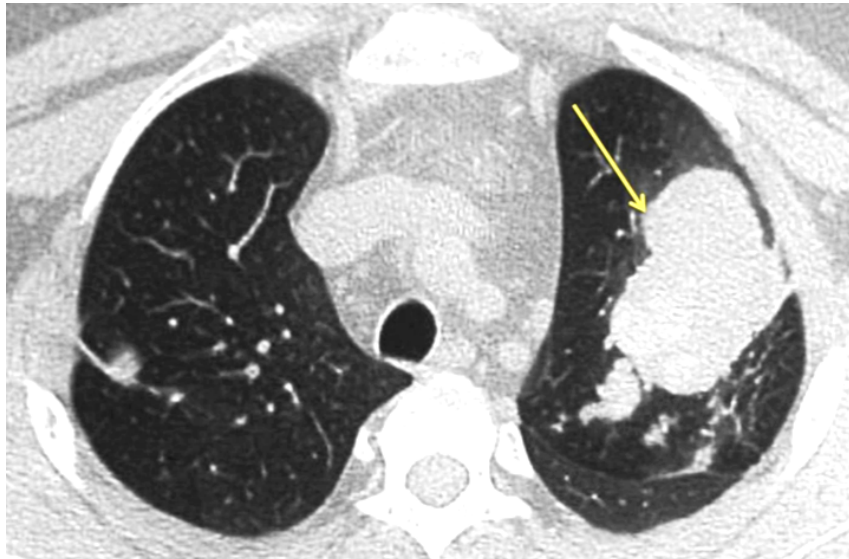
Figure 20c: High attenuation mucous impaction (yellow arrow) (Patel et al. 2019)

3.4.5   Laboratory findings

Blood eosinophils are usually greater than 1000 cells/mm^3; however, authors hypothetize an overreliance on eosinophil count being a significant cause for missed diagnosis of ABPA (Agarwal et al. 2020) and a cut-off of 500 eosinophils/mm^3 would be more appropriate. In ABPA, the pulmonary eosinophilia is far greater than peripheral blood (Agarwal et al. 2013). Serum levels of total IgE may be particularly elevated (variable cut-off depending authors, 417 IU/L or 1000 IU/L) (Agarwal et al. 2013).

An immediate cutaneous hypersensitivity to *A. fumigatus* antigens (either crude or recombinant) is hallmark of ABPA, and represents the presence of IgE antibodies specific to *A. fumigatus*. The test can be performed either using a skin prick test or an intradermal injection. The sensitivity of a positive result in diagnosis of ABPA is about 90% (Agarwal et al. 2013). An elevated level of serum IgE antibodies specific to *A. fumigatus* is considered a characteristic finding of ABPA and is currently the most sensitive investigation in the diagnosis of ABPA (Agarwal et al. 2013, Shah & Panjabi 2016, Patel et al. 2019, Agarwal et al. 2020). Detection of *Aspergillus*-specific serum IgG may not be specific for ABPA as high levels are encountered in other forms of aspergillosis (Agarwal et al. 2013).

Culture of *A. fumigatus* in sputum is supportive but not diagnostic of ABPA because the fungus can also be grown in other pulmonary diseases due to ubiquitous nature of the fungi (Agarwal et al. 2013, Patel et al. 2019, Agarwal et al. 2020). Lastly, serum or BALF galactomannan estimation has a limited role in the diagnostic workup of patients with ABPA (Agarwal et al. 2015a).

3.4.6   Diagnostic evaluation

Uniform criteria for a diagnosis of ABPA have been lacking but increased efforts have been made to create a standardized approach for testing. In 2013, The International Society for Human and Animal Mycology (ISHAM) created a working group to formulate a revised set of diagnostic criteria (Figure 21).

Predisposing conditions: asthma or cystic fibrosis
Obligatory criteria (both must be present)
Aspergillus skin test positivity or elevated IgE levels against *A. fumigatus* (>0,35kUA/l)
Elevated total IgE concentration (typically > 1000 IU/mL, but if the patient meets other criteria, then an IgE value of <1000 IU/mL is acceptable)
Other criteria (at least 2 must be present)
Precipitating serum antibodies to *A. fumigatus* or elevated serum IgG-*A. fumigatus*
Radiographic pulmonary opacities consistent with ABPA
Total eosinophil count of >500 cells/µL in patients who are steroid naive

Figure 21: Acceptable diagnostic criteria of Allergic bronchopulmonary Aspergillosis (from International Society for Human and Animal Mycology Working Group) (Agarwal et al. 2013, Shah & Panjabi 2016, Patel et al. 2019).

3.4.7   Treatment and outcome

Goals of treatment for ABPA includes the management of asthma exacerbations and prevention of progression to bronchiectasis, cavitations and severe fibrotic lung disease while minimizing corticosteroid side effects (Agarwal et al. 2013, Shah & Panjabi 2016).

Currently, systemic glucocorticoids remain the most effective drugs for treating ABPA. Despite its therapeutic effectiveness, the optimal dosing schedule for prednisolone is currently not known (Agarwal et al. 2013, Shah & Panjabi 2016, Patel et al. 2019). Long-term oral corticosteroids are maintained only in patients with frequent symptomatic attacks or evidence of progressive lung damage. Inhaled corticosteroids alone help achieve asthma control, but neither do they prevent symptomatic exacerbations of ABPA nor delay progression of lung damage (Shah & Panjabi 2016, Patel et al. 2019).

The exact role of antifungal agents in the treatment of ABPA is still debated (Agarwal et al. 2013, Shah & Panjabi 2016, Agarwal et al 2020). By reducing the fungal load, antifungal agents help control the antigenic stimulus and thus decrease the inflammatory response (Shah & Panjabi 2016). Oral itraconazole or voriconazole is a useful adjunct to corticosteroids for several months, allowing steroid

dose reductions, decreasing the frequency of exacerbations and improving outcome (Shah & Panjabi 2016, Patel et al. 2019). It is particularly indicated in patients who are unable to taper oral prednisolone (Agarwal et al. 2013, Agarwal et al 2020). Nebulized amphotericin B is also described with decreasing the frequency of exacerbations in patients with ABPA complicating asthma (Ram et al. 2016).

Omalizumab is a humanized monoclonal antibody that works against IgE. Past studies have suggested that omalizumab may be used in the treatment of ABPA, especially in patients with asthma. Despite this, the use of omalizumab in ABPA patients with cystic fibrosis requires more definitive clinical trials (Agarwal et al. 2013, Shah & Panjabi 2016, Agarwal et al 2020).

Nebulized hypertonic saline can be used to reduce the viscosity of sputum to ease expectoration of mucus plugs (Agarwal et al. 2013). However, the first dose should be administered under supervision and preceded by salbutamol because of risk of bronchospasm. Long-term azithromycin therapy can be used to decrease cough and expectoration in patients with bronchiectasis and frequent exacerbations (Agarwal et al. 2013). Therapeutic bronchoscopy should be considered in patients who have proximal collapse, which persists after 3– 4 weeks of oral steroid therapy in patients compliant with therapy. The removal of mucus plugs can cause significant improvement in symptoms and lung function (Agarwal et al. 2013).

Anecdotal patient evidence suggests that some exacerbations are driven by large environmental exposures; thus, activities that could result in inhalation of large numbers of *Aspergillus* conidia (gardening, agricultural and farm-related activities, exposure to home or other building renovations, housing close to a composting site and cleaning old dusty environments) should be avoided. If activities are unavoidable, use of surgical masks may minimize spore inhalation (Agarwal et al 2020).

Total serum IgE level is often used to monitor clinical response to glucocorticoid treatment. Serum IgE levels should have a decrease of 25% after one month of treatment and about 60% after two months. A total serum IgE level decrease of 35% is considered a good therapeutic response. If the serum total IgE levels double at any point then it is an indicator of ABPA exacerbation (Agarwal et al. 2013, Shah & Panjabi 2016, Patel et al. 2019, Agarwal et al 2020).

Objectives

Objective 1: Improved diagnosis of *Angiostrongylus vasorum* in order to exclude infection with this worm in dogs with eosinophilic bronchopneumopathy

As idiopathic EBP is an exclusion diagnosis, specific investigation of cardio-pulmonary parasites is needed. *Angiostrongylus vasorum* is one of the major parasites that are able to cause cough and exercise intolerance in youg adult dogs and infestation may thus be clearly confused with canine idiopathic EBP. From 2008, prevalence of *A. vasorum* in Europe is increasing (Bourque et al. 2008, Taubert et al. 2009, Van Doorn et al. 2009, Gredal et al. 2011, Gallagher et al. 2012). Quantitative PCR has been newly developed but only anecdotally performed on broncho-alveolar lavage fluid samples (Jefferies et al. 2011).

➤   The <u>aim of the first study</u> was to investigate *a posteriori* the possibility of previously undiagnosed angiostrongylosis among a series of coughing and healthy dogs living in Belgium using qPCR on collected and stored broncho-alveolar lavage specimens.

An in-house rapid ELISA assay (Angio Detect™ Test©, Idexx Laboratories) has been developed and documented as a valid alternative with sensitivity of 84% and specificity of 100%, when positive Baermann analysis was considered as gold standard (Schnyder et al. 2014). However, respective clinical usefulness of different diagnostic methods (faecal analysis, rapid device and qPCR) was not investigated in clinical setting.

➤   The <u>objective of the second article</u> was to compare several diagnostic tools including a rapid assay and qPCR on BAL material in a small series of dogs diagnosed with angiostrongylosis.

Objective 2: Contribution to the understanding of the pathogenesis of the idiopathic eosinophilic bronchopneumopathy in dogs

1. **Improved diagnosis of *Bordetella bronchiseptica* and investigation of infectious etiologic agents in dogs with idiopathic eosinophilic bronchopneumopathy**

To date, specific investigation of bacterial infection in dogs with EBP was restricted to conventional bacterial culture on BALF (Clercx et al. 2000, Johnson et al. 2019a, Casamian-Sorrosal et al. 2020). However, some canine respiratory bacterial pathogens are fastidious, including *Bordetella bronchiseptica* (*Bb*) and *Mycoplasma* species. They may require additional more sensitive techniques to be detected such as quantitative PCR. In human medicine, infections with *M. pneumoniae* and *B. pertussis* have been associated with asthma for decades (Kraft et al. 2002, Hansbro et al. 2004, Harju et al. 2006, Blanchard & Raherison 2010, Atkinson 2013). However, as *Bb* has been isolated in clinically healthy dogs by quantitative PCR (qPCR) from nasal and pharyngeal swabs (Schulz et al. 2014), it is unclear how a positive qPCR result from BALF should be interpreted.

➤ In a <u>preliminary study (Study 3)</u>, comparative performance of qPCR analysis, bacterial culture of BALF and cytological examination of BALF in the diagnosis of canine *Bb* infection was described. Additionally, in order to investigate whether a positive qPCR result for *Bb* might possibly either indicate a carrier state or result from contamination during the procedure, qPCR analysis was performed on BALF from 10 healthy dogs and on fifteen blank lavages of endoscopic channel.

➤ <u>In Study 4</u>, qPCR on BALF was used to assess presence and bacterial load of *M. canis, M. cynos* and *Bb* in dogs newly-diagnosed with EBP in comparison with dogs having chronic bronchitis and healthy dogs.

2. Investigation of the potential implication of *Aspergillus fumigatus* by qPCR and specific antibodies measurements in dogs with idiopathic eosinophilic bronchopneumopathy

In humans, Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitive reaction to *Aspergillus fumigatus* in uncontrolled asthmatics and in patients with cystic fibrosis (Agarwal et al. 2013, Shah & Panjabi 2016, Page et al. 2015, Cottin 2016) and is characterized by bronchiectasis with mucous impaction. ABPA is basically diagnosed based on evidence of raised *Aspergillus*-specific IgE antibodies (Agarwal et al. 2013, Page et al. 2015, Shah & Panjabi 2016). Similarities between dogs with EBP and humans with ABPA raised the hypothesis of potential implication of *A. fumigatus* as a triggering or exacerbating aeroallergen in such dogs.

➢ The <u>aims of Study 5</u> were to investigate presence of *A. fumigatus* (using qPCR in BALF) and *A. fumigatus*-specific antibodies (Ig E and G, by ELISA testing) in dogs with newly-diagnosed and steroid-naïve EBP in comparison with dogs having chronic bronchitis or healthy dogs.

Objective 3: Long-term clinical benefit and biological tolerance of inhaled steroid therapy in canine idiopathic eosinophilic bronchopneumopathy

1. **Clinical benefit of inhaled steroid therapy as sole treatment in dogs with idiopathic eosinophilic bronchopneumopathy**

The classic and only well-described effective treatment of dogs with idiopathic EBP consists in long-term oral administration of prednisolone (Clercx et al. 2000). Discontinuation of the treatment leads to recurrence within weeks or months after drug cessation in 30 to 70% of cases (Corcoran et al. 1991, Clercx et al. 2000). These dogs frustratingly require long-term oral therapy to maintain clinical remission but chronic oral steroid therapy may unfortunately lead to undesirable effects. Therefore, alternative treatment with inhaled steroid therapy (IST) has been increasingly used in the past years but published clinical response to IST as single therapy in dogs with idiopathic EBP is very limited (Bexfield et al. 2006).

➢ For <u>Study 6</u>, the first aim was to describe the clinical response to long-term IST with fluticasone as single therapy for more than 6 months in dogs with EBP.

2. **Long-term side effects of inhaled steroid therapy in dogs with idiopathic eosinophilic bronchopneumopathy**

Although clinical and endocrine effects of a 3 to 4 weeks period of IST have been previously assessed in healthy dogs (Cohn et al. 2008, Melamies et al. 2012), long-term side effects of IST in dogs suffering from chronic bronchial diseases or more complex chronic lung diseases such as idiopathic EBP have not been evaluated.

➢ The <u>second objective of Study 6</u> was thus to report possible biological inhibition of PAA and related side effects secondary to chronic steroid administration in dogs with EBP.

Experimental section

─────── Experimental section

Part 1 :

Exclusion of infection with *Angiostrongylus vasorum* as a cause of eosinophilic bronchopneumopathy in dogs

Preamble

The first part of the experimental section groups 2 studies related to **evaluation of qPCR on BALF as relevant tool to exclude infestation by *Angiostrongylus vasorum*.**

Idiopathic EBP is an exclusion diagnosis and specific exclusion of infection by *Angiostrongylus vasorum* is essential, particularly because of epidemiological and clinicopathological similarities between angiostrongylosis and canine EBP.

Thus, the first study described the use of qPCR in BALF for definitive exclusion of angiostrongylosis (Study 1); with this retrospective analysis of stored BALF collected from dogs with chronic cough, we found that pulmonary angiostrongylosis was negligible in Belgium until 2013 and previous misdiagnosis of idiopathic EBP was not made. Since its first emergence in Belgium in 2013, angiotrongylosis is progressively considered as endemic condition and its definitive exclusion is essential to validate a final diagnosis of idiopathic EBP. The second study confirmed that qPCR on BALF is the most sensitive technique to definitely rule out angiostrongylosis in dogs with eosinophilic inflammation on BALF, as non-invasive diagnostic tools including faecal analysis with Baermann method and rapid serological device have imperfect sensitivities (Study 2).

Experimental section

Part 1 – Study 1 :

Detection of *Angiostrongylus vasorum* by quantitative PCR in bronchoalveolar lavage fluid of Belgian dogs

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Abstract

Objectives To describe *Angiostrongylus vasorum* infection in a series of clinical cases over a 12-month period, report the use of qPCR on broncho-alveolar lavage fluid and investigate the possibility of previously undiagnosed angiostrongylosis in a retrospective cohort of coughing and healthy dogs.

Methods Pulmonary angiostrongylosis was diagnosed based on compatible clinical signs and positive qPCR on broncho-alveolar lavage fluid and/or positive Baermann examination. qPCR was also performed retrospectively on broncho-alveolar lavage fluid from 65 dogs (55 coughing and 10 healthy dogs), examined between 2008 and 2014; results of Baermann examination were also available in 33.

Results Seven young-adult dogs from Southeastern Belgium with respiratory clinical signs were diagnosed with angiostrongylosis between March 2013 and April 2014. Positive broncho-alveolar lavage fluid qPCR results and positive Baermann examination were obtained in 5/5 and 2/5 dogs respectively. In the remaining 2 dogs, only Baermann analysis was performed. Among the retrospective cohorts, only one broncho-alveolar lavage fluid from a coughing dog was qPCR-positive whereas all faecal samples were negative.

Clinical significance Until recently, canine angiostrongylosis was not reported in Belgium. It should now be included in the differential diagnosis of coughing Belgian dogs. Identification of affected dogs may be aided by qPCR on broncho-alveolar lavage fluid.

Keywords: *Angiostrongylus vasorum*, dogs, bronchoalveolar lavage, qPCR, Belgium.

Introduction

Angiostrongylus vasorum (*A. vasorum*) is a metastrongyloid nematode inhabiting the right cardiac ventricle and the pulmonary arteries of dogs and other wild canids such as the red fox (*Vulpes vulpes*). Parasite infestation can cause coagulopathy and respiratory signs as well as cardio-vascular, central nervous system and, rarely, digestive tract disorders, either singly or in combination (Bolt et al. 1994, Chapman et al. 2004, Jeffery et al. 2004, Bourque et al. 2008, Koch & Willesen 2009, Helm et al. 2010, Gredal et al. 2011, Elsheika et al. 2014). The condition may be fatal if left untreated, particularly in young dogs with a high worm burden and coagulation disorders (Yamakawa et al. 2009, Gredal et al. 2011).

Recent studies highlight an increasing prevalence of this parasitic infection in long-standing endemic areas and an expanding distribution of the parasite in both Europe and North America (Bourque et al. 2008, Yamakawa et al. 2009, Helm et al. 2010, Gredal et al. 2011, Conboy 2011, Gallagher et al. 2012, Traversa et al. 2013). In Germany, a neighbouring country of Belgium, epidemiological surveys have shown a constantly increasing prevalence of faecal detection of the parasite in dogs between 2003 and 2009, particularly in southwestern areas (Taubert et al. 2009, Barutzki & Schaper 2009). Autochthonous infections of *A. vasorum* were also reported in dogs in The Netherlands (Van Doorn et al. 2009).

Faecal detection of the first-stage larvae (L1) is the most frequently used method to diagnose *A. vasorum* infection (Bolt et al. 1994, Chapman et al. 2004, Koch & Willesen 2009, Elsheika et al. 2014). A direct faecal smear can be easily used in general practice. However, despite its attractive potential for rapid diagnosis of severely affected dogs, its sensitivity is poor (Humm & Adamantos 2010). Baermann examination on three consecutive faecal samples is thus considered to be more sensitive. Nevertheless, despite its high specificity, the sensitivity of this test is questionable, mainly because of irregular faecal shedding of L1 larvae (Bolt et al. 1994, Elsheika et al. 2014, Oliveira-Junior et al. 2006); indeed, larvae have been detected by broncho-alveolar lavage in experimentally-infected dogs before faecal detection by Baermann examination (Barçante et al. 2008). Therefore, diagnostic techniques more sensitive than faecal examination are needed. Detection of specific antibodies by ELISA assays is highly sensitive but not commercially available and only used for epidemiological studies (Schucan et al. 2012, Guardone et al. 2013, Schnyder et al. 2013). Parasite circulating antigens can be detected in whole blood using a highly specific and sensitive sandwich-ELISA assay which has been documented to be a valid alternative for early, reliable diagnosis and for follow-up after treatment (Verzberger-Epshtein et al. 2008, Schnyder et al. 2011). A new in-house rapid device for detection of highly specific circulating antigen is now commercially available in several European countries and been recently evaluated. High sensitivity (84%) was found in a population of 39 naturally infected dogs when Baermann examination

of faeces was considered as the gold standard (Angio Detect^TM Test, *Idexx Laboratories*, Schnyder et al. 2014). However, this study provides no information about infected cases that are Baermann-negative and also reported lower sensitivity when compared to the Baermann test for experimentally infected cases.

Lastly, quantitative PCR tests (qPCR) have recently been made commercially available and appear promising, although, so far, qPCR has been only anecdotally performed on broncho-alveolar lavage fluid samples (Jefferies et al. 2011). Until now, the presence and the prevalence of *A. vasorum* infection amongst coughing dogs have not been investigated in Belgium although a fatal neurological case was reported very recently (Jolly et al. 2014). The aims of the present study were: (1) to describe the clinical use of qPCR on broncho-alveolar lavage fluid in a series of dogs diagnosed with pulmonary angiostronglosis over a 12-month period; and (2) to investigate *a posteriori* the possibility of previously undiagnosed angiostrongylosis among a series of coughing and healthy dogs using both newly available qPCR on collected and stored broncho-alveolar lavage specimens and Baermann examination of faeces.

Materials and Methods

1. <u>Clinical series</u>

Client-owned dogs presented with respiratory clinical signs at the Liège University Veterinary Small Animal Teaching Hospital between March 2013 and April 2014 and diagnosed with pulmonary angiostrongylosis were prospectively recruited.

Pulmonary angiostrongylosis was suspected based on compatible respiratory clinical signs, radiologic findings, bronchoscopy and cytological examination of broncho-alveolar lavage fluid; diagnosis was definitively confirmed by a positive result of qPCR on broncho-alveolar lavage fluid and/or a faecal detection of L1 larvae by Baermann examination. In two dogs, only faecal samples were available because bronchoscopy was not performed owing to financial limitations.

Bronchoscopy, bronchoalveolar lavage and laboratory processing of broncho-alveolar lavage fluid were performed as previously described in detail (Dehard *et al.* 2008). All dogs were anesthetized, using various anesthetic protocols. Premedication with intramuscular or intravenous administration of medetomidine (5-10 μg/kg, Sedator©, *Eurovet Animal Health*) and/or butorphanol (0,2-0,3 mg/kg, Dolorex©, *Intervet Int. via MSD Animal Health*), and/or midazolam (0,3 mg/kg, Dormicum©, *Roche Laboratoire*) was followed by IV administration of propofol up to 6 mg/kg for induction, and with boluses of 0.1–0.2 mg/kg for maintenance (Diprivan©, *AstraZeneca*). In most dogs, a 5-minute preoxygenation period was used. Animals were initially not intubated: oxygen saturation was controlled during the procedure and subsequent recovery using continuous pulse oximetry monitoring. All dogs received supplementary oxygen by flow-by; two of them were intubated during or after the bronchoscopy due to moderate hypoxemia.

Broncho-alveolar lavage fluid analysis consisted of total cell count determination (Thomas' cell), manual differential cell counts calculation, cytological examination of cyto-centrifugated smears and semi-quantitative bacterial culture. qPCR analysis was performed on uncentrifugated specimens. Genomic DNA (gDNA) was purified from 200 μl of uncentrifugated broncho-alveolar lavage fluid using the DNeasy Blood and Tissue Kit (QIAGEN, Manchester, UK), with the DNA eluted in 100 μl, and qPCR analysis performed on 5μl gDNA as described previously (Jefferies *et al.* 2011).

Baermann examination was performed on three consecutive defecations in 5 out of 7 dogs, as previously described (Forey 1989). Faecal samples were stored at 4°C during the collection process, and then processed immediately by the laboratory upon receipt.

2. <u>Retrospective study in coughing and healthy dogs</u>

In order to retrospectively investigate the presence of the parasite among coughing and healthy dogs and evaluate whether or not we previously failed to diagnose pulmonary angiostrongylosis, newly available qPCR analysis was *a posteriori* performed on 65 broncho-alveolar lavage fluid samples collected between January 2008 to April 2014 from 55 coughing dogs and 10 healthy dogs. At the time of broncho-alveolar lavage fluid collection in both healthy and coughing dogs, Baermann examination was also performed in 33 of them; faecal samples were collected over one (healthy dogs and some coughing dogs) or 3 consecutive defecations (majority of coughing dogs) and stored and processed as for clinical cases.

In the retrospective analysis of broncho-alveolar lavage fluid of coughing and healthy dogs, all collected samples were immediately processed in a standardized manner after withdrawal of a minimal amount of fluid for total cell calculation, cytospin preparation and bacterial culture, part of the collected fluid was centrifugated to obtain pelleted cells and supernatant. Supernatant and pellets were frozen directly at – 80°. For the retrospective qPCR analysis, the frozen pelleted cells were thawed and send to the laboratory for analysis after re-suspention in a small volume of sterile saline (NaCl) and analysed as the fresh samples.

Results

1. <u>Clinical series</u>

Pulmonary angiostrongylosis was diagnosed in seven small or medium sized dogs with a median age of 2.3 years [range 0.4-9.0]. All of them were living in Southern or Eastern Belgium and diagnosed over a 12-month period, between March 2013 and April 2014 and represented 3.1% of the 225 registered canine respiratory consultations over this period of time. All were presented for daily cough and/or a moderate to severe exercise intolerance and dyspnea. One dog also suffered from an acute central vestibular syndrome (direction-changing nystagmus, unilateral proprioceptive deficit). No dog had a history of travel to well-recognised endemic areas during the 6 preceding months.

Mild eosinophilia, neutrophilia and regenerative anemia were occasionally observed (in 2, 2 and 1 dogs respectively). All dogs had some radiological changes compatible with pulmonary angiostrongylosis (moderate to severe bronchointerstitial or alveolointerstitial pattern, often more pronounced in peripheral and/or caudo-dorsal areas).

Bronchoscopy was performed in 5 dogs: diffuse mucosal congestion and oedema were present in all and blood-containing secretions originating from the main bronchi were observed in 3 dogs (Figure 1).
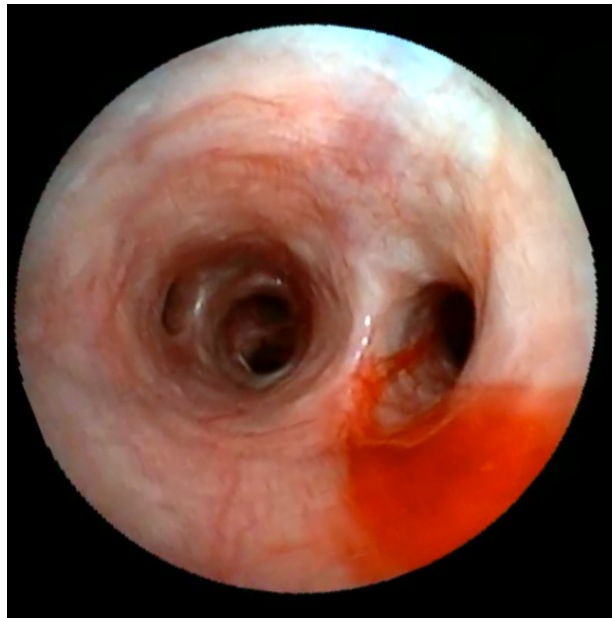
Figure 1: Bronchoscopic view of tracheobronchic bifurcation showing congestion of the tracheobronchial mucosa and blood-containing secretions from the left main bronchus.

No coagulation parameters were measured. Cytological examination of cytocentrifugated MGG-stained smears of broncho-alveolar lavage fluid showed neutrophilic (5 dogs) or mixed neutrophilic and eosinophilic airway inflammation (2 dogs) (Figure 2) and identified L1 larvae (Figure 3) in 2 dogs. There were no complications as a result from the bronchoalveolar lavage.
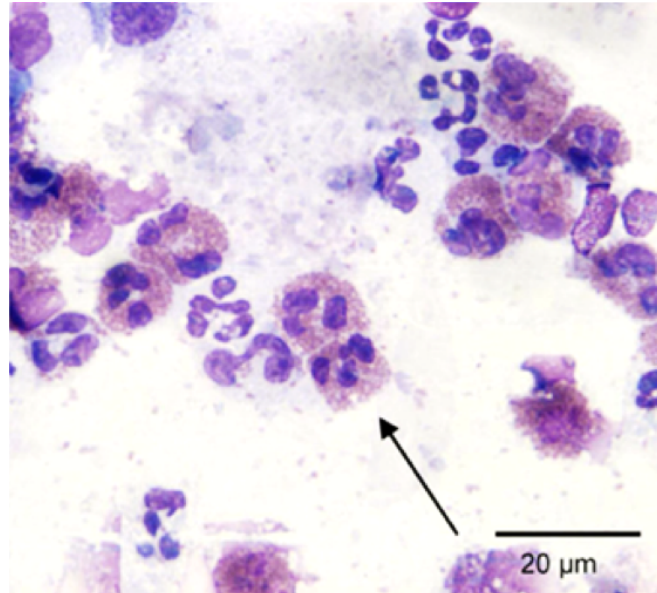
Figure 2: Eosinophilic (arrowed) airway inflammation detected in this cytospin sample of broncho-alveolar lavage fluid in one dog. (May-Grünwald-Giemsa stain, x 1000 magnification)
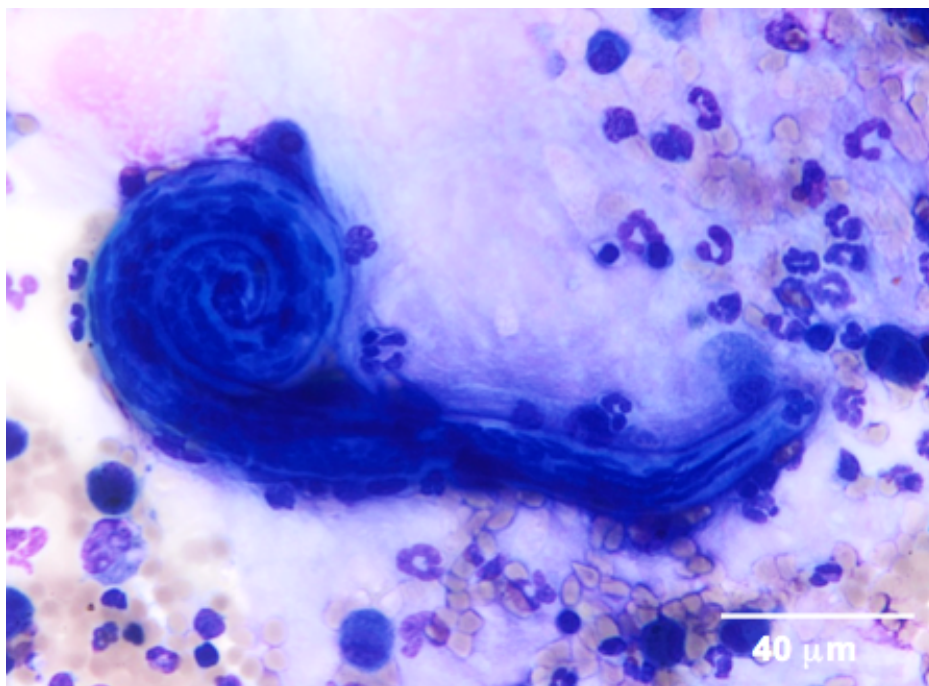
Figure 3: Cytological detection of *Angiostrongylus vasorum* L1 larva in broncho-alveolar lavage fluid from one dog. (May-Grünwald-Giemsa stain, x 400 magnification).

In 5 dogs, both broncho-alveolar lavage fluid and faeces were obtained: qPCR on broncho-alveolar lavage fluid was positive in all, at high or moderate level (cycle threshold (Ct) between 26.7 and 29.6) while L1 larvae were detected in the faeces of only 2 animals despite collection of 3 samples over 3 days. In the remaining two dogs, no broncho-alveolar lavage fluid was collected, but Baermann analysis of faeces consecutively collected over 3 days were positive.

All dogs were treated with a 3-week course of fenbendazole (50 mg/kg/day, Panacur© *Intervet International* via *MSD Animal Health*) as classically advised (Koch & Willesen 2009); in two dogs, two additional spot-on applications of moxidectin (2.5 mg/kg, Advocate Spot-On© *Bayer*) with a one-month interval were prescribed because of persistent mild radiographical lesions after treatment with fenbendazole. In two dogs, anti-inflammatory doses of prednisolone (0.5 mg/kg q24h, Prednisolone© *Kela Laboratoria*) were prescribed for a short period of time (less than a week) because of the initial clinical severity in one dog and the worsening of cough after initiation of the anthelminthic treatment in another. One dog was co-infected with *B. bronchiseptica* diagnosed by both cytological observation of coccibaccili adhering to the cilia of epithelial cells of broncho-alveolar lavage fluid and positive qPCR on broncho-alveolar lavage fluid. Another dog was diagnosed with concomitant *Crenosoma vulpis* infestation, diagnosed by Baermann examination. Both of these dogs with bacterial and parasitic coinfection were treated accordingly (at the same time of the treatment for angiostrongylosis), with nebulized gentamicine (GENTA-kel 5 %© *Kela Laboratoria*) and four weekly oral doses of milbemycin oxime (1 mg/kg, Milbemax© *Novartis*). Clinical resolution was noticed in all dogs. No Baermann examination was available during the follow-up.

2.   Retrospective study in coughing and healthy dogs

The healthy dogs were urban or suburban dogs with neither history nor clinical signs of respiratory problems. Broncho-alveolar lavage fluid had been previously collected and stored for earlier research studies for which ethical approval had been previously obtained from the Liege University local Ethical Committee; total cell counts and cytological examination were available and were within normal limits. Coughing dogs were diagnosed with various respiratory diseases such as eosinophilic bronchopneumopathy, *Bordetella bronchiseptica* infection, bacterial bronchopneumonia and chronic bronchitis. qPCR was negative in the broncho-alveolar lavage fluid from all healthy dogs and yielded a positive result (Ct = 22.0) in only, one broncho-alveolar lavage fluid from a dog originally diagnosed with idiopathic eosinophilic bronchopneumopathy in 2011. In this dog, a short course of fenbendazole had been prescribed at the time of the initial diagnosis, despite a negative result of the Baermann analysis. Baermann examination was negative in all healthy and coughing dogs.

Discussion

The present series represents the first clinical description of canine pulmonary angiostrongylosis in dogs presented with respiratory signs in Belgium. In this series of cases, diagnosis was essentially confirmed by qPCR on broncho-alveolar lavage fluid samples. Based on the recent cases diagnosed in Belgium (2013-2014) and the present retrospective investigation amongst coughing dogs over the last seven years (2008 to 2014), it can be assumed that pulmonary angiostrongylosis is now an emerging disease in Belgium. Since its discovery in France in the middle of the twentieth century (Doby et al. 1970), *A. vasorum* has been found to be endemic in several European and North American countries (Bolt et al. 1992, Bolt et al. 1994, Chapman et al. 2004, Conboy 2011). However, recent reports emphasize that the disease is spreading into several new geographical areas (Bourque et al. 2008, Yamakawa et al. 2009, Helm et al. 2010, Gredal et al. 2011, Conboy 2011, Gallagher et al. 2012, Traversa et al. 2013). Movements in animal populations, climatic global warming and changes in vector epidemiology are presumed to be mainly responsible for this spread (Morgan et al. 2009, Traversa et al. 2010). Seasonal breeding patterns and activity of the gastropod mollusc intermediate hosts (snails and slugs) are strongly sensitive to temperature and moisture (Morgan et al. 2009). In this respect, Belgium is part of a vast area in Western Europe considered highly suitable for the maintenance of *A. vasorum* life cycle. Furthermore, increasing densities of red foxes (Vulpes vulpes), the main wild reservoir of this nematode, in urban and periurban areas are likely to increase the risk for *A. vasorum* transmission to dogs (Bolt et al. 1992, Franssen et al. 2014). Based on results of previous studies, this nematode is increasingly detected in faeces of foxes from almost all countries neighboring Belgium (Elsheikha et al. 2014, Demiaszkiewicz et al. 2014). Lastly, accessibility to accurate recently-developed diagnostic methods (qPCR, immunochromatography test for serological in-clinic diagnosis, Angio Detect[TM] Test, Idexx Laboratories (Schnyder et al. 2014)) and heightened clinical awareness could also account for disease emergence.

Results of the present study support the use of qPCR as an adequate and reliable diagnostic technique to detect *A. vasorum* in broncho-alveolar lavage fluid in coughing dogs. In previous studies using qPCR analysis focused on analysis of blood or faeces, whereas broncho-alveolar lavage fluid was tested in only 2 dogs initially diagnosed through Baermann examination (Jefferies et al. 2011). The present series documents for the first-time positive qPCR results on broncho-alveolar lavage fluid in Baermann-negative dogs. Additionally, in experimentally infected dogs, L1 larvae were found by cytological examination of broncho-alveolar lavage fluid in all dogs at 60 days post infection whereas only 85% of dogs had positive Baermann examination at the same time (Barcante et al. 2008). Therefore, broncho-alveolar lavage fluid might possibly allow detection of L1 larvae either by qPCR or by standard cytological examination before faecal larval shedding. Furthermore broncho-alveolar lavage fluid can yield other useful diagnostic information, such as the existence of bacterial or parasitic co-infection,

which may require specific or alternative therapeutic management. This was the case in two dogs in the present study. In addition, as none of the healthy dogs had positive qPCR results from broncho-alveolar lavage fluid the risk of false positive test results (from endoscope contamination for example) were considered unlikely.

Inclusion criteria used in the present retrospective study may fail to provide an estimation of the prevalence of canine angiostrongylosis in Belgium since only dogs with respiratory clinical signs (i.e cough and /or dyspnoea) were considered and patients with other clinical signs, such as cardiac and central nervous system involvement or coagulopathy, were not considered. Besides, inclusion of large populations of healthy dogs would have been also required because asymptomatic infection can be detected in up to 2% of pets dogs in some endemic areas (Elsheikha et al. 2014).

Although bronchoscopy and bronchoalveolar lavage are fairly safe procedures, the main disadvantage for collecting broncho-alveolar lavage fluid in coughing dogs is the need for anesthesia, which can be particular risky in some dyspneic dogs with angiostrongylosis. Inherently safer tests are currently available to help to make a diagnosis (Baermann examination or in-house rapid device for circulating antigen) and should be included in the diagnostic approach as first-line tests, even though they have drawbacks (especially sensitivity) as previously alluded to. The financial cost of this procedure may also limit its use as a first-choice diagnostic procedure. Therefore, it would be interesting to prospectively compare the respective diagnostic values of qPCR performed on various samples (broncho-alveolar lavage fluid, faeces, blood) and the non-invasive in-house device for antigen testing, in a series of coughing or dyspneic dogs. Based on the present findings, qPCR on broncho-alveolar lavage fluid is a valuable diagnostic tool and should, at least, be recommended for dogs clinically suspected to have pulmonary angisotrongylosis and that have negative result for Baermann analysis and/or negative result for rapid antigen testing on blood.

Furthermore, until this present study, endoscopy findings in dogs with angiostrongylosis were uncommonly described in detail. Based on our observations, any detection of fresh blood or blood-containing secretions in lower airways during bronchoscopy of coughing or dyspneic dogs should lead clinicians to have the suspicion of angiostrongylosis.

Conclusion

Based on results of Baermann analysis on faeces and qPCR on broncho-alveolar lavage fluid, pulmonary angiostrongylosis in coughing dogs was negligible in Belgium until 2013 but should now be considered as an emerging and endemic condition and included in the differential diagnosis of coughing Belgian dogs of any age, especially when blood-containing bronchial secretions are detected on bronchoscopy.

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Experimental section

Part 1 – Study 2 :

Angiostrongylosis in dogs with negative fecal and in-clinic rapid serological test: 7 cases (2013-2017)

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Abstract

Background: Angiostrongylosis is considered as emerging disease disease in dogs in Belgium. Detection of first-stage larvae in faeces using the Baermann method has an imperfect sensitivity. Therefore, alternative methods were developed. Comparative diagnostic usefulness of these techniques has been mainly investigated in dogs with experimental infection.

Objectives: Investigation of efficacy of non-invasive blood and fecal diagnostic tests in comparison with PCR on bronchoalveolar lavage (BAL) material in a small series of coughing or dyspnoeic dogs naturally infected with Angiostrongylus vasorum.

Animals: Seven dogs with angiostrongylosis.

Methods: Retrospective study. Dogs with cough, exercise intolerance and dyspnea of 2- to 8- week duration. Diagnostic methods used included Baermann analysis, AngioDetect rapid assay, ELISAs for detection of circulating antigen and specific antibodies and qPCR on BAL material.

Results: Baermann analysis, AngioDetect^{TM} rapid assay, antigen ELISA, antibody ELISA and qPCR on BAL material were positive in 3/7, 2/7, 3/6, 6/6 and 7/7 dogs, respectively. ELISA for antibodies or qPCR on BAL material were essential for definitive diagnosis in 3 dogs. Relative sensitivities of AngioDetect^{TM} rapid assay, Baermann analysis and ELISA for antigen detection were lower than 50% compared to ELISA for antibodies or qPCR on BAL material.

Conclusion and clinical importance: In this small clinical series, Baermann analysis and AngioDetectTM rapid assay failed to confirm the diagnosis in some dogs. Therefore, ELISA for antibody detection and qPCR on BAL material should strongly be considered in clinically suspected dogs when antigen detection methods (AngioDetectTM or ELISA) and Baermann analysis are negative.

Keywords: bronchoalveolar lavage, qPCR, serology, AngioDetect^{TM}.

Introduction

Angiostrongylus vasorum (*A. vasorum*) is a metastrongyloid nematode residing in the right cardiac ventricle and the pulmonary arteries of dogs and foxes. The latter wild carnivores are considered as the main reservoir and many gastropod species such as slugs and snails act as intermediate hosts (Bolt et al.1992). Over the last 5 years, several studies confirmed the presence of *A. vasorum* in most countries of western Europe. In Belgium, angiostrongylosis in dogs is now considered as an emerging disease (Jolly et al. 2015, Canonne et al. 2016, Lempereur et al. 2016). Clinical signs are various, nonspecific and potentially fatal if left untreated; thus, an early diagnosis is essential (Chapman et al. 2004, Bourque et al. 2008, Koch & Willesen 2009, Gredal et al. 2011).

An old and still the most widely used method for the diagnosis is based on the detection of first-stage larvae by the Baermann fecal technique and has a suboptimal sensitivity (Foreyt 1989, Oliveira-Junior et al. 2006, McGarry & Morgan 2009, Deplazes et al. 2016). A direct fecal smear can be also used in general practice; but, despite its attractive rapidity, its sensitivity is also poor (Humm & Adamantos 2010). Higher sensitivity is obtained by the FLOTAC technique but it is unfrequently used despite the additional advantage that it can be applied also with older samples (Schnyder et al. 2010).

Other diagnostic methods including serological or molecular assays have been successively developed to improve diagnosis. Detection of specific antibodies and detection of specific circulating *A. vasorum* antigen by ELISA are sensitive and highly specific (Verzberger-Epshtein et al. 2008, Schucan et al. 2012, Guardone et al. 2013, Schnyder et al. 2013). They have been validated for reliable diagnosis (Schnyder et al. 2011, Schnyder et al. 2014) and both, antibody and antigen ELISA, are offered by the the Institute of Parasitology, Vetsuisse Faculty, University of Zurich, Switzerland. An in-clinic rapid ELISA (AngioDetect™ rapid assay, Idexx Laboratories) for antigen detection with a high specificity has then been developed. However, direct comparison with the ELISA detecting circulating antigens showed a delay of approximately 3–4 weeks for antigen detection by the AngioDetect™ rapid assay (Schnyder et al. 2014). Furthermore, only anecdotic information about sensitivity of the AngioDetect™ rapid assay in naturally infected dogs with negative Baermann test is reported (Lempereur et al. 2016, Liu et al. 2017).

Quantitative PCR test (qPCR) has been developed and was successively used on different substrates including blood, feces and tracheal swabs from dogs with experimental infection (Jefferies et al. 2011, Schnyder et al. 2015) and from naturally infected foxes (Houpin et al. 2016). Circulating DNA in the blood was evaluated as an early indicator of infection, though has a low sensitivity (Schnyder et al. 2015, Houpin et al. 2016), while detection of specific circulating parasitic antigens by ELISA was

positive in all dogs from 7 weeks after infection, which corresponds to the period before patency (Schnyder et al. 2015).

Recently, qPCR on bronchoalveolar lavage (BAL) material was described to identify dogs infected with *A. vasorum* in a series of coughing dogs (Canonne et al. 2016); however, its relative diagnostic value in comparison with fecal and serological tests has not been investigated. The aim of the present study was to report and compare results obtained by the Baermann fecal technique, by serological detection of circulating *A. vasorum* antigen adopting a rapid assay (AngioDetect[TM] rapid assay) and ELISA, by serology for detection of specific antibodies against *A. vasorum* by ELISA and by qPCR on BAL material in a small series of dogs diagnosed with angiostrongylosis.

Materials and Methods

Client-owned dogs with signs of respiratory disease, including cough, exercise intolerance or respiratory distress, presented at the University Veterinary Small Animal Teaching Hospital of Liège between March 2013 and April 2017, diagnosed with angiostrongylosis and for which results of different diagnostic methods were available, were retrospectively recruited. The five diagnostic methods included the Baermann technique, the rapid immunochromatography assay on blood or plasma (AngioDetect™ rapid assay, *Idexx Laboratories*), ELISA for detection of circulating antigens of *A. vasorum* and specific antibodies and qPCR performed on BAL material.

Angiostrongylosis was suspected based on compatible clinical signs, radiologic findings, bronchoscopy and cytological examination of BAL material; diagnosis was confirmed by a positive result of qPCR on BAL material and clinical response to anthelmintic treatment including fenbendazole (50 mg/kg q24h PO for 3 weeks) or moxidectin (2,5 mg/kg repeated after 2 to 4 weeks).

The Baermann technique was performed at the Laboratory of Parasitology of the Faculty of Veterinary Medicine, University of Liège, on three consecutive samplings from each dog as previously described; differentiation between first-stage larvae of *A. vasorum* and *Crenosoma vulpis* was based on morphological criteria (characteristic notch feature on the tail for *A. vasorum*) (Foreyt 1989, Oliveira-Junior et al. 2006, McGarry & Morgan 2009, Deplazes et al. 2016).

From each dog, one or two serum samples were frozen at the diagnosis. One aliquot of serum from each dog was retrospectively thawed and a rapid test detecting circulating *A. vasorum* antigen (AngioDetect™ rapid assay, *Idexx Laboratories*, Westbrook, Maine, USA) was performed, according to the manufacturer's instructions. Another aliquot of serum from 6 dogs was sent to the Institute of Parasitology, Vetsuisse Faculty, University of Zurich, Switzerland, and further analysed for detection of specific circulating *A. vasorum* antigen and specific antibodies against *A. vasorum* by previously validated ELISAs. Circulating adult *A. vasorum* antigens were detected by a sandwich-ELISA using monoclonal and polyclonal antibodies, as previously described (Schnyder et al. 2013). Specific antibodies were detected by a sandwich-ELISA using *A. vasorum* adult somatic antigen purified by monoclonal antibodies (mAb Av 5/5) as previously detailed (Schucan *et al.* 2012). For both ELISAs, absorbance values were read at 405 nm with a Multiscan RC ELISA reader (Thermo Labsystems©, Helsinki, Finland). All test runs included a background control, a conjugate control, three positive control sera from three experimentally infected dogs and two negative control sera from uninfected dogs. Cut-off values (OD=0,287 and OD=0,234 for ELISA assay detecting circulating adult antigen and specific antibodies, respectively) were based on the mean value of optical density plus three standard deviations of 2000 randomly selected dog samples (Schnyder M, *unpublished data*).

Bronchoscopy, BAL and laboratory processing of BAL material were performed as previously described (Canonne et al. 2016). All dogs were anaesthetised, using various anaesthetic protocols including premedication, a 5-minute preoxygenation period, IV induction and IV maintenance; oxygen saturation was controlled during procedure and recovery with pulse oximetry monitoring. qPCR analysis was performed on uncentrifugated bronchoalveolar lavage fluid. Genomic DNA (gDNA) was purified from 200 µl of lavage fluid using the DNeasy Blood and Tissue Kit (QIAGEN, Manchester, UK), with the DNA eluted in 100 µl, and qPCR analysis performed on 5µl gDNA as described previously (Canonne et al. 2016, Jefferies et al. 2011). This assay has previously been shown to have 100% specificity for *A. vasorum* when DNA from *C. vulpis, Eucoleus aerophilus, Toxocara canis, Dirofilaria immitis, Dirofilaria repens* and *Angiostrongylus cantonensis* were used as controls.

Results

Seven dogs of various breed, age and sex were included (9 months to 10 years, mean age=5 years, 5 females and 2 males). Owners reported cough, exercise intolerance and respiratory distress of variable severity from 2-week to 2-months duration.

Baermann analysis, in-clinic rapid test (AngioDetect[TM] rapid assay), ELISA for antigen, ELISA for antibodies and qPCR on BAL material for gDNA detection were positive in 3/7, 2/7, 3/6, 6/6 and 7/7 dogs, respectively. In the three dogs with negative in-clinic rapid test and for which ELISA for antigen detection was available (dogs number 4, 5 and 6), ELISA for antigen was also negative. Dog number 3 delivered an invalid result by the in-clinic rapid test, *i.e.* the control line did not repeatedly develop (rapid test repeated twice) but ELISA for antigen was positive for this dog. Samples positive for gDNA detection in BAL material were also positive for detection of specific antibodies. If ELISA for specific antibodies had not been performed, qPCR on BAL material would have been essential for definitive diagnosis in dog numbers 5, 6 and 7, which presented cough and exercise intolerance from 1- to 4-week duration.

All dogs completely recovered with prescribed anthelminthic treatment; in dog numbers 3 and 4 that had positive Baermann analysis, examination of feces collected after the end of the treatment was negative for L1 larvae.

Discussion

The present study reports the investigation of the respective diagnostic usefulness of four non-invasive tests (i.e. on blood or feces) compared to an invasive procedure (i.e. BAL) in dogs with natural infection by *A. vasorum*. Based on this small series of dogs with naturally occurring angiostrongylosis, detection of specific antibodies by ELISA and of gDNA on BAL material had the best relative sensitivities. Indeed, both techniques were essential to confirm diagnosis in 3 cases with clinical signs of short duration, i.e. from 1- to 4-week duration.

Results from the present selection of dogs confirm the lower sensitivity of the in-clinic AngioDetect[TM] rapid assay in dogs with positive Baermann analysis, as previously observed in a validation study (Schnyder et al. 2014). In dogs with positive Baermann analysis, sensitivity of AngioDetect[TM] rapid assay was effectively reported to be of 85%, concomitantly with excellent specificity (100%) regarding different cardio-respiratory helminth infections (Schnyder et al. 2014). In a further study, still in relation to Baermann analysis, AngioDetect[TM] rapid assay had a sensitivity of 97.1% and a specificity of 89% (Liu et al. 2017). Nevertheless, it must be highlighted that positive AngioDetect[TM] rapid assay results were reported in some Belgian dogs with negative Baermann analysis and without any clinical signs compatible with angiostrongylosis (Lempereur et al. 2016): it could suggest possible asymptomatic infestation in some dogs (low worm burden ?) or, more simply, false-positive results. Moreover, this in-clinic test can remain positive in up to 7 weeks after start of anthelmintic treatment (Schnyder et al. 2014), which might reflect either incomplete clearance of infection or slow elimination of antigens from dead adult worms. These last observations can make the interpretation of the in-clinic test challenging.

Direct comparison between the two assays detecting circulating antigen revealed a delay of 3-4 weeks for antigen detection by AngioDetect[TM] rapid assay compared to the ELISA (Schnyder et al. 2014). In experimental conditions, ELISA for antigens and AngioDetect[TM] were respectively positive in all dogs at 11 weeks and 14 weeks after inoculation whereas fecal Baermann analysis was positive in all cases from 8 weeks (Schnyder et al. 2014). In the present population, all dogs with negative AngioDetect[TM] rapid assay were also seronegative for antigen-ELISA detection and negative for Baermann analysis with one exception and all dogs presented signs for 1 to 8 weeks. Therefore, based on results from the present small series, pursuing with antigen-ELISA assay in suspected dogs with negative AngioDetect[TM] rapid assay would have been be unhelpful. Interestingly, both antigen-ELISA and AngioDetect[TM] rapid assay were negative in dog number 2, whereas the Baermann analysis was positive. This result might be explained by formation of antigen-antibody complexes, which inhibit detection of circulating antigens (Schnyder et al. 2014, Gillis-Germitsch & Schnyder 2017). In order to test this hypothesis, serum could have been treated by heat as described but this was not performed in

this dog. Moreover, it would have been interesting to test sera from dogs numbers 4-5-6 after heat treatment.

ELISA for detection of specific antibodies has better sensitivity than both serologic tests detecting circulating antigen. Sensitivity and specificity of ELISA detecting specific antibodies are 86% and 99%, respectively (Schucan et al. 2012). In the present cases, relative sensitivity of this test was excellent if positive qPCR on BAL material is to be considered as the gold standard for diagnosis. In experimentally infected dogs, specific antibodies were detected from week 3 in some dogs and from week 5 in all dogs and was the most promising test for identifying dogs at an early stage of infection (Schnyder et al. 2015). Seropositivity for antibodies in dogs with negative antigenemia (AngioDetect[TM] rapid assay or ELISA) has thus been observed previously (Schnyder et al. 2014, Schnyder et al. 2015). Detection of specific antibodies by ELISA is, to our knowledge, only offered by the Diagnostic Unit of the Institute of Parasitology, University of Zurich. Moreover, neither Baermann technique nor serological evaluation for antigens and antibodies are suitable tools to evaluate immediate biological response to anthelmintic treatment, because all these tests could, based on data from experimental infections, remain positive for some time: Baermann analysis can persist positive for up to 3 weeks after treatment, while the other tests can be still positive up to 9 weeks, suggesting possible failure to clear the infestation (Schucan et al. 2012, Schnyder et al. 2015). Lastly, the presence of specific antibodies was observed in Belgian dogs without history or physical examination suggestive of angiostrongylosis (Lempereur et al. 2016) and could be interpreted, once more, as asymptomatic infestation, previous infestation or false positive results. It highlights that any diagnostic test should always be interpreted in light of the epidemiological and clinical context.

The present clinical series confirms the clinical usefulness of qPCR on BAL material (Canonne et al. 2016). The main disadvantage for this procedure is that it requires the collection of material under anaesthesia, which can be particular risky in dogs with respiratory distress. The financial cost of bronchoscopy could also limit its use as a first-choice diagnostic procedure. Cytological diagnosis of *A. vasorum* with fine-needle aspiration of the lung under only brief sedation has been reported in occasional case reports in dogs (Gomes et al. 2009, Palic et al. 2017) and in one cat with Aelurostrongylus abstrusus infection (Gambino et al. 2016). However, the sensitivity of this test has not been investigated in clinical series, especially in comparison with qPCR on BAL material; in addition, absence of peripheral pulmonary lesions on radiographs and potential coagulopathies (Sigrist et al. 2017) can preclude the realisation of this procedure. Bronchoalveolar lavage analysis has the advantage to provide additional diagnostic information such as the presence of bacterial co-infection that requires specific therapeutic management or monitoring (Canonne et al. 2016). Moreover, parasitic co-infection with *C. vulpis* can also be detected by fecal analysis and BAL material analysis. *C. vulpis* infection was also recently documented as a regularly occurring parasite in coughing dogs in Belgium and co-infection with *A.*

vasorum occurs (Taubert et al. 2008, Barutzki & Schaper 2009, Canonne et al. 2016, Lempereur et al. 2016). The sensitivity of Baermann analysis for diagnosis of *C. vulpis* infections is to our knowledge not described in the literature but intermittent excretion in analogy with *A. vasorum* can be assumed. Differentiating *C. vulpis* from *A. vasorum* infection is relevant due to the higher pathogenicity of *A. vasorum* especially related to associated bleeding diathesis (Koch & Willesen 2009, Gredal et al. 2011). In this context, treatment of *A. vasorum* infected dogs with tranexamic acid and plasma transfusion could be helpful to normalize hyperfibrinolysis and hypofibrinogenemia occurring in severe cases (Sigrist et al. 2017).

Limitations of the study include the small-sized clinical series and the lack of qPCR performed on blood or feces. Molecular methods on blood or feces are non-invasive and commercially available. Moreover, blood qPCR provided the earliest indicator of infection, *i.e.* 2 weeks post infection in a previous experimental study, however with low sensitivity (Schnyder et al. 2015). Positive blood qPCR with negative antigenemia was previously reported and possible detection of DNA originating from L3 during migration and maturation is hypothesized to explain this observation.

In the present series, positive qPCR on BAL material was used for definitive diagnosis. Only one study has investigated qPCR on BAL material from healthy dogs or dogs with other pulmonary conditions and suggested that false positive results are unlikely (Barutzki & Schaper 2009, Canonne et al. 2016). Furthermore, as each dog from our cohort favourably responded to medical management, including fenbendazole or moxidectin, authors were confident in definitive diagnosis of angiostrongylosis in each case and thus, qPCR on BAL appears as a highly specific test.

Finally, as the first inclusion criteria was the presence of signs of respiratory disease (cough, exercise intolerance or respiratory distress), it is less clear if the qPCR would perform as favourably on BAL from dogs with isolated signs of neurological disease or extra-respiratory hemorrhages secondary to *A. vasorum* infestation. While the life cycle of *A. vasorum* always involve the lungs, some affected dogs fail to present any historical or radiological signs of respiratory lesions; in such situations, performance of qPCR on BAL has not been assessed, even if the procedure is rarely contra-indicated. Accordingly, true sensitivity of qPCR on BAL cannot be assessed based on this small cohort of dogs presented for signs of respiratory disease, the lack of any defined gold standard and because positive qPCR was the first inclusion criteria. However, the present results of four non-invasive tests compared to qPCR on BAL render this analysis as potential gold standard in dogs with angiostrongylosis and signs of respiratory disease.

Conclusion

In conclusion, this study compares four non-invasive diagnostic procedures with the BAL material analysis in naturally infected coughing dogs. While ELISA for detection of specific antibodies and qPCR on BAL material gave highly valuable results, the best procedure for diagnosing angiostrongylosis in dogs that combines non-invasiveness, promptness, sensitivity and specificity has to be chosen depending on the individual situation. Although Baermann analysis and the in-clinic rapid test should be used as first-line tools in clinically suspect dogs because of their availability, cost-effectiveness and inherent non-invasiveness, they might be of lower sensitivity than detection of specific antibodies and qPCR on BAL material, especially in cases of early infection. Based on the comparative usefulness of qPCR on BAL material and ELISA for detection of specific antibodies in the present case series, effort should be accomplished for increased execution of tests detecting specific antibodies or by potentially empowering antigen detection methods through heat treatments of sera in dogs suspected of *A. vasorum* infection but negative by first-line tools, prioritizing non-invasive methods.

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Experimental section

Part 2 :

Contribution to the understanding of the pathogenesis of the idiopathic eosinophilic bronchopneumopathy in dogs

Preamble

The second part of the experimental section groups 3 studies related to **investigation of potential triggering or exacerbating role of infectious agents** in canine EBP.

Before assessing potential presence and implication of *Bordetella bronchiseptica* (*Bb*) in dogs with EBP, we have assessed the relevance of qPCR on BALF. As compared to conventional bacterial culture, qPCR was highlighted as being the most sensitive method for *Bb* confirmation, specially in referred dogs previously-treated with antibiotics (Study 3). Using highly sensitive qPCR on BALF in dogs with EBP, dogs with chronic bronchitis and healthy dogs, no potential role of *M. canis* and *M. cynos* in the pathogenesis of EBP was supported. Nevertheless, in EBP dogs, *Bb* was detected more frequently in dogs with more severe clinical signs and moderate or high DNA loads were observed only in dogs with EBP (Study 4).

Lastly, potential implication of *Aspergillus fumigatus* was investigated by using qPCR on BALF and measuring specific antibodies by a homemade ELISA assay on serum and BALF in dogs with EBP, compared to dogs with chronic bronchitis and healthy dogs. Results of qPCR on BALF and specific serum Ig E were not different between groups but higher concentrations of serum Ig G for *A. fumigatus* at several dilutions were found in dogs with EBP compared to dogs with chronic bronchitis and healthy dogs (Study 5).

——————— Experimental section

Part 2 – Study 3 :

Quantitative PCR and cytology of bronchoalveolar lavage fluid in dogs

with *Bordetella bronchiseptica* infection

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Abstract

Background: The use of quantitative PCR (qPCR) for *Bordetella bronchiseptica* (*Bb*) in bronchoalveolar lavage fluid (BALF) has not been investigated. The diagnostic value of visualisation of bacteria adhering to ciliated epithelial cells in BALF or bronchial brushing fluid (BBF) has not been assessed in a series of affected dogs. Co-infections may worsen the clinical severity in bordetellosis, but the specific association with *M. cynos* has not been evaluated.

Objectives: To assess the utility of culture, qPCR and cytological examination of cytospin preparations in the diagnosis of canine bordetellosis and the influence of co-infection by *Mycoplasma cynos* on disease severity.

Animals: Twenty-four referred and/or previously treated dogs with *Bb* infection and 10 healthy dogs.

Methods: Retrospective case series. qPCR (*Bb* and *M cynos*) and culture results from BALF were recorded. Cytospin preparations from BALF and/or BBF were reviewed. qPCR on BALF from 10 healthy dogs were used as negative control.

Results: BALF culture and qPCR detected *Bb* in 14/24 and 18/18 dogs, respectively. Coccobacilli were found adhering to ciliated epithelial cells in 20 of the 21 BALF cytological preparations where epithelial cells were found, and 2/3 BBF cytological preparations. qPCR detected a low level of *Bb* in one healthy dog. The frequency of detection of *M. cynos* was not significantly different in *Bb* (9/17 dogs) compared with healthy dogs (2/10 dogs).

Conclusion-clinical importance: qPCR detection of *Bb* in BALF appears to be a useful diagnostic tool. Cytological examination of BALF or BBF, when positive, allows a rapid and reliable diagnosis.

Keywords: Bronchial brushing fluid; Clinical pathology; Cytology; Mycoplasma cynos; qPCR

Introduction

Bordetella bronchiseptica (*Bb*) is a small aerobic gram-negative rod with coccobacillary appearance, which is regarded as one of the primary causative agents of canine infectious respiratory disease (CIRD or « *kennel cough* »). Despite the widespread availability of *Bb* vaccines, the infection is still commonly seen in young dogs, especially in boarding kennels. This contagious disease is often self-limiting, but a wide range of respiratory signs are described, from mild illness to severe pneumonia and death, depending on factors such as presence of concomitant viral or other bacterial pathogens and immune and vaccination status (Bemis 1992, Keil & Fenwick 1998, Ford 2006). *Bb* can exist in the respiratory tract as either a commensal or pathogen (Chalker et al. 2003, Schulz et al. 2014). Although the bordetellosis is a relatively common infectious disease in young dogs, large and recent descriptive clinical studies of confirmed cases, including referral cases with prior therapy, are not available.

Bb can be detected using bacterial culture, and more recently by PCR analysis of various samples such as pharyngeal swabs (Schulz et al. 2014), bronchoalveolar lavage fluid (BALF) or transtracheal wash (Viitanen et al. 2015). As *Bb* has been isolated regularly in clinically healthy dogs, either by culture from the upper respiratory tracts and lungs (Ford 2006, Chalker et al. 2003) or by quantitative PCR from nasal and pharyngeal swabs (Schulz et al. 2014), it is unclear how a positive quantitative PCR (qPCR) result from BALF should be interpreted. Although qPCR has the potential to provide quantitative results based on the cycle threshold (Ct) value, it is unknown whether Ct values can help differentiate a carrier state from a pathological infection.

Furthermore, adherence of *Bb* to cilia via adhesion molecules such as fimbriae and filamentous hemagglutinin, induction of ciliostasis and damage to ciliated epithelium have been implicated in bordetellosis pathogenesis (Bemis et al. 1977, Anderton et al. 2004). Accordingly, pleiomorphic cocci or coccobacilli adhering to cilia of the epithelial cells were occasionally reported as characteristic cytological features in human beings (Tuomanen & Hendley 1983) and anecdotally reported in dogs diagnosed with bordetellosis (Burkhard & Millward 2001). Currently, the diagnostic accuracy of cytological demonstration of *Bb* organisms in cytological preparations, such as cytocentrifuged smears of BALF or bronchial brushing fluid (BBF), has not been reported.

Moreover, although *M. cynos* was recently identified as an emerging and possibly lethal pathogen in CIRD (Rycroft et al. 2007, Zeugswetter *et* al. 2007, Priestnall et al. 2014), the role of *Mycoplasma spp.* as primary respiratory pathogens remains unclear (Chandler & Lappin 2002, Chalker et al. 2004, Chan et al. 2013, Johnson et al. 2013). In dogs with CIRD, the frequency of co-infections with *Mycoplasma spp.,* in particular *M. cynos*, is unknown.

The aims of the present study were: 1) to retrospectively report the signalment, clinical features and the frequency of co-infection by *M. cynos* in a series of referral and/or previously treated dogs with *Bb* infection and 2) to describe performance of qPCR analysis, bacterial culture of BALF and cytological examination of BALF and/or BBF in the diagnosis of canine *Bb* infection.

Materials and Methods

1. Case selection and data collection

Client-owned dogs referred to the Liège University Veterinary Small Animal Teaching Hospital between January 2006 and September 2014, and diagnosed with *Bb* infection, either by positive culture and/or qPCR of the BALF were retrospectively included in the study. Data were collected from the medical records and included signalment, body weight, clinical signs, type and date of previous vaccination against *Bb*, haematology, thoracic radiographs results, bronchoscopy and BALF analysis results, and bronchial brushing results when available. Bronchoscopy, bronchoalveolar lavage (BAL) procedure and BALF laboratory processing were performed as described earlier (Dehard et al. 2008). Briefly, dogs were anaesthetized using various anaesthetic protocols, according to anesthetist's recommendations and a 5-minute pre-oxygenation period was used. Five to 20 ml aliquots (depending of the body weight) of sterile saline (NaCl 0,9%) were instilled twice into a least two different lung lobes via a flexible paediatric endoscope (FUJINON© Paediatric Video-Bronchoscope EB-530S) under anaesthesia, followed by immediate aspiration by gentle suction. The recovered BALF was immediately processed. Aliquots of naïve BALF were used for quantitative culture (Collard Laboratories, Liège, Belgium), for *Bb* and *M. cynos* qPCR (TDDS Laboratories), for total cell count determination using a haemocytometer, as well as for cytospin preparation (centrifugation at 1400 rpm, 197 g, for 4 minutes at 20°C, Thermo Shandon Cytospin©4). Differential cell counts calculations were established by counting a total of 300 cells at high power field on the cytospin preparation. As in previous study of the authors[19], normal cells counts were considered to be 400-600/µl; BALF cytology was considered normal if <12% of neutrophils. The remaining recovered BALF was centrifuged at 1300 g for 15 minutes at 5 °C. Supernatant and cell pellet issued from the centrifugation were stored separately at -80°C. For bronchial brushing, a 1.3-mm sheathed cytology brush (OLYMPUS © Disposable Cytology Brush BC-203D-2006) was passed through the biopsy channel of the bronchoscope. The brush was extended past the end of the bronchoscope and out of the sheath, rubbed gently back and forth across the mucosal surface of a central airway (a primary or secondary bronchus, depending on the size of the dog), pulled back into the sheath and removed from the bronchoscope. The brush was then re-extended out of the sheath, briskly agitated in 2 mL of sterile saline in a sterile cryotube. Cytocentrifuged smears from BBF were then prepared as for BALF (Thermo Shandon Cytospin©4). Cytological preparations of BALF and BBF were stained with a May-Grünwald-Giemsa stain and each smear was independently examined and re-reviewed by two authors (Tual C. and Ramery E.). Samples were not supposed to be contaminated with oropharyngeal material: no sample contained *Simonsiella* bacteria nor oropharyngeal squamous cells.

2. BALF quantitative culture

Samples were plated onto several agar plates (Chapman's, Mac Conkey's, CNA and TSS agars) at 35°C for isolation of aerobic organisms (Collard Laboratories, Liège, Belgium). Standard biochemical methods were used to identify cultured bacteria. The threshold used to define clinically relevant bacterial growth was 1.7×10^3 colony-forming units per milliliter of BALF (Peeters et al. 2000). Bacterial susceptibility testing was performed according to standards of the French Microbiology Society using disk diffusion method.

3. BALF qPCR for *Bb* and *M. Cynos*

qPCR for *Bb* and *M. cynos* testing were performed by a commercial veterinary diagnostic laboratory. The qPCR results were expressed as Ct values. After obtaining the data, Ct values were further categorized into five groups, based on the *Bb* DNA load, as a helping interpretation guide for clinicians: very high load (Ct<20), high load (20.1 to 24), moderate load (24.1 to 28), low load (Ct 28.1-32) and very low load (>32.1).

4. Control group and blank lavages

In order to investigate whether a positive qPCR result for *Bb* might possibly either indicate a carrier state or result from contamination during the procedure, qPCR analysis was retrospectively performed on BALF samples collected from 10 healthy dogs. BALF samples collected from those healthy dogs were available from previous studies for which ethical approval had been previously obtained from the Liège University Local Ethical Committee. Ten control urban or sub-urban dogs belonging to veterinary staff or students (8) or from shelters (2), with neither history nor clinical signs of respiratory problems, were used. Bronchoscopy, BAL procedure and BALF laboratory processing had been performed as for *Bb* dogs and cytological examination of cytocentrifuged preparations of BALF was within normal limits in each dog. For the retrospective qPCR analysis, the frozen pelleted cells were thawed and re-suspended in a 0.5ml volume of sterile 0.9% NaCl at the time of qPCR testing.

In order to further evaluate the risk of samples contamination during the endoscopic procedure and subsequent false positive results, fifteen blank lavages of the channel of the bronchoscope were performed randomly over the study period after standardized cleaning and disinfection by a trained technician. Briefly, once the endoscope was disinfected, 5ml of sterile saline was flushed into the endoscopic channel used for BALF collection and recovered in a sterile cryotube for qPCR assessment.

Two blank samples were obtained after endoscopic cleaning subsequently to BALF collection in dogs finally diagnosed with *Bb* infection.

5. Statistical analysis

Statistical analyses were performed with a commercially available software (XLstat software). Data were expressed as median and range for continuous variables and as proportion for categorical variables.

Chi-square test (for n>5) and exact Fisher's-test (for n≤5) were used to compare the frequency of qPCR detection of *M. cynos* between healthy and *Bb* dogs.

Values of P ≤ 0.05 were considered significant.

Results

1. Signalment and clinical findings

Clinical records from 24 dogs diagnosed with *Bb* infection between 2006 and 2014 were retrospectively reviewed (table 1).

No	Breed	Sex	Age (y)	Body weight (kg)	Global score index	Duration of clinical signs (months)	BALF cellularity TCC (/µl)	BALF cellularity %Neutro	*Bb* Bacterial culture	*Bb* Cytology Bronchial brushing	*Bb* Cytology BALF	qPCR for *Bb* (Ct levels)	qPCR for *M. cynos* (Ct levels)	Other bacteria (culture)
1	Boxer	m	0.8	20	8	8	39 000	98%	Positive*	NA	Positive	#	NA	*Staphylococcus intermedius*
2	Shiba Inu	m	2.9	12	3	3	500	11%	Negative	NA	Negative	27.2	0	No growth
3	Shar pei	f	0.5	9	9	0.5	6 940	98%	Negative	NA	Positive	19	20.1	No growth
4	Yorkshire Terrier	m	0.4	2.6	6	2	760	76%	Positive*	NA	Positive	21	21.89	No growth
5	Yorkshire Terrier	f	0.4	2	5	2	705	25%	Positive*	NA	Positive	19.7	0	No growth
6	American Staffhordshire Terrier	f	0.2	6	12	0.5	10 015	98%	Negative	NA	Negative	25.4	19.94	No growth
7	King Charles Spaniel	m	0.3	4.3	11	3	3 280	89%	Positive*	NA	Positive	20.5	0	No growth
8	Yorkshire Terrier	f	0.4	1.3	7	4	565	89%	Positive*	NA	Positive	20.6	0	*Staphylococcus pneumoniae*
9	Mixed-breed	m	1.5	16.6	5	1	500	41%	Negative	NA	Positive	21.6	0	No growth
10	French Bulldog	f	0.3	2.1	11	1	1 775	95%	Positive*	NA	Positive	19.2	0	No growth
11	Irish Setter	m	3.9	32	4	48	640	40%	Negative	Positive	Negative	26.7	0	No growth
12	King Charles Spaniel	f	0.3	2.3	10	1	11 100	100%	Positive£	Positive	Negative	27.4	21.7	No growth
13	French Bulldog	m	0.8	10.4	8	7	2 285	63%	Negative	NA	Negative	25.9	33.95	*Klebsiella pneumoniae.*
14	Labrador Retriever	f	0.4	12	8	2	560	54%	Positive*	Negative	Negative	24.3	33.22	No growth
15	French Bulldog	m	0.5	7	11	1	7 330	94%	Negative	NA	Positive	24.3	22.1	*Pseudomonas aeruginosa.*
16	French Bulldog	m	0.4	4.2	11	2	1 100	NA	Negative	NA	Positive	31	33.1	No growth
17	French Bulldog	m	3.8	5	6	5	745	45%	Positive*	NA	Positive	NA	NA	*Streptococcus viridans*
18	King Charles Spaniel	m	0.5	4.2	8	3	2 340	99%	Positive*	NA	Positive	NA	NA	*Escherichia coli*
19	King Charles Spaniel	m	0.3	1.5	7	0.25	3 665	91%	Positive*	NA	Positive	NA	NA	*Streptococcus (D Group)*
20	French Bulldog	m	0.6	4.6	6	4	1 130	43%	Positive*	NA	Positive	NA	NA	No growth
21	Boxer	m	0.5	19.4	6	4	NA	76%	Positive*	NA	Positive	NA	NA	No growth
22	Coton Tulear	f	0.5	3	6	5	4 190	98%	Positive*	NA	Positive	NA	NA	No growth
23	French Bulldog	m	0.5	9	7	2	NA	NA	Negative	NA	Positive	19.2	0	*Pseudomonas aeruginosa*
24	French Bulldog	f	0.3	2.6	11	2	4 145	95%	Positive*	NA	Positive	18	18.5	No growth
MEAN MEDIAN		-	0.88 0.5	8.0 4.8	7.8 7.8	4.6 2.0	4694 2030	74% 89%	-	-	-	23. 21.6	22.5 21.8	-
Standard deviation		-	1.1	7.5	2.5	9.5	8281	28%	-	-	-	3.8	9.9	-

Table 1: Signalment, clinical presentation, BALF cytology and culture, BBF cytology and qPCR results for *Bb* and *M. cynos* *: 100 000 CFU/ml   £: 5000 CFU/ml

Median age was 0.5 years (range 0.2-3.8) and median weight was 4.8 kg (range 1.3-30). French bulldogs, Cavalier King Charles spaniels and Yorkshire terriers accounted for 15/24 cases. Vaccination status was recorded for 22 dogs. Seven of them had been vaccinated against *Bb* infection: two had received intranasal vaccine (IN) [Nobivac BbPi©, *Intervet International*] (No 4, 10) and five subcutaneous (SC) vaccine [Pneumodog©, *Merial Sanofi*] (No 3, 5, 15, 17, 19). Five dogs were

vaccinated within 3 to 6 weeks before onset of clinical sign (No 4, 5, 15, 17, 19) and in the last two dogs, a mild cough was already reported at the time of vaccination (No 3, 10).

Chronic productive harsh and hacking daily cough of at least one week to two year's duration (median = 2 months) was present in all cases. In all but two dogs, cough was reported since acquisition. Laryngo-tracheal reflex (tracheal sensitivity) was positive in all but three dogs. Thirteen dogs were moderately to severely dyspneic; in seven of these 13 dogs, hospitalization and oxygen supplementation were required. A variable degree of lethargy and mild fever (39.1° - 39.6°C) were observed in 12 and four cases respectively. A mild serous or sero-mucous nasal discharge was reported in four dogs. Twenty dogs had been unsuccessfully treated with oral antibiotics including amoxicillin or amoxicillin/clavulanic acid (n=17), doxycycline (n=6), marbofloxacin or enrofloxacin (n=6), cephalexin (n=2) and lincomycin (n=2). Among these dogs, at the time of diagnosis, antibiotics had been stopped for less than 48 hours in 6 dogs and at least 1 week before BALF collection in the 14 remaining dogs.

Mild to moderate neutrophilic leukocytosis was present in 14 dogs (for dogs with leukocytosis, median value = $23.4 \times 10^3/\mu l$, range 15.9 - 45.5]). A mild leukopenia was observed in one dog (No 7, $5.8 \times 10^3/\mu l$). Radiographic features included generalized or caudo-dorsal bronchial or bronchointerstitial pattern (n=14), peri-bronchial pattern (n=4) and alveolar changes (n=11). In almost all dogs with alveolar pattern, lesions were considered severe and predominantly presented as multifocal patches, often more pronounced in ventro-cranial lung areas (figure 1a and 1b).
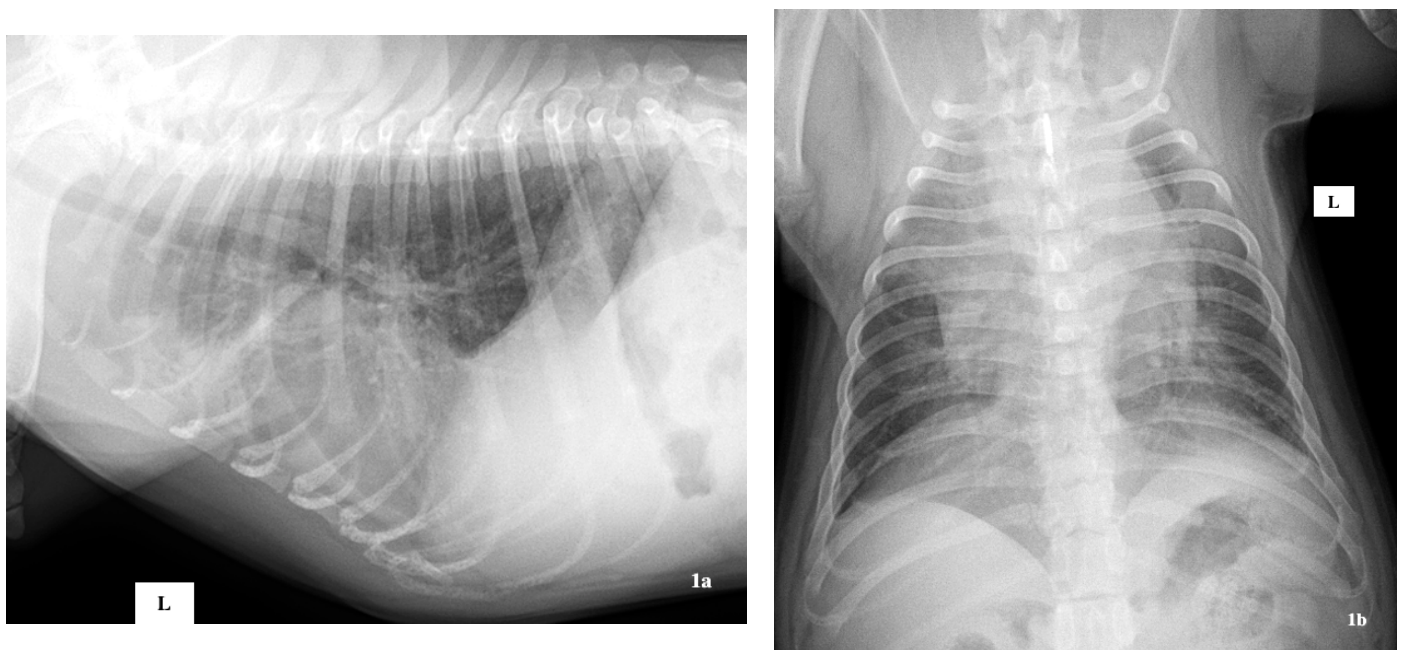
Figure 1 (a,b). Thoracic radiographs showing alveolar lesions in ventro-cranial lobes (Dog n°12, Cavalier King Charles, female, 4 months old).

Bronchoscopy revealed hyperemic and edematous mucosa, thickened bronchi or thick and white-yellow or haemorrhagic material, in moderate to large amounts, within the bronchial and sometimes the tracheal lumens in all dogs (figure 2).
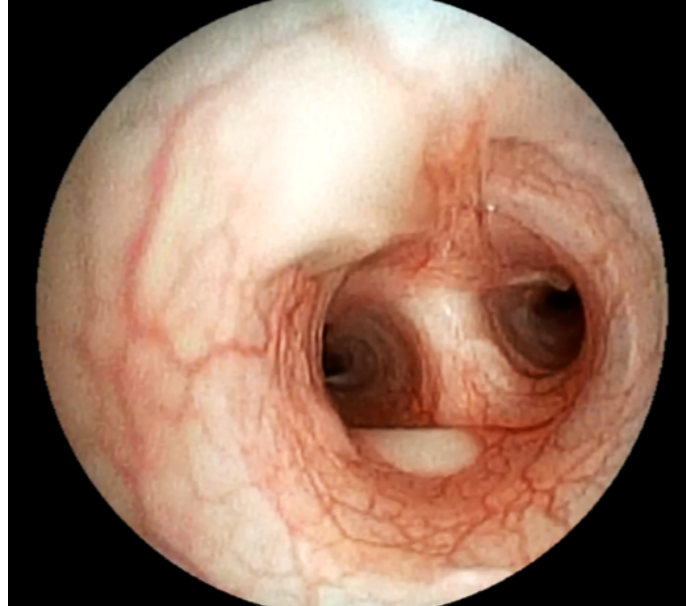
Figure 2. Bronchoscopic view of tracheobronchic bifurcation showing congested mucosa and thick and white-yellow material (Dog n°3, Sharpei, female, 6 months old).

Total cell count of BALF varied from 500 to 39000 cells/µl (median = 2030 cells/µl) with predominantly neutrophilic inflammation in all but one case (dog No 3, cytology showing predominance of macrophages). For 22 dogs, the accurate percentage of BALF neutrophils was available (median 89%, range 11-98%). Neutrophils appeared highly segmented with frequent degenerative changes and sometimes a pyknotic aspect of the nucleus. Ciliated epithelial cells were present in cytological preparations from 21 BALF samples.

2. *Bb* diagnostic test results

Results of culture, qPCR and cytology are presented in table 1. BALF bacterial culture was positive for *Bb* in 14/24 dogs (58.3%) Available antibiotic susceptibility was available in 13 of them, and revealed that all strains were susceptible to gentamicin and fluoroquinolones; 10/13 and 9/13 strains were susceptible to doxycycline and amoxicillin/clavulanic acid, respectively. Only 2/13 strains showed susceptibility to cephalexin and 3rd generation cephalosporins. Among the 13 dogs for which antibiotic

susceptibility was available, 11 dogs were unsuccessfully treated with antibiotic drugs prior BALF collection; 9 out of these 11 dogs received one (1 dog) or two (8 dogs) appropriate drugs, based on the susceptibility patterns of the isolated organisms.

Pleiomorphic cocci or coccobacilli were found adhering to the cilia of the epithelial cells in 18/24 cytocentrifuged BALF preparations (75.0%) and in two cytocentrifuged BBF preparations (figure 3).
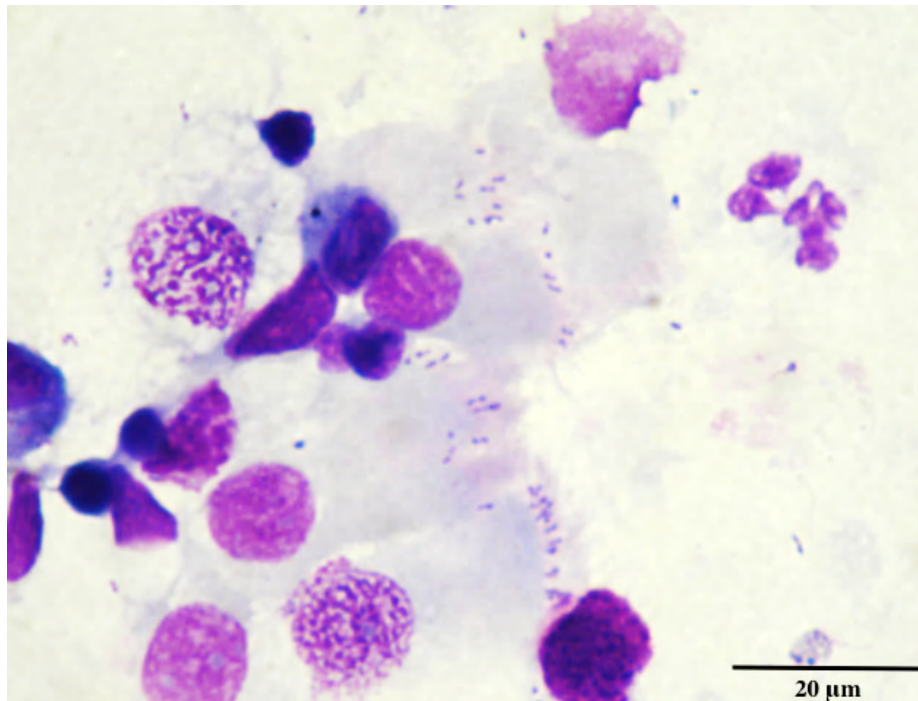
Figure 3. Cytological detection of pleiomorphic cocci or coccobacilli adhering to the cilia of the epithelial cells of BALF (May-Grünwald-Giemsa stain, x 400 magnification, Dog n°8, Yorkshire Terrier, female, 5 months old).

In three of the four dogs for which cytological examination of BALF was negative (No 2, 6, 13), no ciliated epithelial cells were found on the cytospin preparation; BBF was not performed and bacterial culture was also negative. In the fourth dog (No 14), bacterial culture yielded *Bb* growth and identification but both cytological examination of BALF and BBF failed to detect coccobacilli even though ciliated epithelial cells were observed on the cytological preparations.

The qPCR was positive for *Bb* in 18/18 dogs tested (100%); a qPCR Ct-value data for *Bb* was available in all but one of them.

In dogs with high and very high *Bb* DNA loads (*ie* with Ct values lower than 24) (n=9), cytological examination of BALF was always positive whereas culture was positive in 6 of them. In dogs with moderate *Bb* DNA load (*ie* Ct values between 24.1 and 28) (n=7), cytology of BALF or BBF and culture were positive in three and two cases, respectively. One BALF showed a low *Bb* DNA load (*ie* Ct value higher than 28.1) and cytology of BALF from this dog was positive. Dogs with positive cytology had higher *Bb* DNA load (mean Ct = 21,2) compared to dogs with negative cytology (mean Ct = 26,1) (p=0,009).

Among 17 dogs in which qPCR data for *M. cynos* were available, nine dogs (52.9%) were positive (table 1).

Other common bacteria were also cultured in 8/24 (33%) (table 1).

3.   Control group and blank lavages

The control group was composed of ten dogs (six females, four males) of various breeds aged from 2 to 11 years (median = 6 years). BALF culture and cytological examination of cytocentrifuged smears were negative for *Bb* in all control dogs. In one of them, qPCR detected a very low *Bb* DNA load (Ct = 33.44).

M. cynos was detected by qPCR assay in 2 of the control dogs. The frequency of detection of *M. cynos* was not significantly different in *Bb* (9/17 dogs) compared with healthy dogs (2/10 dogs) (p = 0.09).

Among the 15 blank lavages, *Bb* qPCR was positive at very low grade (Ct = 35.6) in only one case. This blank lavage was performed after cleaning of the endoscope subsequently to bronchoscopical examination in a dog ultimately diagnosed with *Bb*.

Discussion

The present study suggests that, in this specific population of affected dogs, *i.e.* referred with mostly chronic clinical signs, a qPCR detection of *Bb* in BALF is a very useful diagnostic test. Indeed, qPCR was positive, with indication of moderate to very high DNA content, in all suspected animals tested, even when bacterial culture or cytological detection were negative. The present study also shows that cytological examination of BALF and/or BBF cytospin preparations allows rapid and reliable suspicion of *Bb* infection in the great majority of dogs; this method seems to be more sensitive than BALF culture.

The present case series confirms that *Bb* infection mainly affects young dogs from small breeds and that clinical presentation can be quite severe. Almost all dogs from the present case series were presented with chronic cough (median = 2 months), while CIRD is classically associated with a shorter duration of respiratory signs (less than two weeks) (Ford 2006). Of interest, Cavalier King Charles spaniels, Yorkshire Terriers and French bulldogs represented more than a half of the study population. Since the majority of the dogs affected in this case series were very young, and since three young French bulldogs developed concomitant generalized demodicosis, an inadequate or impaired immune response can be strongly suspected but was not investigated. In Cavalier King Charles spaniels, previous descriptions of Pneumocystosis jiroveci infection, some of them with concomitant demodicosis, have suggested that immune-incompetence underlies the susceptibility of this breed to infectious diseases (Watson et al. 2006).

Pleiomorphic cocci or coccobacilli were found adhering to the ciliary apexes and inter-ciliary spaces of the epithelial cells in 20/24 cytocentrifuged BALF and/or BBF preparations. In comparison, only 14/24 dogs had positive culture and only one dog had negative cytology despite a positive culture. The pathogenesis of bordetellosis involves adherence of the *Bb* to the cilia of the respiratory tract (Bemis et al. 1977, Anderton et al. 2004). In a previous experimental study using tracheal cell culture models in dogs, electron microscopy allowed observation of bacteria adhering to cilia of tracheal epithelial cells, although optic microscopy was not investigated (Anderton et al. 2004). Until the present study, demonstration of bacteria adhering to ciliated epithelial cells obtained by BAL or per-endoscopic bronchial brushing from dogs with spontaneous *Bb* infection has not been assessed in the diagnosis of canine bordetellosis. Adhered bacteria were observed by standard cytology in all dogs with high and very high *Bb* DNA loads. These results suggest that standard cytological examination of cytocentrifuged preparations of BALF and BBF allows reliable suspicion of *Bb* in the vast majority of dogs. Besides, cytological preparations can be immediately assessed, before results of bacterial culture and/or qPCR analysis are obtained. Although in some cases, *Bb* can be formally identified, based on cytological findings alone, its strict identification may be dependent on the quality of the cytological preparations

as well as on the expertise of the cytologist. In theory, any coccobacillus (such as *Pasteurella* spp. or *Acinetobacter* spp.) superimposed on the apical side of ciliated cells would be falsely diagnosed as *Bb*. In such cases, confirmation is strongly advised, and in this respect, qPCR on BALF appears particularly useful. Furthermore, effects of delay of BALF processing or speed and time of cytocentrifugation on the number of intact ciliated epithelial cells were not anticipated in this study; any factor affecting the integrity of ciliated epithelial cells would reasonably decrease the relative sensibility of the BALF cytology in the diagnosis of *Bb* infection.

In a recent study, BBF was shown to be inferior to BALF for the diagnosis of septic inflammation in dogs (Zhu et al. 2015). However, amongst the 6 dogs with negative BALF cytology, BBF cytology was positive in 2 out of the 3 dogs in which this procedure was performed. This suggests that BBF could be the sample of choice for cytological investigation of *Bb*. This is logical since higher numbers of ciliated tracheobronchial cells are recovered by brushing procedures, compared to BALF, which only contains a low percentage of ciliated epithelial cells (<1%) (Hawkins et al. 1990, Vail et al. 1995, Rajamäki et al. 2001).

In the present study, bacterial culture was negative in 10/24 dogs while qPCR confirmed the presence of *Bb* in 18/18 tested dogs. This is not surprising since sensitivity of bacterial culture for the diagnosis of *Bordetella* spp. infection (*Bb*, *B. pertussis and parapertussis*) has been reported to be lower than PCR in a human study (Reizenstein et al. 1993). Currently available qPCR methods are able to detect as few as 10 genome copies of *Bb*/µl (Koidl et al. 2007). However, the great majority of infected dogs had received various antibiotic drugs before diagnosis and a non-negligible proportion of them had received antimicrobial therapy within the 48 hours prior to the bronchoalveolar lavage, which is susceptible to affect culture results. Because infection and aerosol shedding may persist for weeks (Ford 2006), positive results of both culture and PCR must be interpreted cautiously. In a recent study, *Bb* was detected in up to 45% of healthy dogs by using qPCR analysis on nasal and pharyngeal swabs (Schulz et al. 2014) but bronchial samples were not assessed. Comparative assessment of qPCR results in affected dogs, healthy dogs and blank lavages of the endoscope led us to consider the Ct values, as well as their interpretation into *Bb* DNA loads, as a useful and accurate tool to distinguish true infection from carrier state or environmental contamination. Indeed, moderate to very high loads were found in all but one affected dog while the load was very low in both the single blank and the single control sample, which were positive. We feel confident that the case with low *Bb* DNA load (high Ct value) (No 16) did suffer from bordetellosis since cytology was positive.

Despite appropriate cleaning and disinfection of the endoscopic material, a very low *Bb* DNA load positive qPCR was obtained in one blank lavage sample; this observation highlights the possibility of false positive results due to contamination during the BALF procedure from previously sampled

cases. The question of appropriate disinfection should thus clearly be considered before interpretation of qPCR results of BALF. Therefore, it is unclear how the positive qPCR result from the healthy dog in the present study should be interpreted. Whether it potentially indicates a carrier state or simply results from a contamination is unknown.

According to the results of the present study, positive culture or positive cytology and/or moderate to very high *Bb* DNA loads can be proposed as criteria to establish a definitive diagnosis of bordetellosis in dogs with compatible clinical signs. However, such findings do not rule out the possibility of concurrent infections contributing to clinical presentation. Moreover, the present study included a challenging population of dogs being referred, frequently with prior unsuccessful treatment that may have interfered with culture or cytology more than PCR; in such dogs, qPCR on BALF could be assumed to be more sensitive than culture or cytology. However, BALF analysis should always include semi-quantitative bacterial culture in case of clinical suspicion of *Bb* infection, in order to identify common bacterial co-infections and to obtain antimicrobial susceptibility patterns.

The present study had some limitations. The patient population is comprised of dogs that were referred, and do not represent the majority of dogs with canine infectious respiratory disease complex. Nearly all had received prior antibiotic therapy and the duration of signs was not typical (chronic cough), so these dogs may not reflect typical cases. The second major issue concerns the lack of well-recognized gold standard for the diagnostic of clinical bordetellosis. Besides, due to retrospective nature of the study, prior antibiotic therapy in some dogs can have affected the proportion of dogs with positive quantitative culture for *Bb* or other concomitant bacteria, proportion of dogs with positive qPCR for M. cynos, and results from all complementary examinations were not available for all included dogs. Among *Bb* dogs, BBF was performed in only a limited number of them, preventing an adequate comparison and definitive conclusion concerning the relative benefit of the BBF procedure, compared to the BALF one, for cytological detection of *Bb*. A prospective study including larger groups of infected and healthy dogs in which BALF, BBF, culture and qPCR are performed is warranted. Such study would allow the assessment of the sensitivity and the specificity of each diagnostic test, assuming that qPCR detection of *Bb* at moderate to very high DNA load in the BALF of a dog with compatible clinical signs might be considered as a gold standard for the definitive diagnosis of canine bordetellosis.

Conclusion

This retrospective clinical case series highlights that *Bb* infection can have chronic and sometimes severe and fatal clinical presentations and it commonly affects very young dogs of small breeds. Standard cytological examination of cytocentrifuged preparations of BALF and/or BBF can be considered as a rapid and inexpensive diagnostic modality. Providing that the sample is adequately collected and processed, qPCR detection of *Bb* on BALF, with consideration of the *Bb* DNA load, appears to be the most sensitive method for *Bb* confirmation in the studied population since qPCR detection of *Bb* on BALF was positive in all cases tested, including those with negative cytology and bacteriology results.

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Experimental section

Part 2 – Study 4 :

Detection of specific bacterial agents by quantitative PCR assays in the bronchoalveolar lavage fluid in dogs with eosinophilic bronchopneumopathy versus dogs with chronic bronchitis and healthy dogs

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Abstract

In humans, *Mycoplasma pneumoniae* and *Bordetella pertussis* infections are suggested to trigger or exacerbate asthma. Whether *Mycoplasma* or *Bordetella* are associated with chronic inflammatory bronchial diseases in dogs has not been investigated. The aim of the present study was to assess the potential presence and bacterial load of *Mycoplasma canis (M. canis), M. cynos* and *Bordetella bronchiseptica (Bb),* in dogs with eosinophilic bronchopneumopathy (EBP) and chronic bronchitis (CB), compared with healthy dogs. Specific quantitative PCR (qPCR) analyses for *M. canis, M. cynos* and *Bb* were retrospectively performed on bronchoalveolar lavage fluid collected from 24 dogs with EBP, 21 dogs with CB and 15 healthy dogs. Possible associations between qPCR results and age, lavage cytology or clinical severity scores in EBP dogs were investigated.

Mycoplasma canis, M. cynos and *Bb* were detected in 6, 2 and 6 EBP dogs, 2, 2 and 2 CB dogs and 4, 2 and 2 control dogs, respectively. The proportion of EBP dogs with positive qPCR for *Bb* was higher in dogs with higher clinical severity scores (p=0.014) and positive dogs had higher percentage of neutrophils (p<0.001). Among dogs with positive qPCR for *Bb*, moderate to high loads were only detected in dogs with EBP.

Mycoplasma canis and *M. cynos* do not appear to be associated with EBP or CB disease while higher *Bb* load are only found in EBP dogs with high clinical severity scores. The cause-effect relationship between presence or load of *Bb* and EBP remains unclear.

Keywords: *Bordetella bronchiseptica*, *Mycoplasma*, asthma

Introduction

Idiopathic eosinophilic bronchopneumopathy (EBP) is a chronic disease characterized by eosinophilic infiltration of the lung and bronchial mucosa in young adult dogs (Corcoran et al. 1991, Clercx et al. 2000, Rajamäki et al. 2002). Dogs with chronic bronchitis (CB) also present with chronic cough, but are usually older. The aetiology of both inflammatory conditions remains unclear. In EBP, an underlying immunologic hypersensitivity is highly suspected, although the inciting antigens remain mostly unidentified (Clercx et al. 2002; Peeters et al. 2005, Clercx & Peeters 2007). In both diseases, effective treatment consists of long-term oral and/or inhaled administration of steroids (Clercx et al. 2000, Bexfield et al. 2006, Rozanski 2014, Canonne et al. 2016b).

In humans, infections with *Mycoplasma pneumoniae (M. pneumoniae)* have been associated with asthma for decades (Hansbro et al. 2004, Atkinson 2013, Ye et al. 2014). *M. pneumoniae* infection is associated with acute exacerbation of asthma in adult and future development of asthma in children (Hansbro et al. 2004) and specific treatment improves pulmonary function in asthmatic patients (Kraft et al. 2002). *Bordetella pertussis* (*B. pertussis*) has also been suggested as potential trigger in human inflammatory bronchial disease and asthma (Harju et al. 2006, Wakashin et al. 2008, Nicolai et al. 2013, Yin et al. 2017). Furthermore, in a mouse model of allergic asthma, prior *B. pertussis* infection exacerbated signs of airway disease (Ennis et al. 2004, Kavanagh et al. 2013).

Although *Mycoplasma cynos* (*M. cynos*) was recently identified as an emerging, possibly contagious, and lethal pathogen in dogs with canine infectious respiratory disease ("*kennel cough*" or "*canine infectious tracheobronchitis*") (Rycroft et al. 2007, Zeugswetter et al. 2007, Mannering et al. 2009, Priesnall et al. 2014), the exact role of *M. canis* and *M. cynos* as primary respiratory pathogens remains unclear (Chandler & Lappin 2002, Chalker et al. 2004, Chan et al. 2013). Moreover, a recent study has highlighted the strong impact of oral bacterial contamination on results of *Mycoplasma*-specific PCR from bronchoalveolar lavage (Chan et al. 2013), making the investigation of any primary role of *Mycoplasma* spp. in canine lower respiratory conditions even more complex.

On the other hand, *Bordetella bronchiseptica* (*Bb*) is recognized as one of the primary causative pathogen agents of canine infectious respiratory disease. *Bordetella bronchiseptica* can exist in respiratory tract of dogs either as a commensal, or pathogen (Bemis et al. 1977, Bemis 1992, Schultz et al. 2014). Currently, quantitative polymerase chain reaction assays (qPCR) allow highly sensitive and specific detection of of *Bb*, *M. canis* and *M. cynos* (Chan et al. 2013, Schulz et al. 2014, Lavan et al. 2015, Canonne et al. 2016a). However, the potential roles of these infectious agents in chronic inflammatory bronchial diseases in dogs have not been investigated; whether these specific bacteria could act as triggers or exacerbating agents in dogs with EBP remains unknown.

Therefore, the aim of the present study was to evaluate the presence and bacterial load of *M. canis, M. cynos* and *Bb* in dogs newly-diagnosed (*i.e.,* steroid-naïve) with EBP or non-specific CB and compare these to findings in healthy dogs, using specific qPCR analysis of bronchoalveolar lavage samples. Additionally, we assessed potential association between positive qPCR results for any of these bacterial species and the clinical severity of EBP.

Materials and Methods

1. Dogs

Client-owned dogs presented at the University Companion Animal Hospital between March 2009 and February 2016 and diagnosed with EBP or CB were retrospectively recruited.

Definitive diagnosis of idiopathic EBP and CB was made in accordance with previously described criteria (Clercx *et al.* 2000, Rozanski 2014). Briefly, this was based on compatible respiratory clinical complaints, radiological changes, bronchoscopic findings, analysis of the bronchoalveolar lavage fluid, including culture and standard cytological examination and, in some cases, histopathological examination of endoscopically obtained bronchial mucosal biopsies. In CB dogs, bacterial origin of neutrophilic airway inflammation was excluded based on cytology (lack of degenerative neutrophils, absence of intra-cellular bacteria), culture and positive response to steroids. In EBP dogs, exclusion of other causes of eosinophilic airway inflammation, including cardio-pulmonary parasites, was required to definitively diagnose idiopathic EBP by using faecal and BALF analysis (Baermann method, cytology and qPCR on BALF). All dogs were newly-diagnosed and had not received corticosteroids at the time of airway sampling.

In dogs with final diagnosis of idiopathic EBP, a clinical severity score (CSS, scaled from 1 to 5) was assigned at the time of diagnosis, based on severity and frequency of cough, evidence of exercise intolerance or lethargy, as previously defined (Canonne *et al.* 2016b): cough was scored from 1 to 3 depending on frequency and severity, and presence of retching and exercise intolerance each graded with 1. The numbers obtained from scoring of cough, retching and exercise intolerance were summed.

Bronchoscopy, bronchoalveolar lavage procedure and bronchoalveolar lavage fluid processing were performed as described earlier (Clercx *et al.* 2000). Briefly, dogs were anaesthetized using various anaesthetic protocols after a 5-minute pre-oxygenation period. Five to 20 ml aliquots (depending of the body weight) of sterile saline (NaCl 0,9%) were instilled twice into a same bronchus and a third time into a different lung lobe via a flexible paediatric endoscope (Fujinon© Paediatric video-bronchoscope EB-530S), followed by immediate aspiration by gentle automated suction. The recovered bronchoalveolar lavage was immediately processed: aliquots of naïve bronchoalveolar lavage were used for semi-quantitative bacterial culture (Collard Laboratories, Liège, Belgium, according to methods previously described in details (Peeters *et al.* 2000)), for total cell count determination using a haemocytometer, as well as for cytospin preparation (centrifugation at 1400 rpm, 197 g, for 4 minutes at 20°C, Thermo Shandon Cytospin® 4). Differential cell counts calculations were established by counting a total of 300 cells at high power field on the cytospin preparation with normal cells counts considered to be 400-600/µl; bronchoalveolar lavage cytology was considered normal if neutrophils

accounted for <12% and eosinophils accounted for <7% of total cells. The remaining recovered bronchoalveolar lavage was centrifuged at 1300 g for 15 minutes at 4°C. Supernatant and cell pellet issued from the centrifugation were stored separately at -80°C. Additionally, angiostrongylosis was excluded in all EBP dogs based on a negative quantitative polymerase chain reaction (qPCR) test result in the bronchoalveolar lavage (Canonne *et al.* 2016b). All dogs were treated at time of diagnosis with anthelmintics (fenbendazole 50 mg/kg q24h for 5 days, Panacur® Intervet International via MSD Animal Health, or milbemycin oxime 1 mg/kg once, Milbemax® Novartis), while results were pending.

2. Control group

Bronchoalveolar lavage samples collected from 15 healthy dogs were available from previous studies for which ethical approval had been obtained from the University Local Ethical Committee. Control dogs belonging to veterinary staff or students (n=13) and living in urban or sub-urban areas, or from shelters (n=2), with neither history nor clinical signs of respiratory problems, were recruited. Bronchoscopy, bronchoalveolar lavage procedure and bronchoalveolar lavage laboratory processing and storage had been performed as for dogs with EBP and CB and both total cell counts and cytological examination of cytocentrifuged preparations of bronchoalveolar lavage were within normal limits in each dog.

3. Quantitative PCR analysis

Quantitative PCR analysis was performed on stored bronchoalveolar lavage for *M. canis, M. cynos* and *Bb* in dogs with EBP, CB and healthy dogs. The frozen aliquots were thawed and, after re-suspension in a small volume of sterile saline (0·9% NaCl), samples were sent to the laboratory for qPCR analysis. The qPCR results were expressed as Ct values. Ct values were arbitrarily further categorized into five groups: very high load (Ct<20), high load (20.1 to 24), moderate load (24.1 to 28), low load (Ct 28.1-32) and very low load (>32.1).

4. Statistical analysis

Statistical analyses were performed with a commercially available software (XLstat software). Continuous and categorical variables were expressed as medians [range] and as proportions, respectively.

Chi-square or Fisher's exact (when necessary) tests were used to compare the proportions of qPCR detection of *M. canis, M. cynos* and *Bb* between dogs having EBP and healthy dogs and between EBP and CB dogs. Dogs with EBP were grouped according to the CSS into two groups: dogs with CSS ≤ 3 (mild clinical severity) and dogs with CSS > 3 (moderate-to-severe clinical signs). Proportion of EBP dogs with positive qPCR results for *M. canis, M. cynos* and *Bb* were compared between dogs with CSS ≤ 3 and dogs with CSS > 3 by using exact Fisher's exact test (for n≤5). For each bacterial agent, age of EBP dogs with positive qPCR was compared to age of EBP dogs with negative qPCR by using Wilcoxon - Mann Whitney's test. For each bacterial agent, total cell count and percentage of neutrophils in BALF from EBP dogs with positive qPCR were compared to BALF parameters from EBP dogs with negative qPCR by using Wilcoxon - Mann Whitney's test.

Values of P ≤ 0.05 were considered significant.

Results

The sample populations consisted of 24 middle-aged dogs with EBP (median age = 4.2y [0.9 – 13.2]) and 21 dogs with non-specific CB (median age = 8.0 [0.9 – 14.0]).

The proportions of dogs with positive qPCR result for *M. canis*, *M. cynos* and *Bb* did not differ between dogs with EBP and either of the other groups for any bacteria (Table 1).

	Dogs with EBP	Dogs with CB	Healthy dogs	P
Mycoplasma canis	6/24 (25%)[a,b]	2/21 (10%)[b]	4/15 (13%)[a]	1.00[a] 0.25[b]
Very high load (Ct < 20)	–	–		
High load (20.1–24)	–	–		
Moderate load (24.1–28)	1	–		
Low load (Ct 28.1–32)	3	–	1	
Very low load (>32.1)	2	2	3	
Mycoplasma cynos	2/24 (8%)[a,b]	2/21 (10%)[b]	2/15 (13%)[a]	0.63[a] 1.00[b]
Very high load (Ct < 20)	2	1	–	
High load (20.1–24)	–	–	–	
Moderate load (24.1–28)	–	–	1	
Low load (Ct 28.1–32)	–	–	–	
Very low load (>32.1)	–	1	1	
Bordetella bronchiseptica	6/24 (25%)[a,b]	2/21 (10%)[b]	2/15 (13%)[a]	0.45[a] 0.25[b]
Very high load (Ct < 20)	–	–	–	
High load (20.1–24)	1	–	–	
Moderate load (24.1–28)	2	–	–	
Low load (Ct 28.1–32)	–	–	–	
Very low load (>32.1)	3	2	2	

[a,b]Groups with same letter were compared.

Table 1: Frequency of positive qPCR performed on bronchoalveolar lavage fluid for *Mycoplasma canis, Mycoplasma cynos* and *Bordetella bronchiseptica* in dogs with eosinophilic bronchopneumopathy (EBP), chronic bronchitis (CB) and healthy dogs

Among dogs with positive qPCR for *M. cynos* on bronchoalveolar lavage, Ct values corresponding to very high load were only found in dogs with inflammatory bronchial disease. Among dogs with positive qPCR for *Bb* on bronchoalveolar lavage, moderate or high loads were found in dogs with EBP only.

In dogs with EBP, CSS varied between 2 and 5 at time of diagnosis, total cell counts of BALF from 700 to 6000/ml, percentage of eosinophils from 20% to 85% and percentage of neutrophils from 1% to 30% (table 2). A greater proportion of dogs with CSS >3 (5/9=56%) had Bb detected in lavage than dogs with CSS ≤ 3 (1/15= 7%; P = 0.015). The CSS of dogs with positive and negative mycoplasma qPCR results did not differ (data not shown). The age of EBP dogs with negative *Bb* qPCR did not differ

from that of EBP dogs with positive *Bb* qPCR (3.7 years [0.9 – 13.0] and 5.8 years [0.9 – 13.2] *P* = 0.44); ages of dogs negative and positive for *M. canis* and *M. cynos* also did not differ (for *M. canis,* 4.3 years [0.9 – 13.0] and 2.9 years [1.6 – 13.2] *P* = 0.73; for *M. cynos,* 4.1 years [0.9 – 13.0] and 7.6 years [0.9 – 13.2] *P* = 0.71).

Case	Breed	Sex	Age (years)	M. canis load	M. cynos load	Bb load	CSS	BALF cytology (total cell count/µL, % eosinophils–% neutrophils)	BALF bacterial culture
1	Mixed-breed	M	13	–	–	–	2	1400/µL, 20%–5%	Negative
2	Mixed-breed	F	6	–	–	–	2	4000/µL, 85%–3%	Negative
3	Shar pei	F	3	–	–	–	2	2500/µL, 65%–5%	Negative
4	Shi tzu	M	4	–	–	–	3	3000/µL, 60%–1%	Negative
5	Shi tzu	M	1.2	–	–	–	3	1600/µL, 55%–30%	Negative
6	Mixed-breed	M	12.4	–	VH	–	3	1900/µL, 30%–10%	Negative
7	Beauceron	F	1.6	L	–	–	3	1800/µL, 55%–3%	Negative
8	Shar pei	F	13.2	M	–	M	3	2000/µL, 55%–3%	Negative
9	Mixed-breed	F	3.4	–	–	–	3	1400/µL, 60%–1%	Negative
10	Tervuren	M	2.0	VL	–	–	3	700/µL, 55%–16%	Negative
11	Whippet	F	3.0	L	–	–	3	1100/µL, 20%–3%	Negative
12	Golden retriever	F	3.3	–	–	–	3	1300/µL, 80%–4%	Negative
13	Brittany spaniel	F	7.1	VL	–	–	3	800/µL, 25%–3%	Negative
14	Siberian husky	M	0.9	–	–	–	3	1500/µL, 80%–3%	Negative
15	Mixed-breed	F	5.2	–	–	–	3	4000/µL, 85%–7%	Negative
16	Doberman	M	0.9	–	–	VL	4	2000/µL, 40%–10%	Negative
17	Siberian husky	F	1.0	–	–	–	4	1600/µL, 75%–15%	Negative
18	Basset artesien	M	4.3	–	–	–	4	2500/µL, 35%–3%	Negative
19	Whippet	F	8	–	–	–	4	1700/µL, 40%–15%	Negative
20	Brittany spaniel	F	5.7	–	–	M	5	2500/µL, 70%–20%	Negative
21	Border terrier	M	2.9	L	VH	H	5	6000/µL, 20%–15%	Negative
22	Fox terrier	F	10.0	–	–	VL	5	1800/µL, 58%–20%	Negative
23	Jack Russell terrier	M	4.4	–	–	–	5	3100/µL, 62%–1%	Negative
24	Tervueren	M	6	–	–	VL	5	4500/µL, 60%–15%	Negative

Table 2: Signalment, qPCR results from bronchoalveolar lavage fluid (BALF) for Mycoplasma canis, M. cynos, Bordetella bronchiseptica (Bb), clinical severity scores (CSS) and BALF cytology in dogs with eosinophilic bronchopneumopathy (EBP). M, male; F, female; VH, very high load; H, high load; M, moderate load; L, low load; VL, very low load

In dogs with EBP, for each bacterium, total cell count of the BALF from dogs with negative qPCR did not differ from dogs with positive qPCR (data not shown). The percentages of neutrophils in BALF from dogs with positive PCR for *Bb* and *M. cynos* were higher than from dogs with negative PCR (for *Bb,* 15% [3 – 20] and 3.5% [1 – 30] p < 0.001; for *M. cynos,* 12.5% (10-15) and 3.5% [1 – 30] *P* = 0.02). However, the percentage of neutrophils in BALF from dogs with positive and negative results of qPCR for *M. canis* did not differ (data not shown).

Discussion

The objectives of the present study were to detect the presence and assess the bacterial load of *M. canis, M. cynos* and *Bb* in canine EBP compared with dogs diagnosed with CB and healthy controls, using specific qPCR on bronchoalveolar lavage samples. Our study failed to identify any association between both mycoplasmal species and canine EBP. However, in dogs with EBP, the probability of a positive qPCR result for *Bb* increased with the clinical severity. Moreover, among dogs with positive qPCR for *M. cynos* or *Bb*, we found moderate, high or very high bacterial loads only in dogs with inflammatory bronchial disease, but small-sized numbers of dogs with positive qPCR precluded specific analysis for comparison of bacterial load between groups.

Human asthma and canine EBP are different diseases; for example, bronchial hyper-responsiveness is not a hallmark of canine EBP. However, human asthma and canine EBP are both TH2-driven inflammatory conditions characterized by eosinophilic infiltration. The role of bacteria implicated in induction or exacerbation of asthma in humans has never been investigated in dogs with EBP. In humans, infection with *M. pneumoniae* favours asthma development in predisposed patients and specific treatment with clarithromycin improves pulmonary function (Martin et al. 2001, Kraft et al. 2002, Hansbro et al. 2004, El Sayed Zaki et al. 2009, Atkinson 2013, Ye et al. 2014). The proportion of asthmatic humans who have both IgM and IgG titres for *M. pneumoniae* increases with clinical severity (Iramain et al. 2016, Yin et al. 2017). In mouse models, single exposure to a specific toxin of *M. pneumoniae* was sufficient to cause asthma-like disease with a histological observation of pulmonary eosinophilic inflammation (Medina et al. 2012). Our study fails to support the hypothesis of a similar association between mycoplasmal infection or presence and EBP in dogs. This observation could result from the small-sized population that limits the power of statistical tests for comparison. Moreover, in asthma in humans, the role of *Mycoplasma* spp. has been investigated not only by PCR, but also by the presence of serum *Mycoplasma*-specific antibodies (El Sayed Zaki et al. 2009, Iramain et al. 2016); we did not evaluate such antibodies in the present study. Furthermore, the potential therapeutic benefit of a specific antibiotic treatment, such as doxycycline, macrolide or fluoroquinolone, has not been evaluated in dogs with EBP or CB and positive qPCR results for *M. cynos*. Lastly, involvement of other mycoplasmal species in canine chronic lower airway inflammation could not be excluded; several other species from the *Mycoplasma* genus have been already investigated in dogs in research studies (Chalker et al. 2004) but, currently, no specific and sensitive diagnostic methods such as qPCR are available for these more marginal mycoplasmal species.

Although *Bb* was detected no more frequently in dogs with EBP than in dogs with CB or healthy dogs, the frequency of *Bb*-positive EBP dogs was positively associated with the clinical severity. Moreover, among dogs with positive *Bb* qPCR on bronchoalveolar lavage, we found moderate or high

loads only in EBP dogs. Whether *Bb* is able to trigger eosinophilic lower airway inflammation or whether the inflammation in airways favours *Bb* growth in dogs is unknown. In humans, *Bb* infections are unusual and almost exclusively described in immunocompromised patients with contact with animals. *B. pertussis* is more common and has been discussed as a triggering and exacerbating factor in chronic inflammatory bronchial diseases with *B. pertussis* toxin favouring eosinophilic airway inflammation (Ennis et al. 2004, Harju et al. 2006, Wakashin et al. 2008, Kavanagh et al. 2013, Nicolai et al. 2013, Yin et al. 2017). Despite a large overlap between values, the percentage of neutrophils in BALF was higher in EBP dogs with positive qPCR for *Bb* than in EBP dogs with negative qPCR, while total cell counts did not differ. Consequently, larger studies about the implication of *Bb* in EBP dogs are needed.

The potential influence of any previous *Bb* vaccination on positive samples could not be completely ruled out. However, in dogs with known vaccination status, the number of vaccinated dogs did not differ between the three groups (data not shown). In puppies vaccinated with a single dose of modified live topical vaccine, *Bb* can be detected by qualitative PCR during at least 1 month after vaccination (Ruch-Gallie et al. 2016). No study has investigated the impact of previous vaccination on qPCR performed on BALF. Nevertheless, no dog was vaccinated within the 2 months prior to diagnosis. Therefore, we considered the impact of vaccination on qPCR for *Bb* in BALF as negligible.

As all EBP and CB dogs were referred cases, an impact of any previous antimicrobial pre-treatment, such as doxycycline or fluoroquinolones, on qPCR results presented in this study cannot be completely ruled out; this might have caused possible underestimation of the presence of each agent in diseased compared to healthy dogs. This potential risk was considered quite low because a wash-out period of two or more days was usually respected before BAL procedure.

The last minor limitation of this study is the lack of validation of CSS that was used to score clinical signs in EBP dogs. To limit this drawback, CSS was attributed to each EBP dogs by only one author (AMC), who remained blinded to qPCR results. Intra and inter-observer variabilities in CSS evaluation should be assessed before it can be used on a larger scale in EBP dogs.

Conclusion

The present investigation by qPCR on bronchoalveolar lavage does not support any role of *M. canis* and *M. cynos* in the pathogenesis of EBP in dogs. Nevertheless, in EBP dogs, *Bb* was detected more frequently in dogs with more severe clinical signs. Moreover, among dogs with positive qPCR for *Bb*, moderate or high loads were observed in only dogs with EBP. Despite both observations, a cause-and-effect relationship between presence and bacterial load of *Bb* and severe canine EBP remains unclear. However, since EBP dogs could potentially act as *Bb* carriers and a source of infection for susceptible dogs, this pathogen should be systematically searched for in dogs newly-diagnosed as having EBP, particularly in case of severe clinical presentation, high percentage of neutrophils in BALF or acute exacerbation in previously diagnosed cases; such positive dogs should presumably be treated accordingly with close monitoring of the respiratory signs.

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Part 2 – Study 5 :

Potential implication of *Aspergillus fumigatus* investigated by qPCR and specific antibodies in dogs with idiopathic eosinophilic bronchopneumopathy

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Abstract

Background Canine eosinophilic bronchopneumopathy (EBP) is an idiopathic airway disease presumably caused by a hypersensitivity reaction but causative allergens remain unidentified. In humans, allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity to *Aspergillus fumigatus* in uncontrolled asthmatics.

Objectives To investigate potential implication of *A. fumigatus* in dogs with EBP compared to healthy dogs and dogs with chronic bronchitis (CB).

Animals Paired serum and bronchoalveolar lavage fluid (BALF) collected from dogs with EBP or CB and healthy dogs were retrospectively evaluated.

Methods For all dogs, quantitative PCR (qPCR) for *A. fumigatus* was performed on BALF and *A. fumigatus*-specific antibodies were searched by a homemade validated ELISA test on serum and BALF. For comparison, an ELISA test was also performed to evaluate house-dust-mites-(HDM)-specific antibodies in serum and BALF.

Results *A. fumigatus* qPCR was weakly positive in BALF from 2 out of 23 EBP dogs and negative in all other dogs. Concentrations of *A.fumigatus*-specific immunoglobulins E in serum and BALF were very low and not different between groups. Concentrations of *A.fumigatus*-specific serum IgG were higher in EBP dogs than in CB dogs and healthy dogs ($p<0.01$) but concentrations in BALF were not different. Concentrations of *HDM*-specific IgG were higher in both serum and BALF in EBP dogs compared with CB dogs ($p<0.01$).

Conclusion and clinical significance: Higher concentrations of serum IgG for *A. fumigatus* and HDM observed in EBP dogs might suggest multiple IgG -producing reactions. According to the results of this study, EBP is not mediated by *Aspergillus*-specific IgE.

Keywords: bronchoalveolar lavage fluid, bronchoscopy, ELISA, immunoglobulin, *Aspergillus*, house-dust mites, eosinophilic bronchopneumopathy, chronic bronchitis

Introduction

Canine idiopathic eosinophilic bronchopneumopathy (EBP) is a chronic and poorly understood inflammatory airway disease characterised by eosinophilic infiltration of the lung and bronchial mucosa in young to middle-aged adult dogs (Corcoran et al. 1991, Clercx et al. 2000, Rajamäki et al. 2002).

Diagnosis of EBP is based on compatible clinical signs (chronic cough with retching, variable lethargy or respiratory difficulties, such as exercise intolerance, tachypnoea, dyspnoea...), diagnostic imaging and bronchoscopy findings, demonstration of eosinophilic inflammation by cytological examination of bronchoalveolar lavage fluid (BALF) or histopathologic examination of bronchial biopsies, and through exclusion of other causes of eosinophilic infiltration of the lower airways such as parasitic diseases (Clercx et al. 2000, Clercx & Peeters 2007). More recently, computed tomodensitometry findings have been described with severe bronchiectasis and multifocal obstructive accumulation of fluid or tissue in some dogs (Meler et al. 2010, Mesquita et al. 2015). The cause of canine EBP remains unclear. A Th2-dominant immune response with increased numbers of CD4^{+} T-cells has been described (Peeters et al. 2005, Peeters et al. 2006), suggesting a hypersensitivity reaction to aerosolized antigens but exact causative allergens remain presently unidentified (Clercx et al. 2002, Clercx & Peeters 2007). Previous investigations failed to confirm a definitive role for neither *Mycoplasma canis, Mycoplasma cynos* nor *Bordetella bronchiseptica* in triggering and/or exacerbation of EBP in dogs (Canonne et al. 2018a). Similarly, *Aspergillus* species was only occasionally detected by mycotic culture of BALF of EBP dogs (Clercx et al. 2000, Johnson et al. 2019a); in both studies, *Aspergillus* was considered contaminant or incidental rather than causative.

In humans, eosinophilic bronchial and pulmonary diseases are heterogeneous groups of diseases characterized by the prominent infiltration of bronchi, pulmonary interstitium and/or alveolar space by polymorphonuclear eosinophils. Diseases are generally classified as eosinophilic lung diseases of unknown (such as acute and chronic eosinophilic pneumonia) and known causes including allergic bronchopulmonary aspergillosis (ABPA) (Kousha et al. 2011, Akuthota & Weller 2012b, Agarwal et al. 2013, Cottin 2016, Shah & Panjabi 2016, Lai et al. 2017, Weissler 2017, Allen & Wert 2018, Suzuki & Suda 2019). ABPA is a rare lung disorder linked to Th2 CD 4[+]T-cell-mediated hypersensitivity to antigens of *A. fumigatus* in non-immunocompromised patients having a history of asthma or cystic fibrosis. It is characterized by recurrent exacerbation of cough and breathlessness, chronic bronchial obstruction, and the development of pathognomonic central cylindrical bronchiectasis with high attenuation mucous impaction. (Agarwal et al. 2013, Shah & Panjabi 2016, Patel et al. 2019, Agarwal et al. 2020). A definitive diagnosis of ABPA requires elevated serum *Aspergillus*-specific immunoglobulins E (IgE) and elevated total IgE in a patient with asthma or cystic fibrosis. Additional

criteria (at least 2 criteria have to be present) include presence of serum precipiting antibodies to *A. fumigatus* or elevated serum *A. fumigatus* IgG, radiographical lung opacities consistent with ABPA and total eosinophil count >500 cells/mL in patients who are steroid naïve while culture and serum galactomamnan measurements have little role in the diagnosis (International Society for Human and Animal Mycology Working Group, Agarwal et al. 2013, Agarwal et al. 2015, Page et al. 2015, Shah & Panjabi 2016, Agarwal et al. 2020).

Similarities in immunologic investigation and tomodensitometry findings between dogs with EBP and humans with ABPA raised the hypothesis of potential implication of *A. fumigatus* as a triggering or exacerbating aeroallergen in dogs with EBP. Quantification of *A. fumigatus* DNA by qPCR in BALF of dogs with EBP has not been previously performed. At the exception of sparse data reported in an abstract published by Peyron et al. in 2009, the presence of *A. fumigatus*-specific antibodies in the sera of dogs with EBP has not been investigated.

The aims of the present study were 1) to investigate the presence of *A. fumigatus* DNA by qPCR in BALF from dogs with newly-diagnosed and steroid-naïve EBP in comparison with BALF collected from dogs with CB or healthy dogs and 2) to quantitatively evaluate presence of *A. fumigatus*-specific immunoglobulins (IgE and G) by ELISA testing in serum and BALF from dogs with EBP in comparison with serum and BALF samples from dogs with CB and healthy dogs. Investigation of house-dust-mites (HDM)-specific IgG was also performed by ELISA testing on serum and BALF collected from each dog.

Materials and Methods

1.   *EBP Dogs*

Client-owned dogs presented at *masked for review* between 2009 and 2016 with newly-diagnosed and steroid-naive EBP were retrospectively researched.

Definitive diagnosis of idiopathic EBP was made in accordance with criteria previously described (Clercx et al. 2000). Briefly, this was based on compatible respiratory clinical complaints, radiological changes, bronchoscopic findings, analysis of the BALF including culture and standard cytological examination and, for some cases, histopathological examination of per-endoscopic bronchial mucosal biopsies. Exclusion of other causes of eosinophilic airway inflammation, including cardio-pulmonary parasites, was required to definitively diagnose idiopathic EBP by using faecal and/or BALF analysis (faecal analysis by Baermann method, cytology and qPCR on BALF based on previous studies [Canonne et al. 2016, Canonne et al. 2018b]). Besides, all dogs were treated at time of diagnosis with anthelmintics (fenbendazole 50 mg/kg q24h for five days, Panacur Intervet International via MSD Animal Health, or milbemycin oxime 1 mg/kg once, Milbemax Novartis), while results were pending. Definitive diagnosis was also validated by subsequent clinical improvement with steroid therapy in each dog.

Bronchoscopy, BALF procedure and processing were performed as described earlier (Clercx et al. 2000). Briefly, dogs were anaesthetized using various anaesthetic protocols after a five-minute pre-oxygenation period. Five to 20 mL aliquots (depending of the body weight) of sterile saline (NaCl 0.9%) were instilled twice into a same bronchus and a third time into a different lung lobe via a flexible paediatric endoscope (Fujinon, Paediatric video-bronchoscope EB-530S), followed by immediate aspiration by gentle automated suction. The recovered BALF was immediately processed: aliquots of naïve BALF were used for semi-quantitative bacterial culture (SYNLAB Laboratories, Liège, Belgium, according to methods previously described in details (Peeters et al. 2000)), for total cell count determination using a haemocytometer, as well as for cytospin preparation (centrifugation at 1400 rpm, 197 g, for 4 minutes at 20°C, Thermo Shandon Cytospin 4). Differential cell count calculations were established by counting a total of 300 cells at high power field on the cytospin preparation; BALF cytology was considered normal if <12% of neutrophils and <7% of eosinophils. The remaining recovered BALF was centrifuged at 1300 g for 15 minutes at 4°C. Supernatant and cell pellet issued from the centrifugation were stored separately at -80°C.

2.   Control groups

Stored serum and BALF samples collected from dogs with CB and from healthy dogs presented at *masked for review* were available from previous studies for which ethical approval had been previously obtained from the University Local Ethical Committee. Bronchoscopy, BALF procedure and laboratory processing and storage had been performed as for dogs with EBP.

Diagnosis of CB was made in accordance with criteria previously described (Rozanski 2014). Briefly, this was based on compatible respiratory clinical complaints, radiological changes, bronchoscopy findings, analysis of the BALF including culture and standard cytological examination; for this group of dogs, bacterial origin of neutrophilic airway inflammation was excluded based on cytology (lack of degenerative neutrophils, absence of intra-cellular bacteria), quantitative culture and positive response to inhaled or oral steroids.

Dogs with no history or clinical signs of respiratory problems, normal CBC results and for which stored serum and BALF were available were included in the group of healthy control dogs.

3.   qPCR analysis on BALF

Quantification of *A. fumigatus* and *Aspergillus spp*. DNA was *a posteriori* performed on stored BALF from dogs with EBP, dogs with CB and healthy dogs (VPG Exeter (formerly TDDS), United Kingdom). The frozen pelleted cells were thawed and, after re-suspension in a small volume of sterile saline (0.9% NaCl), samples were sent to the laboratory for qPCR analysis. The qPCR results were expressed as cycle threshold (Ct). Ct values were arbitrarily further categorized into five groups: very high load (Ct<20), high load (20.1 to 24), moderate load (24.1 to 28), low load (Ct 28.1-32) and very low load (>32.1).

4. *Serological testing for Aspergillus fumigatus in serum and BALF*

A ELISA test was designed in the laboratory to measure *A.fumigatus*-specific IgG and E in serum and BALF. Archived paired serum and BALF from dogs with EBP, dogs with CB and healthy dogs were used for ELISA testing.

A pure culture of *A. fumigatus* was obtained from a nasal sample of a dog with sinonasal aspergillosis. Fungal colonies were dissolved in a culture broth; the mycelium was then separated by centrifugation and supernatant was dialysed in a PBS solution. Dialysate was purified with several filtrations, concentrated and then stored at -20°C (solution A).

BalBC-Mice were intra-peritoneally and subcutaneously immunized with the final and pure solution containing *A.fumigatus* antigens (solution A). For 3 mice, alum adjuvant was added to the solution A to specifically promote production of specific IgE. On the other hand, for 3 other mice, Freund's adjuvant was used to specifically promote production of specific IgG. Four control mice receiving saline injections with adjuvants were also included. Boosters were injected 2 and 4 weeks after the first administration. Six weeks after first immunization, mice were sacrificed and sera were collected. An ELISA testing confirmed presence of *A.fumigatus*-specific IgG and IgE in sera of immunized mice. Briefly, ELISA plates were coated with the pure solution of *A.fumigatus* antigens (solution A). Sera of mice were then incubated and secondary anti-mouse antibodies were used to detect specific IgG and IgE (ThermoFisher Scientific Waltham, Massachusetts, United States).

As the home-designed ELISA test successfully detected *A.fumigatus*-specific antibodies in mice, the solution A was then used for coating ELISA plates and to test for the presence of specific IgE and IgG in sera and BALF collected from dogs. Briefly, sera and BALF of dogs with EBP, dogs with CB and healthy dogs were tested as the same manner for IgG and IgE. For this purpose, sera were incubated on ELISA plates previously-coated at 4°C overnight with solution A (100 microL per well) and then a goat anti-Dog IgE antibody (HRP conjugated, ThermoFisher ND) or a goat anti-Dog IgG antibody (Biotin conjugated, ThermoFisher ND) were used. At each step, plates were washed with PBS-T (PBS containing 0,2% Tween 20). Serum of dogs were tested at several dilutions. For IgE, sera were assessed undiluted and after dilution at 1/30, 1/100 and 1/300 and for IgG, sera were assessed after dilution at 1/30, 1/100, 1/1000 and 1/10 000. For both IgG and IgE, BALF were tested undiluted and after dilution at 1/100 and 1/1000.

Inter-assay and intra-assay precisions were assessed. For inter-assay precision, all ELISA tests were reproduced twice for each sample, a few days apart. For intra-essay precision, some ELISA tests were conducted in duplicate over the same plate.

5. *Serological testing for HDM-specific Ig G in serum and BALF*

Sera and BALF of the same dogs were tested for HDM-specific IgG. ELISA plates were coated with a HDM solution at 4°C overnight. Sera and BALF were incubated as described above and a goat anti-Dog IgG antibody (Biotin conjugated, ThermoFisher ND) was subsequently used. Sera were assessed after dilution at 1/1000 and 1/10 000 and BALF were tested undiluted and after dilution at 1/100 and 1/1000.

6. *Statistical analysis*

Statistical analyses were performed with a commercially available software (GraphPad Prism 9 software).

To compare the proportions of qPCR positive results for *A.fumigatus* et *Aspergillus spp.* in BALF between dogs having EBP, CB and healthy dogs, Fisher's test (n<5) was used.

Optical density values for IgG and IgE in both sera and BALF were analyzed to determine whether distribution was normal or not. If results were not normally-distributed, optical density values would be compared between dogs with EBP, dogs with CB and healthy dogs with non-parametric test (Kruskall Wallis test and MannWhitney test). Values of $P \leq 0.05$ were considered significant.

In dogs with EBP, correlation between optical density values for *A.fumigatus*-specific IgG and HDM-specific IgG was evaluated with Spearman correlation test at both 1/1000 and 1/10000 dilutions. Values of $P \leq 0.05$ were considered significant.

Results

1. *qPCR results*

For qPCR testing for *Aspergillus* spp and *A. fumigatus* in BALF, stored samples collected from 23 fogs with EBP, 21 dogs with CB and 14 healthy dogs were retrospectively selected.

Aspergillus spp. qPCR yielded weak positive results in two EBP dogs (Ct = 34 and 33), 1 dog with CB (Ct = 36) and 1 healthy dog (Ct = 33), while *Aspergillus fumigatus* qPCR was only positive in the same 2 EBP dogs (Ct = 32). Proportions of qPCR positive results were not statistically different between groups.

2. *Serological studies for Aspergillus-specific immunoglobulins G and E in serum*

For ELISA testing in serum and BALF, 17 dogs with EBP, 9 dogs with CB and 10 healthy dogs having archived paired serum and BALF were recruited.

Comparative absorbance results for *A.fumigatus*-specific IgG in sera from dogs with CB and dogs with EBP at 1/30, 1/100, 1/1000 and 1/10 000 dilutions are shown in Figure 1. For all dilutions, absorbance values were significantly higher in dogs with EBP compared to dogs with CB and healthy dogs ($p<0.01$ and $p<0.001$, respectively).

Figure 1: ELISA absorbance results for *A.fumigatus*-specific IgG in serum from dogs with chronic bronchitis (CB) and dogs with eosinophilic bronchopneumopathy (EBP) at 1/30, 1/100, 1/1000 and 1/10000 dilutions. For all dilutions, differences were significant between dogs with CB and dogs with EBP. (** : $p<0.01$; *** : $p<0.001$; **** : $p<0.0001$)

Comparative absorbance results for *A.fumigatus*-specific IgE in sera from dogs with CB and dogs with EBP are shown in Figure 2 and were not statistically different between groups.

Figure 2: ELISA absorbance results for ***A.fumigatus*-specific IgE in serum** from dogs with chronic bronchitis (CB) and dogs with eosinophilic bronchopneumopathy (EBP) for undiluted sera and at 1/100 dilution. No significant difference (ns) was detected between groups.

3.   *Serological studies for Aspergillus-specific immunoglobulin G and E in BALF*

Comparative absorbance results for *A.fumigatus*-specific IgG in BALF from dogs with CB and dogs with EBP are shown in Figure 3. For all dilutions (1/100 and 1/1000), no significant difference was detected between groups. No difference was detected for IgE (in BALF) between the three groups (Figure 3 bis).

Figure 3: ELISA absorbance results for *A.fumigatus*-**specific IgG in BALF** from dogs with chronic bronchitis (CB) and dogs with eosinophilic bronchopneumopathy (EBP) at 1/100 and 1/1000 dilutions. No significant difference (ns) was detected between groups.
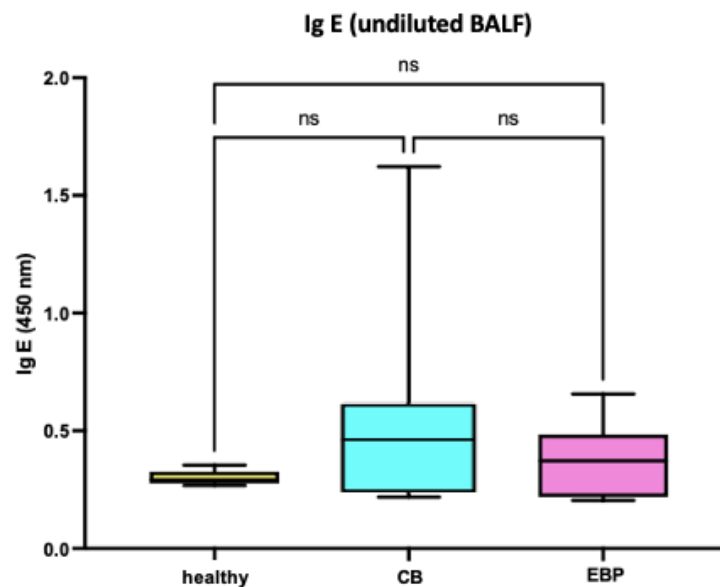
Figure 3bis : ELISA absorbance results for *A.fumigatus*-**specific IgE in BALF** from healthy dogs, dogs with chronic bronchitis (CB) and dogs with eosinophilic bronchopneumopathy (EBP) (undiluted semaples). No significant difference (ns) was detected between groups.

4. *Serological studies for HDM-specific immunoglobulin G in serum and BALF*

Comparative absorbance results for *HDM*-specific IgG in sera and BALF from dogs with CB and dogs with EBP are shown in Figure 4. For all tested dilutions, ELISA optical density values were significantly higher in both serum ($p<0.01$ and $p<0.0001$) and BALF ($p<0.05$ and $p<0.01$) in EBP dogs compared to samples from CB dogs.
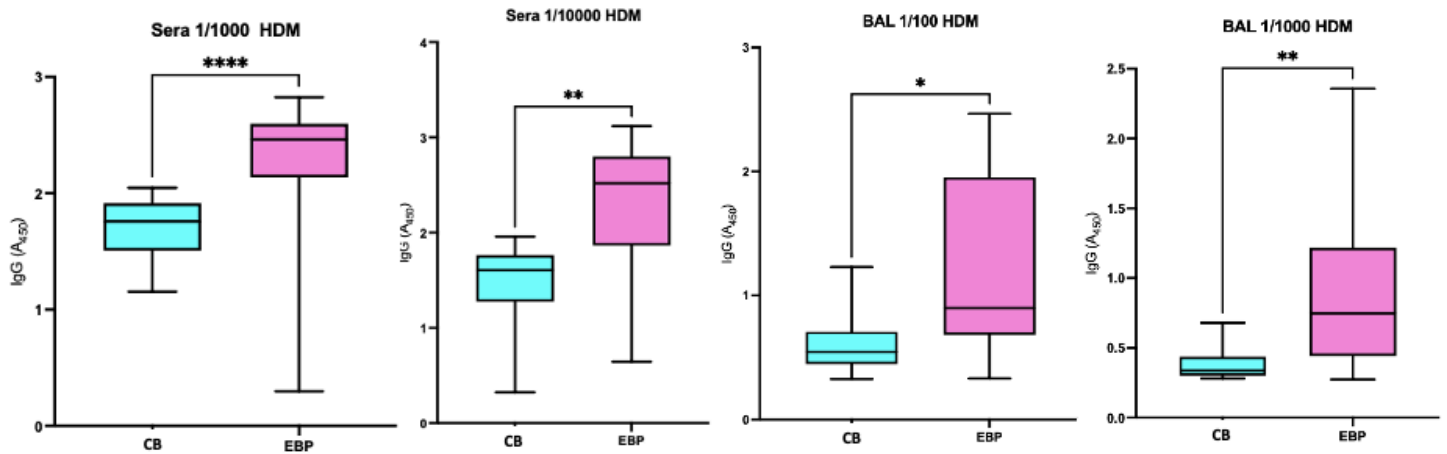
Figure 4: ELISA absorbance results for ***HDM*-specific IgG in sera and BALF** from dogs with chronic bronchitis (CB) and dogs with eosinophilic bronchopneumopathy (EBP) at 1/1000 and 1/10000 dilutions for sera and at 1/100 and 1/1000 dilutions for BALF. For all comparisons, differences were significant between dogs with CB and dogs with EBP (* : $p<0.05$; ** : $p<0.01$; *** : $p<0.001$; **** : $p<0.0001$).

5. *Correlation between optical density values for Aspergillus-specific IgG and for HDM-specific IgG in EBP dogs*

Optical density values of serum Aspergillus-specific IgG are correlated to serum values of HDM-specific IgG in dogs with EBP at 1/10000 dilution (Figure 5) (r=0.51, p=0.037). Correlation was not found for 1/1000 dilution.
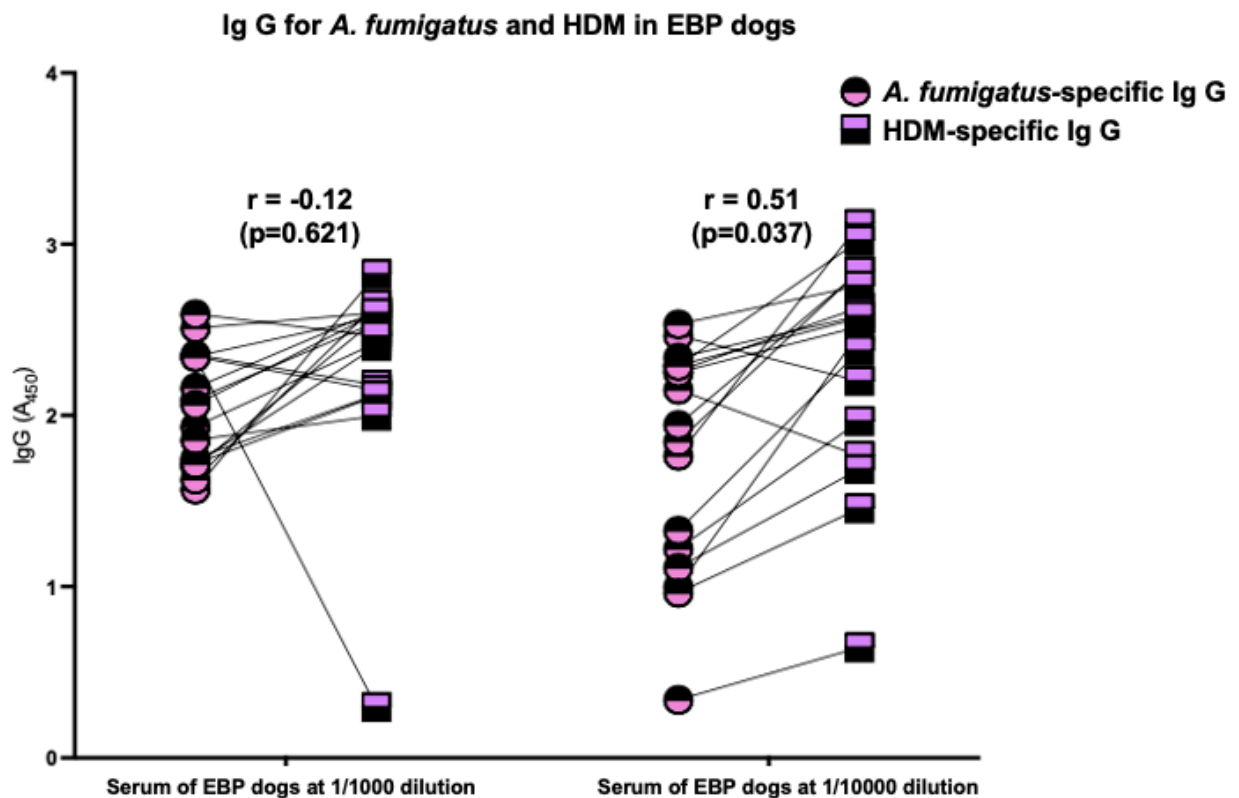
Figure 5: Optical density values for **serum *Aspergillus*-specific and HDM-specific IgG in each dog with eosinophilic bronchopneumopathy** (EBP) at 1/1000 and 1/10000 dilutions.

r : Spearman coefficient.

Correlation was significant for 1/10000 dilution (r = 0.51, p = 0.037).

6. *Inter- and intra-assay precisions*

Absorbance results obtained in duplicate for IgG in sera from dogs with EBP and dogs with CB are presented in Figure 6 at dilutions 1/1000 and 1/10000.

Absorbance results obtained in duplicate for IgG in BALF from dogs with EBP and dogs with CB are presented in Figure 7 at dilutions 1/100 and 1/1000.
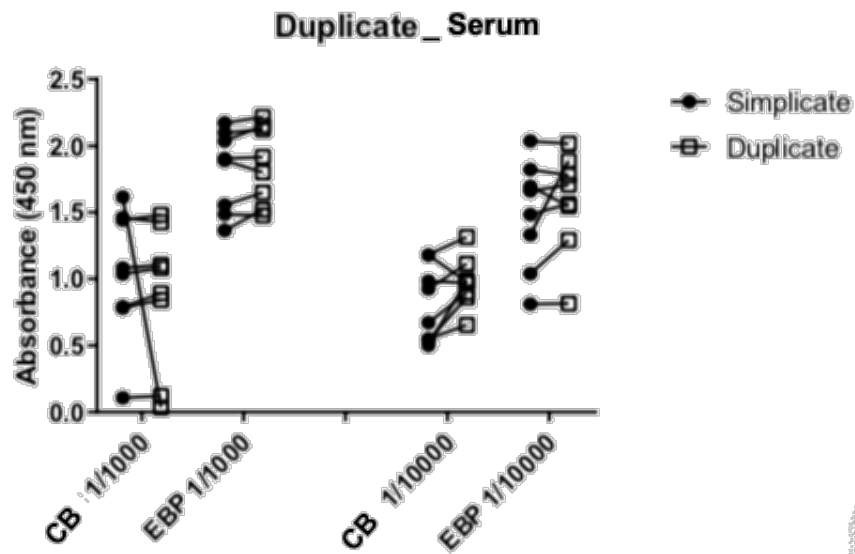
Figure 6: **Intra-assay precision**. ELISA absorbance results in **duplicate for *A.fumigatus*-specific IgG in serum** from dogs with chronic bronchitis (CB) and dogs with eosinophilic bronchopneumopathy (EBP) at 1/1000 and 1/10000 dilutions.
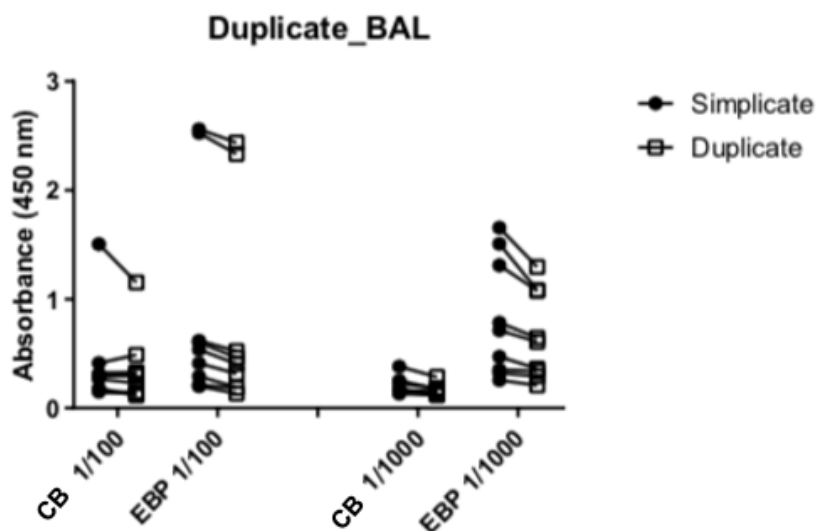
Figure 7 : **Intra-assay precision**. ELISA absorbance results in **duplicate for *A.fumigatus*-specific IgG** in **BALF** from dogs with chronic bronchitis (CB) and dogs with eosinophilic bronchopneumopathy (EBP) at 1/100 and 1/1000 dilutions.

Figure 8 reports ELISA absorbance results obtained twice, in few days apart, for *A.fumigatus*-specific IgG in serum from dogs with CB and dogs with EBP at dilution 1/1000 and 1/10000.

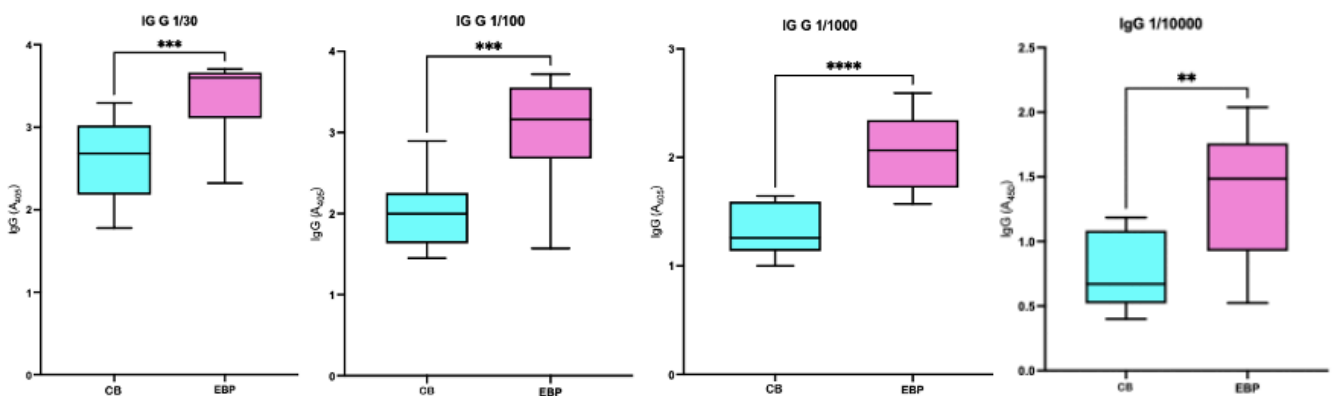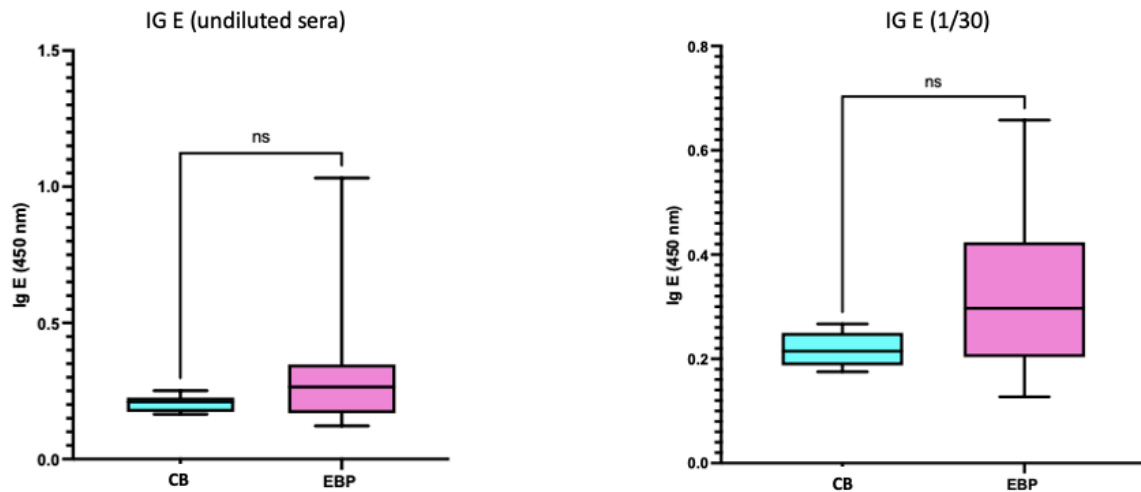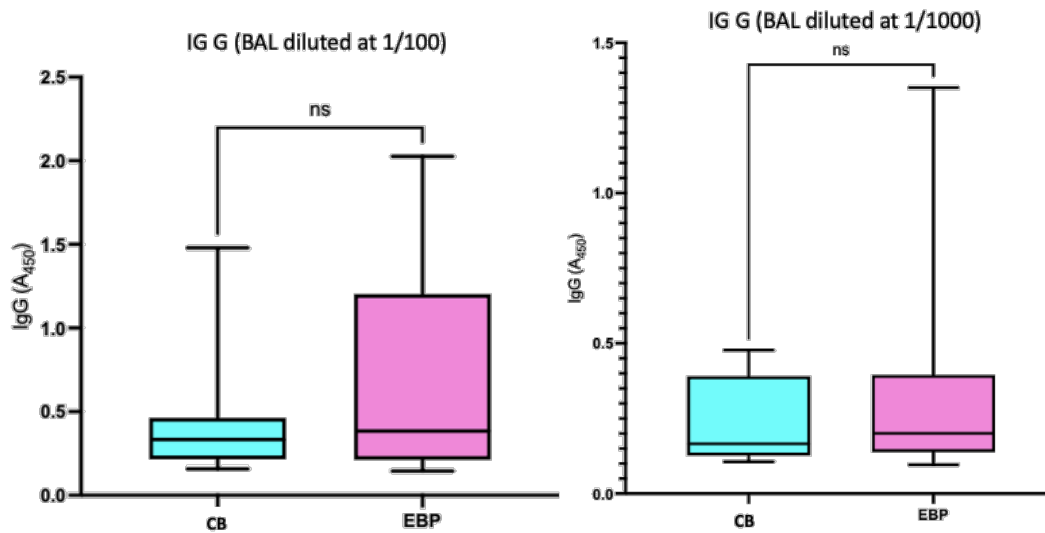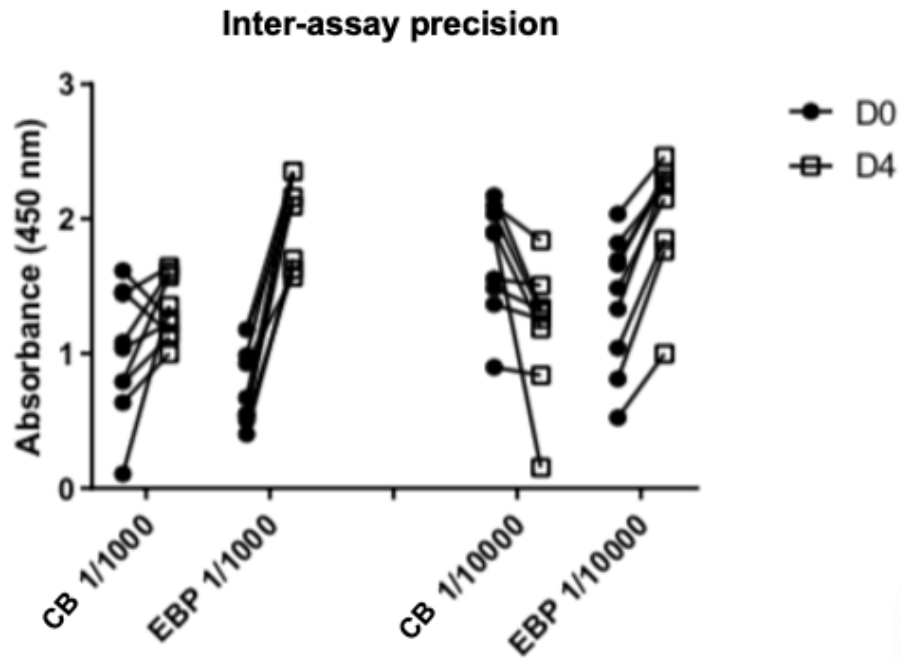Figure 8 **Inter-assay precision**. ELISA absorbance results in **few days apart** for *A.fumigatus*-specific IgG in serum from dogs with chronic bronchitis (CB) and dogs with eosinophilic bronchopneumopathy (EBP) at 1/1000 and 1/10000 dilutions.

Discussion

The present study constitutes the first detailed investigation about the potential implication of *Aspergillus fumigatus* in the unelucidated pathogenesis of canine EBP. Weak positive qPCR results in BALF from EBP dogs were occasionally observed but proportion of positive results was not statistically different in comparison with dogs with CB and healthy dogs. Nevertheless, serum levels of *Aspergillus fumigatus*-specific IgG were higher in EBP dogs compared to CB dogs or healthy dogs and EBP dogs had also higher levels of HDM-specific IgG in both serum and BALF compared to both other groups. Finally, levels of *Aspergillus fumigatus*-specific IgE were particularly low and not different between groups of dogs.

No significant difference was found between EBP dogs, CB dogs and healthy dogs regarding *Aspergillus* spp or *Aspergillus fumigatus* DNA quantification in BALF. In dogs having EBP and a positive qPCR result, DNA was detected only in weak quantity; these few dogs had a moderate clinical score index (CSI previously defined in Canonne et al. 2016) (CSI of 3/5 (data not shown)) while, among dogs with EBP and having negative qPCR result in BALF, some patients had a more severe clinical presentation. The first large case series about canine EBP reported positive *Aspergillus* spp culture in 2/22 dogs (Clercx et al. 2000). Moreover, in a recent study, a single colony of *Aspergillus* was also detected in a dog with EBP (Johnson et al. 2019a). For both studies, given the small numbers of dogs with positive fungal culture, authors could not conclude about any causal implication of *Aspergillus* in canine EBP. This fungus was thus rather considered as contaminant or incidental in these publications. and we cannot exclude that the few positive qPCR results on BALF observed in the dogs of the present study actually represent contaminants. On the other hand, comparison between the three groups may be underpowered because of small sized groups. In humans with ABPA, although positive culture or PCR in airway sampling is not required for definitive diagnosis, authors report higher sensitivity of PCR on sputum, bronchial and tracheal samples compared with BALF, probably because of dilution (Denning 2021). Nonetheless, the greater probability to detect fungal contaminant in upper samples is also legitimately questionable. Additional evaluation of qPCR performed on more proximal airway samples such as bronchial or tracheal secretions might be of interest in dogs with EBP.

Based on previous immunological investigation in dogs with EBP suggesting implication of Th2 pathway (Peeters et al 2005, Peeters et al. 2006), we could have expected to observe difference in optical density values for *Aspergillus*-specific IgE in both sera and BALF between dogs having EBP and others. Despite some immunological and imaging similarities between canine EBP nd human ABPA, the failure to detect any concentration of *A.fumigatus*-specific IgE in dogs with EBP does not support the hypothesis that canine EBP can be compared to this human disease. However, recent reviews in human

literature are suggesting a higher performance (better sensitivity) of serological assays by using recombinant and more immunogenic antigens such as Asp f1 and f2 (Page et al. 2015, Carsin et al. 2017, Muthu et al. 2018, Alghamdi et al. 2019, Muthu et al. 2020). Thus, we cannot exclude that our home-made ELISA test might lack of sensitivity and complementary serological assays for IgE in dogs with EBP should be performed with ELISA plates coated with recombinant *A. fumigatus* antigens.

On the contrary, significantly higher levels of serum *A. fumigatus* IgG were found at several dilutions in dogs with EBP compared to dogs with CB and healthy dogs. In humans, raised *A. fumigatus*-specific IgG are observed in *Aspergillus* bronchitis which is usually diagnosed on the basis of positive culture or PCR on BALF or sputum (Page et al. 2015) while specific imaging findings of chronic pulmonary aspergillosis or ABPA are absent. Therefore, as almost all dogs with EBP had negative qPCR on BAFF,canine EBP do not resemble *Aspergillus* bronchitis neither. Lastly, extrinsic allergic alveolitis or hypersensitivity pneumonitis are other human lung diseases that may be associated with elevated *A.fumigatus*-specific IgG (Denning 2021) but they are defined by dominant lymphocytosis on BALF, which is not found in EBP cases.

Optical density values for HDM-specific IgG were significantly higher in serum and BALF from dogs with EBP compared to samples from dogs with CB. Crude fungal extracts as used in solution A for ELISA plates coating may contain cross-reactive allergenic proteins (pan-allergens) (Fukutomi et al. 2016). A recent study identified a specific antigen of HDM that shares epitopes with *A. fumigatus* (ElRamlawy et al. 2016).

Another hypothesis to explain higher serum values for *A.fumigatus*-IgG and HDM-IgG in dogs having EBP might be the occurrence of multiple IgG-mediated hypersensitivity reactions with implication of several aeroallergens. In humans, *Alternaria alternata* and *Candida albicans* are reported to be the most prevalent causal fungi of allergic bronchopulmonary mycosis induced by fungi other than *A. fumigatus* (Ishiguro et al. 2014, Fukutomi et al. 2016, Bush 2020). Again, additional serological investigations should be done in dogs with EBP with specific investigation of IgG against other fungi as well as other environmental mites to further strengthen our presumption.

Conclusion

Dogs with EBP have higher levels of serum A.*fumigatus* IgG than dogs with CB and healthy dogs while *Aspergillus* DNA detection on BALF and levels of *A.fumigatus* IgE are not different between groups. Dogs with EBP have also higher levels of HDM-IgG in both serum and BALF compared to dogs with CB. These results refute the initial hypothesis that canine EBP may be compared to human ABPA. Nevertheless, additional serological assays using more specific recombinant *A.fumigatus* antigens, as well as antigens from *Altermaria alternata*, *Candida albicans* and other mites, are needed to definitively determine whether IgG-mediated hypersensitivities actually occur in dogs with EBP.

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Experimental section

Part 3 :

Long-term clinical and biological tolerance of inhaled steroid therapy in canine idiopathic eosinophilic bronchopneumopathy

Preamble

The third and last part of the experimental section was dedicated to the **evaluation of long-term clinical benefit, tolerance and potential side effects of inhaled steroid therapy (IST) with fluticasone as sole treatment** in dogs **with idiopathic** EBP.

Historically, the treatment of choice for canine idiopathic EBP is oral corticosteroid therapy but early or more delayed relapses are frequent if oral steroids are discontinued (Clercx et al. 2000, Rajamaki et al. 2002a, Casamian-Sorrosal et al. 2020). In practice, such dogs may require prolonged oral steroid therapy over years and undesirable side effects may develop. In such cases, IST could be beneficial. Even though this option is frequently proposed in practice, no study has investigated the long-term response of a series of dogs with EBP. Moreover, negative impact of IST on pituitary-adrenal-axis (PAA) in dogs treated over months to years was not yet explored.

Unfortunately, while IST using facemask was well accepted in all dogs, long-term fluticasone monotherapy fails to control cough in part of them, in which oral treatment is ultimately required (Study 6). Furthermore, inhibition of the PAA was also confirmed in two dogs treated with IST for 48 months (Study 6) and one of them developed progressive clinical signs of iatrogenic hyperadrenocorticism.

Experimental section

Part 3 – Study 6 :

Long-term follow-up in dogs with idiopathic eosinophilic bronchopneumopathy treated with inhaled steroid therapy

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Abstract

Background Treatment of canine idiopathic eosinophilic bronchopneumopathy mainly consists of long-term oral corticosteroid therapy. To avoid side effects, inhaled steroid therapy has been increasingly used; however long-term clinical response and potential side effects are sparsely described.

Objectives Description of clinical response and side effects with long-term fluticasone in dogs with eosinophilic bronchopneumopathy.

Methods Case series of dogs with eosinophilic bronchopneumopathy and treated with fluticasone monotherapy for at least 6 months. Clinical response and side effects assessed by physical examination, standardized questionnaire and ACTH stimulation test.

Results Eight dogs were treated for 6 months up to 5 years. Cough initially improved in all of them. Subsequently two dogs remained asymptomatic, three were well controlled while three severely relapsed. Pituitary-adrenal axis inhibition occurred in two dogs treated with fluticasone monotherapy for more than 2 years; only one dog had clinical signs of iatrogenic hyperadrenocorticism.

Clinical significance Fluticasone monotherapy allows initial improvement or remission in the majority of dogs. However, long-term treatment fails to resolve the cough in some dogs. Besides, such therapy may induce pituitary-adrenal axis inhibition. Prospective larger and randomized studies including both fluticasone and orally treated dogs are needed to define the optimal treatment.

Keywords: eosinophilic bronchopneumopathy, dogs, inhaled steroid therapy, fluticasone, iatrogenic hyperadrenocorticism

Introduction

Idiopathic eosinophilic bronchopneumopathy (EBP) is a chronic disease characterized by eosinophilic infiltration of the lung and bronchial mucosa that has traditionally been referred to as pulmonary infiltrates with eosinophilia (PIE) or pulmonary eosinophilia (PE) (Corcoran et al. 1991, Clercx et al. 2000). The disease is considered to be a manifestation of immunologic hypersensitivity, while responsible antigens are generally not identified (Clercx et al. 2002, Peeters et al. 2005). The classic and only well-described effective treatment of dogs with idiopathic EBP consists in long-term oral administration of steroids such as prednisolone (Clercx et al. 2000). Oral steroid therapy provides a rapid and significant positive clinical response but discontinuation of the treatment leads to recurrence of clinical signs within weeks or months after drug cessation in 30 to 70 % of cases (Corcoran et al. 1991, Clercx et al. 2000); these dogs frustratingly require long-term oral therapy to maintain clinical remission.

Chronic oral steroid therapy may unfortunately lead to iatrogenic hyperadrenocorticism and its use may also be contraindicated in dogs with concurrent diseases such as diabetes mellitus, obesity or cardiac disease. Therefore, alternative treatment with inhaled steroid therapy (IST) has been increasingly used in the past years, with the suggested advantages to provide both high drug concentrations within the airways and less side effects due to reduced systemic absorption. In human beings, IST is currently recommended as first-line treatment for chronic bronchial diseases (Singh & Loke 2010, Pandya et al. 2014). In cats, IST is also used for the management of experimental asthma (Cohn et al. 2010) and spontaneous chronic bronchial disease (Allerton et al. 2013). In dogs, despite its widespread and regular use by clinicians in practice, published clinical response to IST as single therapy in dogs with idiopathic EBP is limited to one case treated with fluticasone (Bexfield et al. 2006). Furthermore, although clinical and endocrine effects of a 3 to 4 weeks period of IST have been previously assessed in healthy dogs (Cohn et al. 2008, Melamies et al. 2012), long-term side effects of IST in dogs suffering from chronic bronchial diseases or more complex chronic lung diseases such as idiopathic EBP have not been evaluated.

Therefore, the aims of the present study were (1) to describe the clinical response to long-term IST with fluticasone as single therapy in coughing dogs with EBP and (2) to report possible biological inhibition of the pituitary–adrenal axis (PAA) and related side effects secondary to chronic steroid administration.

Materials and methods

1. Dogs

Client-owned dogs presented at the University Companion Animal Hospital between March 2009 and February 2014 and diagnosed with EBP were retrospectively recruited; only dogs treated with IST with fluticasone as single therapy for at least 6 months were included. Dogs that were treated with oral corticosteroids before the start of IST with fluticasone were not excluded, as long as fluticasone was administered alone for the minimal defined period (*i.e.*, 6 months).

Diagnosis of EBP was made in accordance with criteria described by Clercx and others (Clercx et al. 2000). Briefly, diagnosis was based on compatible respiratory clinical complaints, radiographic changes, bronchoscopic findings and standard cytological examination of the bronchoalveolar lavage fluid (BALF) and/or histopathological examination of per-endoscopic bronchial mucosal biopsies.

At the time of diagnosis, the clinical severity was assessed based on severity and frequency of cough, presence and severity of exercise intolerance or evidence of lethargy. A complete blood count was performed to investigate the presence of eosinophilia. Three view thoracic radiographs were performed and blindly reviewed by a board-certified radiologist (GB) in order to assess the predominant radiographic changes (bronchial, interstitial, or broncho-interstitial pattern), the presence of peribronchial cuffing, alveolar pattern, bronchiectasis, nodule(s) and/or tracheobronchial lymph node enlargement.

Bronchoscopy, bronchoalveolar lavage and laboratory processing of BALF were performed as described elsewhere (Clercx et al. 2000, Dehard et al. 2008). BALF analysis included total cell count determination using a Thomas' cell, manual differential cell counts (analysis of 200 cells on cyto-centrifugated smears), cytological examination of cyto-centrifugated smears, and semi-quantitative bacterial culture. In one dog with severe focal bronchiectasis (No 3), additional fungal culture was performed in order to exclude bronchopulmonary aspergillosis. Histopathological examination of bronchial mucosal biopsies was available in 2 dogs. Conventional faecal flotation and Baermann sedimentation were negative in all except 2 dogs in which feces could not be sampled. Additionally, angiostrongylosis was excluded in all dogs based on a negative quantitative polymerase chain reaction (qPCR) result in the BALF (Canonne et al. 2014). Besides, all dogs were treated at time of diagnosis with anthelmintics (fenbendazole 50 mg/kg q24h for 5 days, Panacur© *Intervet International* via *MSD Animal Health,* or milbemycin oxime 1 mg/kg once, Milbemax© *Novartis*). As none of the dogs had travelled outside Belgium, they were presumed to be free of heartworm disease and systemic fungal infection.

2. Treatment

IST with fluticasone propionate (Flixotide© 50 and 250 µg/dose *Glaxosmithkline Laboratory*) was used as single therapy for at least 6 months. It was administered using a propellant-driven pressurized metered dose inhaler (pMDI) connected to a valved spacer device and face mask (AeroDawg© Canine Aerosol Chamber, *Trudell Medical International*). For each administration, the pMDI was discharged (one or two actuations) into the spacer device and after placing the mask over the dog's face, the dog had to breathe into the device at least 7 times as classically proposed in earlier studies (Bexfield et al. 2006, Melamies et al. 2012). The mask size was chosen to adequately accommodate the muzzle of each dog (size 0 to 3). The initial dosage varied from 100 to 250 µg BID and was further adapted at the discretion of the clinician depending on initial clinical severity, size of the dog and clinical response. Dogs weighing more than 10 kg classically received 250 µg BID according to previous studies (Cohn et al 2008, Melamies et al. 2012). Owners were instructed by verbal communication and demonstration on their own dog for the first treatment. No oral steroid drug was concurrently given until the main recheck (*cf infra*).

3. Clinical response and potential side effects

Follow up consisted of a first recheck and a main recheck in all dogs, and an additional recheck in all but one of them.

During the first recheck, clinical response was assessed by phone consultation or physical examination 3 to 4 weeks after start of IST monotherapy. In case of clinical remission, owners were then instructed to decrease the dose per administration (one puff instead of 2) or, alternatively, the frequency of inhalations (from twice to once daily).

Until the main recheck, the follow up of dogs was then ensured either by our clinic or by the referring veterinarian. No other therapy was added during that period.

The main recheck occurred as late as possible after the start of the treatment and not before 6 months of IST. At that time, clinical response and potential side effects to IST were concurrently evaluated. Therefore, body weight and history were assessed and physical examination, complete blood count, serum biochemistry profile, standard urinalysis, thoracic radiography and an intravenous ACTH stimulation test were performed. A standardized medical questionnaire was completed. It included questions about the frequency and severity of respiratory clinical signs (cough, retching, dyspnoea, exercise intolerance) at that time compared with the clinical signs before IST, and about treatment administration (potential difficulties, compliance...). Other questions concerned the dog's appetite,

intake of water (any change in the volume of water or in the frequency of water needed to be replaced), urination (frequency and volume), presence of skin problems (particularly alopecia and scaling/seborrheic dermatitis), presence of abdominal distension and general demeanour. The intravenous ACTH stimulation test was conducted early in the morning prior to any other potentially stressful manipulation. Blood was collected before and 60 minutes after intravenous injection of 5 µg/kg synthetic ACTH (Synacthen[©], *Sigma-Tau Laboratory, France*) as previously described for diagnosis of iatrogenic and naturally-occurring hyperadrenocorticism (Ginel *et al.* 2012). Cortisol was assayed using an immunoassay suitable validated in the canine species (Immulite Analyser©, automated immunoassay, *Siemens Healthcare Diagnostics*). Inhibition of PAA was defined as pre- and post-ACTH cortisol less than 83 nmol/L (*i.e* 3 µg/dL); this value was taken because it was the highest post-ACTH cortisol concentration reported within the case series of Huang and others (1999).

An additional recheck was available in 7 dogs; a second ACTH stimulation test was performed in one of them.

Results

Eight dogs (female n=5; male n=3) diagnosed with EBP met the inclusion criteria; mean age at diagnosis was 47 months [range 11 – 120] (table 1). In 5 cases, the previous use of oral prednisolone prescribed over a short period of time typically had led to a good clinical improvement; however, all dogs had relapsed after treatment discontinuation (No 1, 2, 4, 6, 7). At the time of diagnosis, none of the dogs received any treatment. Mean duration of clinical signs was 6.5 months [range 2 – 24] (table 1). Only one dog had significant peripheral eosinophilia (No 7, table 1). A mixed moderate to severe generalized bronchial and/or broncho-interstitial pattern was most frequently observed on thoracic radiographs without evidence of alveolar or nodular pattern or tracheobronchial lymph node enlargement. Total cell counts in BALF varied from 275 to 8910 cells/µl (median=1472 cells/µl), percentage of eosinophils varied from 15 to 75 % (table 1) and semi-quantitative bacterial cultures was negative in all dogs. The initial dosage of IST with fluticasone varied from 100 to 250 µg BID (table 1).

No	Sign.	At diagnosis				At main recheck					At additional recheck	
		CBC eosinophils (/µl) ref 100-1350/µl	BALF eosinophils (%) ref < 7%	Duration of clinical signs	Initial IST dosage	Interval diagnosis – main recheck	IST dosage	Basal cortisol (nmol/L)	ACTH-stim cortisol (nmol/L)	Treatment modification	Interval diagnosis – additional recheck	Treatment modification
1	Whippet F, 3y	854	14	9 months	100 µg BID	6 months	100 µg SID	<28	348	No modification	28 months	No modification
2	Brittany spaniel F, 5y8m	752	30	6 months	250 µg BID	48 months	250 µg SID	<28	<28	250 µg BID + oral predni.	49 months	Tapering dosage of oral predni.
3	Border terrier M, 3y	1380	38	12 months	250 µg BID	48 months	250 µg SID	<28	<28	100 µg SID	-	-
4	Whippet M, 4y10m	149	20	24 months	250 µg BID	41 months	250 µg SID	<28	2150	100 µg SID	60 months	250 µg SID + oral predni.
5	Husky F, 1y	1359	75	5 months	250 µg BID	22 months	250 µg SID	353	450	No modification	42 months	No modification
6	Brittany spaniel F, 7y1m	810	25	9 months	100 µg BID	6 months	50 µg SID	193	510	50 µg EOD	20 months	No modification
7	Husky 11m	7428	67	4 months	250 µg BID	14 months	250 µg SID	NA	NA	250 µg EOD	18 months	No modification
8	Fox Terrier F, 10y	980	15	2 months	250 µg BID	14 months	250 µg SID	NA	NA	250 µg SID + oral predni.	15 months	Tapering dosage of oral predni.

Table 1: Signalement, clinical presentation at diagnosis, ACTH-stimulation test results and therapeutic adaptations at rechecks. Sign: signalement, F: female, M: male, *y*: years, *m*: months

Ref: reference interval

Predni: prednisolone, SID: once a day, BID: twice a day, EOD: every other day

1. Clinical responses

At first recheck 3 to 4 weeks after start of IST monotherapy (recheck by phone consultation in n=2 dogs and physical examination in n=6 dogs), all dogs showed a positive clinical response to IST, including clinical remission or significant reduction in frequency and severity of the cough.

The main recheck occurred 6 months to 48 months after initiation of IST (median = 18.0 months, mean = 24.9 months). At that time, all dogs were treated with once daily IST (table 1). Owners' compliance was considered satisfactory and all dogs easily accepted the treatment procedure. After 6 months of IST, two dogs (No 6, 7) were considered asymptomatic. In two other cases (No 3, 4), cough reappeared as soon as owners moved on to every-other-day therapy. Owners agreed on reducing the dose of the IST in these four dogs. Three other dogs showed persistent cough at low (No 1, 5) or moderate frequency (No 8) (three to five times a week and once a day, respectively). After an initial positive response of several months, the remaining dog (No 2) presented a progressive clinical deterioration, despite the maintenance of the same fluticasone dosage, and was coughing more than 10 times a day with progressive development of exercise intolerance at the time of the main recheck. Therefore, additional oral prednisolone was temporarily prescribed for the last two dogs (No 2, 8). At the main recheck, radiological lesions were unchanged (2 dogs) or improved (3 dogs) or worsened (2 dogs) with more severe bronchial or interstitial pattern, presence of peribronchial cuffing and/or bronchiectasis.

An additional recheck was available for 7 dogs (table 1). Two dogs (No 2, 8) were rechecked one month after initiation of oral prednisolone: one dog showed a significant decrease in frequency and severity of the cough (No 2) and the other was considered asymptomatic (No 8). The five other dogs were rechecked from 18 to 60 months after the start of IST. In 4 dogs, cough was stable in severity and frequency in comparison with the main recheck. The remaining dog (No 4) suffered from a moderate to severe clinical relapse of cough; oral prednisolone was thus initiated and led to clinical improvement of the frequency and the severity of the cough within a few days. In total, 3 out of 8 dogs required additional oral steroid therapy at least 14 months after start of IST monotherapy (table 1).

2. Side effects

At the main recheck, the intravenous ACTH stimulation test was performed in 6 dogs (No 1 to 6). The ACTH stimulation test revealed low pre- and post-stimulation cortisol levels compatible with inhibition of PAA in two dogs (No 2, 3) that were both treated with IST for 4 years (table 1). Only one of them (No 2) developed progressive clinical signs of iatrogenic hyperadrenocorticism after 45 months of IST monotherapy, including weight gain, polyuria and polydipsia, diffuse symmetric alopecia, skin thinning, severe abdominal distension, hepatomegaly and panting and moderate mature neutrophilic leukocytosis (22 500 neutrophils/µl). Despite these overt adverse and deleterious effects, respiratory clinical signs were concurrently relapsed. In the two Whippet dogs (No 1, 4), only pre-stimulated cortisol level was lower than reference value (table 1). An additional recheck was performed in one of them 60 months after start of IST (No 4); at that time, the ACTH stimulation test was repeated and PAA inhibition was still absent (data not shown).

Serum biochemistry profile and urinalysis were within normal reference in all dogs (data not shown). Complete blood count was also unremarkable in all except the dog No 2.

Discussion

The purposes of this clinical study were to assess the clinical benefit of long-term IST with fluticasone as a single therapy for more than 6 months in canine EBP and to investigate consequence of long-term IST on PAA. Results of the present study show that inhaled medication using facemask was well tolerated in all cases. Despite of initial clinical improvement or remission in the majority of dogs, data suggest that long-term IST used as a single therapy failed to provide optimal control of the cough in 3 out of 8 dogs, in which oral steroid therapy was ultimately required after at least 14 months of IST. Besides, long-term IST may induce inhibition of PAA in dogs, but this inhibition is not associated with clinical iatrogenic hyperadrenocorticism in all dogs.

Current literature on management of EBP is scarce but suggests that a large proportion of dogs unfortunately requires long-term oral steroid therapy, which may lead to clinical signs of iatrogenic hyperadrenocorticism (Corcoran et al. 1991, Clercx et al. 2000). So far, IST has been widespread adopted in small animal respiratory medicine without clear evidence to support this practice. Clinicians commonly consider IST as a practical therapy in clinical practice and have experience of good owners' obedience in dogs. Actually, the present study confirmed a satisfactory compliance with long-term IST; indeed, only one EBP dog was discarded from inclusion in the study because the owners had preferred to give additional oral therapy. On the other hand, expert opinion and limited published information suggest that IST may be considered as an alternative therapy when the use of oral corticosteroids is contraindicated. In the present series, a significant decrease in frequency and severity of the cough was observed within the first months of administration of IST in all dogs and owners agreed on reducing the dose of the IST after 6 months in four dogs. However, mild to moderate residual cough actually persisted or recurred in 6/8 of them after 6 months. In 2 dogs, clinical severity worsened after a long period of stabilisation; for both of them, transition to oral medication led to a clear clinical improvement of the cough. Besides, unlike reports about oral treatment in dogs with EBP (Clercx et al. 2000), none of the dogs treated with IST could be weaned off medication. Although a direct comparison cannot be made with previous descriptions including orally-treated dogs (Corcoran et al. 1991, Clercx et al. 2000), the relative frequencies of remission and clinical relapse might be less promising with inhaled fluticasone monotherapy. Alternatively, population in the present series might have included dogs with a more refractory disease state.

Although inhaled therapies are increasingly being used to treat respiratory diseases in dogs, there is a paucity of information about pharmacokinetics of inhaled steroids in this species. In people, scintigraphic quantification of lower airways deposition of radiolabelled aerosols is classically

performed in pharmacokinetic studies. However, in healthy humans using pMDI, only 12% of inhaled radiolabelled fluticasone was shown to actually deposit in lungs and deposition was shown to be primarily in large central and intermediate airways (Leach et al. 2002). This low pulmonary deposition is related to the particle size of the drug delivered by inhalers, with larger particles providing the least lung deposition and greatest oro-pharyngeal deposition (Leach et al. 2002). In dogs, scintigraphic evaluation of lung deposition of radiolabelled fluticasone delivered by pMDI was recently performed in ten healthy dogs. Lung deposition of fluticasone in this group seems to be very low (2%) (Chow et al. 2014). Moreover, it might be inappropriate to use these preliminary data from healthy dogs to predict the deposition and absorption of fluticasone in dogs with chronic bronchial diseases or diseases associated with pathophysiological changes in the lung, such as EBP. Indeed, deposition of fluticasone in the lungs of patients with asthma was demonstrated to be even lower than in healthy humans (Daley-Yates et al. 2000). This difference is probably caused by narrowed small airways; besides, presence of mucus promotes mucociliary clearance of lipophilic drugs, such as fluticasone, which slowly dissolve in airway lining fluid (Mortimer et al. 2006, Singh & Loke 2010).

To date, doses of inhaled fluticasone are not standardized in dogs and largely extrapolated from human medicine. Only one study assessed three different doses in a model a feline asthma and demonstrated that airway inflammation was similarly improved regardless the dose of inhaled fluticasone (Cohn et al. 2010). Based on this observation and regarding the pharmacokinetic considerations in dogs and humans discussed above, getting a better clinical response by increasing dosage in dogs with EBP could not be excluded but appears unlikely.

Asymptomatic and symptomatic depressions of PAA have been well described in human asthmatic patients treated with nebulized fluticasone propionate (Iles et al. 2008, Masoli et al. 2006, Pandya et al. 2014, Wlodarczyk et al. 2008). The type of inhaled molecule, dose and duration of drug administration, and expiratory volume influence the severity of the inhibition of PAA in people (Masoli et al. 2006, Pandya et al. 2014, Singh & Loke 2010, Wlodarczyk et al. 2008). In healthy dogs, asymptomatic inhibition of PAA has been shown to occur after 3 to 4 weeks treatment with inhaled fluticasone propionate (Cohn et al. 2008, Melamies et al. 2012). So far, long-term side effects of IST in dogs suffering from chronic bronchial diseases such as idiopathic EBP have not been accurately evaluated. Results of the present study confirmed inhibition of the PAA in two dogs treated with IST for 48 months, which might suggest a possible correlation of inhibition of PAA with duration of treatment, although another dog treated for 60 months had normal pre- and post-stimulation cortisol level. Despite a poor lung deposition after inhalation (12%), fluticasone has a much higher distribution (78%) to the oropharynx in people (Leach et al. 2002). This substantial portion of the drug may be then swallowed, systemically absorbed through the gastric mucosa and further responsible for well-known side effects, which can develop despite a moderate to poor improvement of the cough. Besides,

individual susceptibility to side effects of oral corticosteroid therapy is well known in dogs (Reusch 2015) and it might apply to inhaled steroid medication as well. In humans, polymorphisms in glucocorticoid receptor genes have been found to be associated with both increased and decreased sensitivity to exogenous steroids and possibly in a tissue-specific manner in the same patient (Charmandari 2011).

In the present study, we used an ACTH-stimulated cortisol concentration less than 83 nmol/L to define inhibition of PAA; however, no single cut-off has been validated to characterize suppression of PAA (Reusch 2015). We also used the dose classically advised for intravenous ACTH stimulation test in dogs (Ginel et al. 2012). However, in human medicine, a lower dose of ACTH (*i.e* 1 μg) can be used with reported better sensitivity (Bernstein & Allen 2007). Therefore, mild degree of depression of PAA might have been underestimated in this clinical series.

The small population size is a clear limitation in the present study. Moreover, clinical severity varied among dogs at the time of diagnosis. Orally-treated dogs were not included in the present study, making statistical comparison with dogs treated with only inhaled fluticasone impossible. Moreover, the inclusion criteria of the present study were strict, limiting the number of dogs in the study. These criteria required that dogs were treated only with fluticasone monotherapy and for at least 6 months and that owner's compliance was considered as satisfactory, *i.e.,* strict obedience to the prescribed protocol (neither discontinuation nor other concomitant treatment allowed) and availability for the rechecks.

Undoubtfully, repeated bronchoscopy and BALF analysis during the course of the treatment could have provided helpful additional data, especially in relapsing or refractory cases. One additional bronchoscopy and BALF cytology and bacterial culture were available in only two dogs for which bacterial infection was excluded as the cause of the treatment failure 48 and 60 months after start of fluticasone monotherapy (No 2 and 4, data not shown).

Therefore, a larger study with prospective inclusion of dogs randomized in different comparative groups of treatment (*i.e* IST *versus* oral therapy in newly diagnosed and/or relapsing dogs) is warranted to support the present preliminary findings and to provide statistical data to determine the optimal treatment of dogs with EBP. In this respect, it would also be interesting to assess the clinical response to a mixed protocol combining initial bitherapy (*i.e.,* oral prednisolone and inhaled fluticasone) followed by tapering of oral therapy together with long-term maintenance of IST. A multicentre approach would be required to optimize the number of recruited dogs.

Scintigraphic evaluation of lung deposition of inhaled radiolabelled fluticasone in dogs diagnosed with EBP would also afford additional helpful information to explain the poor long-term clinical response of some dogs. Lastly, in dogs with poor clinical response to IST and adverse

effects/contraindications of steroid therapy, investigation of novel therapeutic options with more clinical efficacy and less systemic side effects are also warranted. Ciclesonide, currently used with pMDI to treat severe cases of human asthma, was observed to have an improved therapeutic margin compared with some other currently available inhaled steroid drugs without effect on the PAA function (Pandya et al. 2014); however, to date, the effect of this drug has not been assessed in the canine species.

Conclusion

Inhaled fluticasone as single treatment seems to be a practical therapy in clinical practice and allows initial clinical improvement or remission in the majority of dogs diagnosed with EBP. However, long-term fluticasone monotherapy fails to control cough in part of the dogs, in which oral treatment is ultimately required. Furthermore, long term IST may induce biological and clinical inhibition of PAA. In dogs diagnosed with EBP and successfully treated with long-term IST, both clinical monitoring and regular evaluation of potential PAA inhibition should thus be advised. A further prospective trial including a larger number of dogs as well as a comparison with a group of EBP dogs treated with oral steroids would be helpful to support our observations.

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Discussion - Perspectives

In this work, we firstly aimed to **improve diagnosis of EBP by focusing in exclusion of the endemic helminth _Angiostrongylus vasorum_.**

The shared objective of the first two studies was to highlight the relevance of quantitative PCR on BALF to definitively exclude infestation by _Angiostrongylus vasorum_ once eosinophilic airway inflammation is demonstrated in a dog.

With the retrospective analysis on stored BALF collected in coughing dogs, Study 1 also demonstrated the new emergence of pulmonary angiostrongylosis in Belgium in 2016. From 2008 to 2016, more and more publications emphasize the spreading of canine angiostrongylosis into several areas (Bourque et al. 2008, Yamakawa et al. 2009, Helm et al. 2010, Gredal et al. 2011, Conboy 2011, Gallagher et al. 2012, Traversa et al. 2013). From the time that Study 1 was performed (2016) up to now (2022), publications have increasingly reported the presence of _A. vasorum_ in sick or asymptomatic dogs from north-baltic, eastern and southern European countries including Estonia, Finland, Romania, Austria, Bulgaria, Slovakia, Portugal, Spain, Greece (Alho et al. 2016, Deak et al. 2019, Tiškina et al. 2019, Angelou et al. 2020, Carreton et al. 2020, Iliev et al. 2020, Fuehrer et al. 2021, Globokar et al. 2021, Morchon et al. 2021, Oborina et al. 2021, Šmigová et al. 2021). To date, almost no European country seems spared from the circulation of this parasite. Several factors are presumed to contribute to this expansion: movements in animal populations, climatic global warming, changes in vector epidemiology and increasing densities of red foxes (_Vulpes vulpes_) in urban and periurban areas (Bolt et al. 1992, Morgan et al. 2009, Traversa et al. 2010, Franssen et al. 2014).

It is therefore particularly crucial to rule out this largely-endemic disease which can result in highly variable clinical pictures (Colombo et al. 2021), especially in a young or middle-aged one and if eosinophilic airway inflammation was detected.

Blood-containing bronchial secretions detected on bronchoscopy are suggestive of angiostrongylosis (Study 1) whereas thick yellow-green mucus or mucopurulent material and mucosal polypoid proliferation are bronchoscopic hallmarks of EBP. However, in both diseases, bronchoscopy changes may be mild and analysis of BALF is required to differentiate both conditions. For this purpose, qPCR on BALF is particularly useful. Study 1 documents for the first-time positive qPCR results on broncho-alveolar lavage fluid in Baermann-negative dogs and Study 2 confirmed that qPCR is the sole available method having the best relative sensitivity compared to non-invasive techniques including faecal analysis and in-clinic rapid assay. Analysis of BALF might thus allow detection of L1 larvae either by qPCR or by standard cytological examination before faecal larval shedding. Although Baermann analysis and the in-clinic rapid test should be used as first-line tools in clinically suspect dogs because of their availability, cost-effectiveness and inherent non-invasiveness, they might be of lower sensitivity than detection of specific antibodies and qPCR on BAL material, especially in cases of early

infection. Moreover, as none of the healthy dogs had positive qPCR results from broncho-alveolar lavage fluid the risk of false positive test results (from endoscope contamination for example) were considered unlikely (Study 1).

In experimental studies, in-clinic rapid assay was positive 14 weeks after inoculation (Schnyder et al. 2014. In dogs with positive faecal analysis by Baermann, in-clinic rapid assay was positive in only 85% of dogs (Schnyder et al. 2014). Based on these results and observations from Study 2, a negative in-clinic rapid assay cannot rule out angiotronsgylosis. The moderate sensitivity might be explained by formation of antigen-antibody complexes, which inhibit detection of circulating antigens. Gilis-Germitsch et al. described heat treatment of serum to increase sensitivity of rapid assay and this procedure was not performed in seronegative dogs included in Study 2. However, impact of heat on seropositivity depends on the delay from infestation and still remains unperfect, so authors don't recommend systematic heating of sera (Gilis-Germitsch et al. 2017).

More recently, we assessed the diagnostic utility of in-clinic rapid assay performed on BALF samples in dogs with confirmed angiostrongylosis that tested seronegative (Roels et al. 2021). Even if results are immediately available unlike qPCR, performing the rapid device on BALF samples was of no added diagnostic values as all results were negative.

Circulating DNA in the blood was not assessed in our Study 2; this non-invasive tool was suggested as an early indicator of infection, *i.e.* 2 weeks post infection in a previous experimental study, but has a very low sensitivity in experimentally-infected dogs as in foxes (Jefferies et al. 2011, Schnyder et al. 2015, Houpin et al. 2016). In a small recent and unpublished series, we performed qPCR on blood in 5 dogs having angiostrongylosis and tested seronegative and qPCR on blood was only positive in 2 out of them (Roels et al. 2021).

Quantitative PCR on feces was not performed in Study 2; even if it could be more sensitive than Baermann faecal analysis, testing on feces is unable to detect prepatent lung infestation.

Lastly, ELISA for detection of specific antibodies had similar sensitivity to qPCR on BALF (Study 2); it is a non-invasive technique being the most promising test for identifying dogs at an early stage of infection (Schnyder et al. 2015); unfortunately it is currently not commercially-available.

In conclusion of the part 1, definitive diagnosis of idiopathic EBP requires exclusion of presence of endemic helminth *Angiostrongylus vasorum* which is spread over Belgium as over all other European countries. To date, the highest sensitive test to rule out angiostrongylosis in coughing dogs having eosinophilic inflammation on BALF seems to be qPCR on BALF. As detection of specific antibodies is of comparable sensitivity, efforts should be made to develop a rapid test to easily and non-invasively detect specific antibodies in coughing dogs; however, unlike qPCR on BALF, antibody seropositivity does not distinguish current or past infection.

We secondly aimed to **better understand pathogenesis of canine EBP in particular by investigating potential triggering or exacerbating role of infectious agents including *Bordetella bronchiseptica, Mycoplasma canis, Mycoplasma cynos* and *Aspergillus fumigatus*.**

To date, large clinical case series describing dogs with EBP failed to identify specific bacteria as causative agent for EBP (Clercx et al. 2000, Johnson et al. 2019a, Casamian-Sorrosal et al. 2020) but specific investigation was restricted to conventional bacterial culture on BALF. In human medicine, infections with *Mycoplasma pneumoniae* and *B. pertussis* have been associated with asthma for decades (Kraft et al. 2002, Hansbro et al. 2004, Harju et al. 2006, Blanchard & Raherison 2010, Atkinson 2013, Rubin & Glazer 2018, Ondari et al. 2021) but the role of canine species of *Mycoplasma* and *B. bronchiseptica* as triggers or exacerbating agents, has never been investigated in canine EBP using qPCR on BALF.

B. bronchiseptica has been isolated in clinically healthy dogs by qPCR from nasal and pharyngeal swabs (Schulz et al. 2014); thus, it is unclear how a positive qPCR result from BALF should be interpreted. According results of Study 3 describing use of qPCR on BALF collected from dogs having bordetellosis, qPCR analysis on BALF, with consideration of *B. bronchiseptica* DNA load, is highly sensitive and reliable method for *B. bronchiseptica* confirmation, especially in referred dogs that are frequently previously treated with antibiotics that could result in negative bacteriology results.

When *B. bronchiseptica* was searched in dogs with EBP, chronic bronchitis and healthy dogs, proportion of positive results were non different between groups (Study 4). However, in dogs with EBP, the probability of a positive qPCR result for *B. bronchiseptica* increased with the clinical severity. Median percentage of neutrophils in BALF from dogs with positive PCR for *B. bronchiseptica* were also significantly higher than median value from dogs with negative PCR. Moreover, among dogs with positive qPCR for *B. bronchiseptica*, moderate or high loads were observed only in dogs with EBP.

In puppies vaccinated with a single dose of modified live topical vaccine, *B. bronchiseptica* can be detected by qualitative PCR during 1 month after vaccination (Ruch-Gallie et al. 2016). The potential influence of previous vaccination on qPCR performed on BALF was unknown in dogs but as no dogs with EBP was vaccinated within the 2 months prior to diagnosis, we assumed the impact of vaccination on qPCR for *B. bronchiseptica* in BALF as negligible. Moreover, Study 3 highlighted that false positive qPCR results is very unlikely when standardized protocol of cleaning and disinfection by a trained technician is respected so all positive qPCR results observed in Study 4 were considered as true-positive.

However, Study 4 had some limitations. Underestimation of *B. bronchiseptica* in BALF from dogs with EBP cannot be ruled out as included dogs were referred and some of them had received some antimicrobial drugs before diagnosis and BALF collection. Moreover, clinical severity of dogs with EBP was assessed by a non-validated clinical severity score; however, score was blindly attributed by a single observer.

A cause-and-effect relationship between presence and bacterial load of *B. bronchiseptica* and severe canine EBP remains unclear. We can hypothesize as suggested by human literature about *B. pertussis*, that *B. bronchiseptica* is able to trigger eosinophilic lower airway inflammation in young or middled-aged dogs. Several experimental publications confirmed *B. pertussis* and its toxins as being potent adjuvants, inducing asthma and allergic sensitization in animal models and human disease; furthermore, in children, a history of whooping cough increase the risk of asthma and allergic sensitization (Dong & Gilmour 2003, Ennis et al. 2004, Hansbro et al. 2004, Ennis et al. 2005, Harju et al. 2006, Atkinson 2013, Vogt et al. 2014, Rubin & Glazer 2018, Connelly et al. 2021, Ondari 2021). Lastly, the asthma epidemic in the United States parallels the surge in *B. pertussis* incidence and relationship between subclinical *B. pertussis* colonization and allergic sensitization disease is strong (Rubin & Glazer 2018). A longitudinal study following dogs with *Bb* infection over years to document if such dogs are eventually predisposed to later develop canine idiopathic EBP is needed. On the contrary, an opposite scenario could also be that the eosinophilic airways inflammation favours *B. bronchiseptica* growth in dogs.

In dogs, the exact role of *M. canis* and *M. cynos* as primary respiratory pathogens still remains unelucidated (Chandler & Lappin 2002, Chalker et al. 2004, Chan et al. 2013). Moreover, oral bacterial contamination can result in false-positive results of *Mycoplasma*-specific PCR from bronchoalveolar lavage (Chan et al. 2013), making the evaluation of any primary role of *Mycoplasma* spp. in canine lower respiratory conditions even more confusing. Proportions of positive qPCR results for both *M. cynos* and *M. canis* were not different on BALF between dogs with EBP, dogs with chronic bronchitis and healthy dogs. However, among dogs with positive qPCR for *M. cynos* on BALF, DNA levels corresponding to very high load were only found in dogs with EBP or chronic bronchitis. Unlike our observations with *Bb*, dogs with EBP that were qPCR positive for *M. cynos* did not have a higher clinical severity than negative dogs.

In humans, infection with *M. pneumoniae* favours asthma development in predisposed patients and specific treatment with clarithromycin improves pulmonary function (Martin et al. 2001, Kraft et al. 2002, Hansbro et al. 2004, El Sayed Zaki et al. 2009, Atkinson 2013, Ye et al. 2014, Kassisse et al. 2018, Liu et al. 2021). The proportion of asthmatic humans who have both IgM and IgG titres for *M.*

pneumoniae increases with clinical severity (Iramain et al. 2016, Yin et al. 2017, Kumar et al. 2019, Liu et al. 2021). In mouse models, single exposure to a specific toxin of *M. pneumoniae* ("Community-Acquired Respiratory Distress Syndrome Toxin": CARD-toxin) was sufficient to generate functional IgE and cause asthma-like disease and pulmonary eosinophilic inflammation (Medina et al. 2012, Medina et al. 2017). In children infected by *M. pneumoniae*, patients with more clinical signs had excessive secretion of IL-4 and IL-5 and overdifferentiation of Th0 cells into Th2 cells; these interleukins and Th2 response are well-known to contribute to eosinophilic inflammation (Ye et al. 2018). The Study 4 failed to suggest a similar connection between canine mycoplasmal species and development of EBP in dogs. However, the small-sized study population may have limited the statistical power of analysis. In humans, implication fo *M. pneumoniae* could also be explored by assessment of specific antibodies (El Sayed Zaki et al. 2009, Iramain et al. 2016, Kumar et al. 2019, Liu et al. 2021) which were not measured in our study. Rycroft et al. documented elevated serum antibodies to *M. cynos* in dogs entering the re-homing kennel and antibody response was positively related to the development of clinical respiratory disease (Rycroft et al. 2007) but such serological tests are not commercially-available. Moreover, other mycoplasmal species such as *M. edwardii M. spumans* and *Ureaplasma* were not investigated in dogs with EBP or chronic bronchitis in the Study 4. These atypical bacteria have been previously detected by PCR in dogs with CIRD (Chalker et al. 2004) but prevalence in tracheal and bronchial lavage were not significantlty different from those observed in healthy dogs (Chalker et al. 2004). According to this single study, their specific role in canine respiratory diseases might thus be negligible but additional investigation is needed.

In humans, there is also strong evidence for an association between infection by *Chlamydia pneumoniae* (*C. pneumoniae*) and pathogenesis and exacerbation of asthma (Hansbro et al. 2004, Iramain et al. 2016, Paróczai et al. 2020, Smith-Norowitz et al. 2020, Calmes et al. 2021). *C. pneumoniae*-specific IgE levels are higher in asthmatics compared with non-asthmatics, despite negative upper respiratory PCR (Smith-Norowitz et al. 2020). Moreover, asthmatics with IgA and IgG against *C. pneumoniae* have more severe disease with increased airway obstruction and need higher doses of IST (Calmes et al. 2021). Accordingly, future studies assessing the potential impact of atypical bacteria in the pathogenesis or exacerbation of canine EBP should probably include the specific evaluation of *Chlamydia* spp. To date, there is limited information about the seroprevalence of *Chlamydia* spp. and the risk factors associated with *Chlamydia* spp. infection in dogs in the world. Two independent serological surveys were undertaken in China and concordant seroprevalence among pet dogs was found (15%) (Wu et al. 2013, Tian et al. 2014). Nevertheless, no specific study in dogs having respiratory disease has been yet conducted.

Lastly, in humans, there is also growing evidence that the microbiomes of the upper or lower airways and the gut play a major role in the development of asthma (Lu et al. 2017, Zhang et al. 2017,

Kozik et al. 2019, Sharma et al. 2019, Barcik et al. 2020, Hufnagl et al. 2020, Yang et al. 2022). In both children and adults, digestive and airway microbiome dysbiosis may contribute to both the inception and progression of asthma and to corticosteroid resistance in asthma (Sharma et al. 2019). To date, literature about the nasal and lung microbiota in dogs is sparse. Lung microbiota has been evaluated in dogs with chronic bronchitis (Ericsson et al. 2020). While inter-sample diversity differed significantly between samples from dogs with chronic bronchitis and dogs without chronic bronchitis, the variability within both groups made it difficult to discern reproducible bacterial classifiers of disease. Modifications of lung microbiome have been also shown in canine non specific bacterial pneumonia (Vientós-Plotts et al. 2019) and in dogs with *B. bronchiseptica* infection (Fastres et al. 2020). Potential implication of digestive and airway microbiome dysbiosis in the pathogenesis of EBP has to be investigated.

In conclusion of <u>Study 4</u>, whatever the exact connection between eosinophilic airway inflammation and presence of *B. bronchiseptica* in dogs is, our observations invite to systematically search for *B. bronchiseptica* in dogs newly-diagnosed as having EBP, particularly in case of severe clinical presentation, high percentage of neutrophils in BALF or acute exacerbation in previously diagnosed cases and specific treatment should be prescribed accordingly. Additional studies such as longitudinal follow-up of dogs infected by *B. bronchiseptica* and evaluation of further development of EBP may be relevant. Concerning potential impact of mycoplasmal bacteria, other explorations such as research of other marginal species (such as *M. edwardii* or *M. spumans*) and testing for *Mycoplasma*-specific antibodies in dogs with EBP would be interesting. Moreover, in dogs with EBP, specific evaluation of *Chlamydia* spp. by qPCR on BALF and serum specific antibodies as well as description of digestive and airway microbiome might also be interesting perspectives.

The figure 22 summarizes the diagnostic approach for a dog presenting a chronic cough; successive steps for the confirmation of an idiopathic EBP are detailed. Results from Studies 2, 3 and 4 regarding qPCR analysis on BALF have been used to produce this diagram.
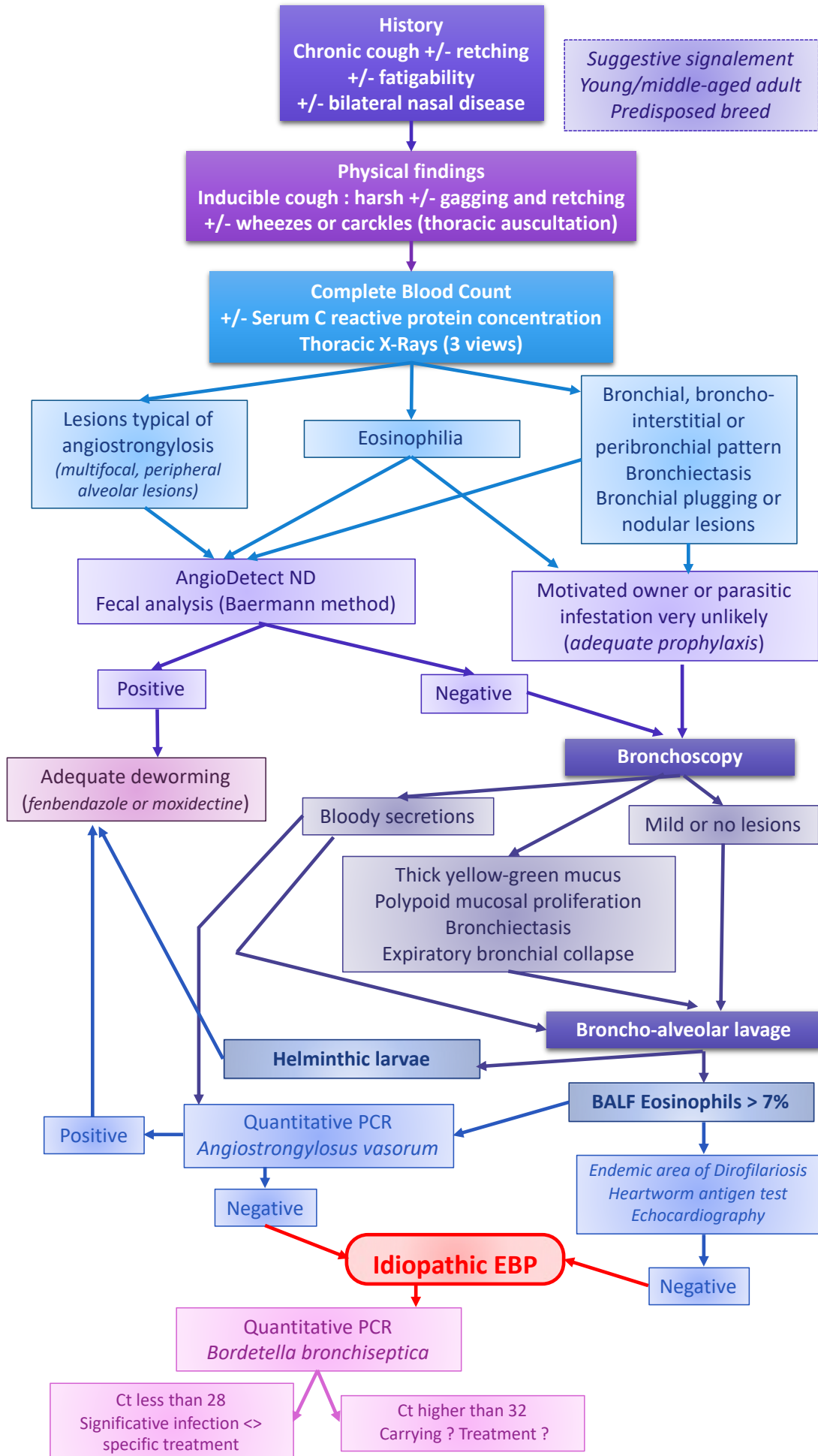
Figure 22: Proposed diagnostic approach for a chronic cough in a dog ; successive steps to confirm idiopathic eosinophilic bronchopneumopathy

Some similarities regarding immunologic reactions and tomodensitometric lesions exist between canine EBP and human ABPA. Thus, the **last study of the second part of this work aimed to investigate the presence of *A. fumigatus* (by qPCR in BALF) and *A. fumigatus*-specific antibodies (Ig E and G, by ELISA testing) in dogs with newly-diagnosed and steroid-naïve EBP** in comparison with dogs having chronic bronchitis and healthy dogs.

The main result of <u>Study 5</u> is the higher level of serum *A.fumigatus*-specific Ig G in dogs with EBP compared to dogs with chronic bronchitis or healthy dogs, while serum or BALF *A.fumigatus*-specific Ig E and qPCR on BALF were not different between the three groups of dogs.

Concerning qPCR on BALF, 2 dogs with EBP were positive at low DNA level and these dogs had clinical signs of moderate severity. Dogs having EBP with more severe clinical signs were all qPCR negative on BALF. Thus, presence of *A. fumigatus* wouldn't be associated with development or exacerbation of canine EBP. However, comparison between groups might be underpowered because of small-sized groups and additional larger studies may be interesting. Moreover, in humans, sputum, bronchial and tracheal samples are known to be more sensitive for PCR than BALF, probably because of dilution (Denning 2021). Nevertheless, in humans with ABPA, presence of *A. fumigatus* (culture or PCR) in airway sampling is not needed for diagnosis and evaluation of specific antibodies is required. In our recruited dogs having EBP, we failed to show higher levels of specific Ig E in serum or BALF compared to healthy dogs or dogs with chronic bronchitis. Thus, we conclude that canine EBP is a well-distinct entity from human ABPA. For ELISA assays of <u>Study 5</u>, we used a pure *A.fumigatus* culture with mechanical disintegration and filtration as it is performed in humans for serological testing. This technique provides somatic and extra-cellular antigens and may lack of sensitivity. Recent reviews suggest higher performance of serological assays by using recombinant antigens (Page et al. 2015) but contradictive results were observed regarding the best recombinant antigen for testing (Asp f1, 2, 3, 4, 6) (Page et al. 2015). Recombinant antigens Asp f1 and f2 may have the most immunogenic potential (Carsin et al. 2017, Alghamdi et al. 2019). Repeating serological assays for Ig E in dogs with EBP should be considered with coating ELISA plates with a solution of recombinant antigens.

Nevertheless, dogs with EBP had higher level of serum *A.fumigatus*-specific Ig G at several dilution EBP compared to dogs with chronic bronchitis or healthy dogs. In humans, elevated *A.fumigatus*-specific Ig G are central to diagnosis of chronic pulmonary aspergillosis which is characterized by a progressive lung destruction by fibrosis and cavitations infected by *A fumigatus* and five-year mortality is particularly high (Page et al. 2015). Such this human entity does not share any clinical or imaging findings with canine EBP. In humans, raised *A. fumigatus*-specific Ig G levels are also observed in *Aspergillus* bronchitis; however, in this disease, fungal culture or PCR on BALF or

sputum has to be positive (Page et al. 2015). Again, *Aspergillus* bronchitis does not seem to completely resemble canine EBP. Lastly, other lung diseases associated with elevated *A.fumigatus*-specific Ig G are described in humans and included, among others, extrinsic allergic alveolitis (Denning 2021). However, this disease and other hypersensitivity pneumonitis are defined by dominant lymphocytosis on BALF which is not observed, by definition, in canine EBP.

Additionnally, in <u>Study 5</u>, for all tested dilutions, ELISA optical density values for House-Dust-Mites (HDM)-specific Ig G were significantly higher in both serum and BALF in dogs with EBP compared to samples from dogs with chronic bronchitis. In serum, optical density values were also higher in dogs with EBP than in healthy dogs. Cross-reactivity may be questioned; crude fungal extracts may contain cross-reactive allergenic proteins (pan-allergens), which are highly conserved molecules with similar functions present in widely different species that belong to the same protein family (Fukutomi et al. 2016). A previous study identified a specific antigen of HDM, Der f34, which belongs to a highly conserved family of imine deaminases, showing cross-reactivity with *A.fumigatus* (El Ramlawy et al. 2016); in this study, ELISA inhibition analysis showed that Der f34 shared its immunoglobulin-binding epitopes with those of *A.fumigatus*, indicating that Der F34 could be an important cross-reactive allergen. Recombinant antigens of *A.fumigatus* such as Asp f1 and f2 can be considered specific allergen components whereas Asp f3 and f6 are considered cross-reactive allergen components ; thus, as suggested above, repeating ELISA assays while coating ELISA plates with a solution of Asp f1 or f2 would be particularly relevant in our three grous of dogs.

On the other hand, higher serum levels of *A.fumigatus*- and HDM- Ig G in dogs with EBP might also mean that multiple Ig G-mediated hypersensitivity reactions with implication of several aeroallergens are involved in canine EBP. In humans, *Candida albicans* is reported to be the most prevalent causal fungi of allergic bronchopulmonary mycosis induced by fungi other than A*. fumigatus* (Ishiguro et al. 2014, Agarwal et al. 2015, Fukutomi et al. 2016, Bush 2020). Moreover, recombinant antigens of *Alternaria alternata* are also commercially-available and allergens rAlt a1 seems to be particularly specific (Gabriel et al*.* 2016). Thus, in order to exhaustively complete our observations, further ELISA assays should be conducted in our three groups of dogs by coating plates with recombinant antigens of *A.fumigatus* (Asp f1 or f2), recombinant antigens of *Alternaria alternata* (rAlt a1) and a pure solution of *Candida albicans*.

In people, some genetics risk factors are known for the development of ABPA such as specific HLA genotypes or polymorphism in toll-like receptors. Patients carrying HLA-DR2 or HLA-DR5 phenotype are more susceptible to ABPA. The hypothesized mechanism is an increased production of regulatory IL 10 from dendritic cells expressing HLA-DR2/DR5 with downstream inhibition of Th1 and

Th17 type responses, which are instrumental for an effective response to *A.fumigatus* (Carsin et al. 2017). Because such genetic susceptibilities have not been investigated so far, we cannot actually exclude that *Aspergillus* may be implicated in the development of EBP in some dogs. Additional studies assessing polymorphisms in DLA genotypes or toll-like receptors in dogs with EBP compared to dogs with chronic bronchitis would be warranted.

Lastly, while the bacterial microbiome of the upper and lower airways has been the focus of many recent studies (Whiteside et al. 2021, Chioma et al. 2021, Gokulan et al. 2022), the contribution of fungal microbiota to asthma is an emerging research interest (Zhang et al. 2017, Goldman et al. 2018, Sharma et al. 2019, Barcik et al. 2020, Bush 2020, Yang et al. 2022). Moreover, murine models have provided experimental evidence that fungal microbiota in peripheral organs, notably the gastrointestinal tract, influence pulmonary health (Zhang et al. 2017). Emerging evidence suggests that dysbiotic fungal microbiota interact to drive or exacerbate chronic airway inflammatory disease (Zhang et al. 2017, Sharma et al. 2019, Yang et al. 2022). Thus, evaluation of fungal microbiota should be further considered in dogs with EBP.

In conclusion of <u>Study 5</u>, dogs with EBP have higher levels of serum *A.fumigatus* Ig G than dogs with chronic bronchitis and healthy dogs while qPCR on BALF and levels of Ig E are not different between groups. Dogs with EBP have also higher levels of HDM-Ig G in both serum and BALF compared to dogs with chronic bronchitis. These results lead to conclude that canine EBP obviously differs from human ABPA and is a very distinct entity that does not exist in humans. Additional investigations are crucial to understand if seropositivities for *A.fumigatus* and HDM in dogs with EBP are reflecting a cross-reactivity or multiple Ig G-mediated hypersensitivity reactions with implication of several aeroallergens. For this purpose, use of recombinant fungal antigens would be relevant for further ELISA testing. Moreover, as genetic predispositions are known in patients sensitized to fungi, polymorphisms in DLA genotypes or toll-like receptors in dogs with EBP having high serum *Aspergillus*-specific Ig G would also be warranted.

The last purpose of the present work (<u>Study 6</u>) was to describe the long term follow up of dogs with idiopathic EBP and receiving IST as sole therapy.

Prompt and dramatic improvement of clinical signs is achieved with oral corticosteroid therapy. However, relapses frequently occur when discontinuation is attempted (Clercx et al. 2000, Rajamaki et al. 2002a, Casamian-Sorrosal et al. 2020). Dogs with idiopathic EBP are young-aged and may require long-term systemic steroid therapy for years but deleterious side effects can then impact quality of life and general health. In such cases, IST could be more beneficial.

<u>Study 6</u> was the first study assessing tolerance, efficacy and inhibition of PAA in dogs with EBP treated with IST as sole treatment and followed more than 6 months.

Inhaled medication using facemask is well tolerated by dogs and satisfactory compliance with long-term IST is confirmed.

Although all included dogs improved within 4 weeks after the start of IST, mild to moderate signs persisted or recurred within the 6 months, and some dogs required additional oral steroid therapy to improve. In comparison with previous descriptions of orally-treated dogs (Corcoran et al. 1991, Clercx et al. 2000), IST might be less promising in the long term for EBP dogs and unlike to oral treatment, none of the dogs treated with IST could be weaned off medication.

Since the time of publication of our study 6, a larger serie including 70 dogs treated with oral or dual therapy (oral combined to IST) was published and has corroborated our observations (Casamian-Sorrosal et al. 2020). In this cohort, only 3 dogs were treated with IST monotherapy and long-term follow up of these dogs was unfortunately not available. For the 67 remaining dogs, 25 were treated with a combination of oral corticosteroid therapy and IST. Only 6 out of them (24%) had their oral corticosteroid therapy discontinued and were successfully managed solely with IST. Authors compared remission rate between dogs treated with dual therapy and dogs treated only with oral treatment and difference was not different but no comparison was possible between dogs only treated with IST and the others. Moreover, among dogs treated with oral treatment, Casamian-Sorrosal et al. investigated by univariate analysis any potential factor that may impact remission rate such as presence of bronchiectasis, presence of blood eosinophilia, bronchoscopic score or eosinophilic grade on BALF and no difference was observed between any subgroup. We can therefore hypothesize that criteria reflecting clinical severity did not underestimate the clinical response to IST that we observed in <u>Study 6</u>.

Information about pharmacokinetics of inhaled steroids in dogs is unfortunately scarce. However, in healthy humans using pMDI, only 12% of inhaled radiolabelled fluticasone was shown to actually deposit in lungs and deposition was shown to be primarily in large central and intermediate airways (Leach et al. 2002). In dogs, scintigraphic evaluation of lung deposition of radiolabelled fluticasone delivered by pMDI was recently performed in ten healthy dogs. Lung deposition of fluticasone in this group seems to be very low (2%) and comparable to observations in children (Chow et al. 2017). In dogs with EBP, comparable pharmakocinetic study has not yet been done but lower lung deposition may be hypothesized as presence of mucus within airways promotes mucociliary clearance of lipophilic drugs, such as fluticasone. In patients with asthma, deposition of fluticasone in the lungs was therefore demonstrated to be even lower than in healthy humans (Daley-Yates et al. 2000)

The second aim of Study 6 was to assess the potential impact of prolonged administration of IST on PAA inhibition. In healthy dogs, asymptomatic inhibition of PAA has been shown to occur after 3 to 4 weeks treatment with inhaled fluticasone propionate (Cohn et al. 2008, Melamies et al. 2012). Depressions of PAA have been well described in human asthmatic patients treated with fluticasone and Addisonian's crisis are also well described in case of brutal discontinuation of daily high-dose of IST (Masoli et al. 2006, Iles et al. 2008, Wlodarczyk et al. 2008, Pandya et al. 2014). The type of inhaled molecule, dose and duration of drug administration, and expiratory volume influence the severity of the inhibition of PAA in people (Masoli et al. 2006, Wlodarczyk et al. 2008, Singh & Loke 2010, Pandya et al. 2014). Fluticasone has a higher distribution (78%) to the oropharynx in people (Leach et al. 2002). This substantial portion may be then swallowed, absorbed and further responsible for side effects, despite a moderate to poor improvement of the cough. Inhibition of the pituitary-adrenal-axis was also confirmed in two dogs treated with IST for 48 months in our Study 6 and one of them developed progressive clinical signs of iatrogenic hyperadrenocorticism, including weight gain, polyuria and polydipsia, diffuse symmetric alopecia, skin thinning, severe abdominal distension, hepatomegaly and panting.

Study 6 has some limitations. The study population was small- sized because of long period of follow up as inclusion criteria. The clinical severity also varied among included dogs at the time of diagnosis and selected dogs might have a more refractory disease state than dogs seen in general practice. Moreover, mild degree of depression of PAA might have been underestimated in our study; in human, lower dose of ACTH is used to detect iatrogenic hyperadrenocorticism with better sensitivity (Bernstein & Allen 2007).

In conclusion of the part 3 of this work, our observations highlight the limited clinical benefice of inhaled fluticasone at long term for dogs; it may not be considered as an effective alternative to oral steroids when their use is contraindicated. However, all inhaled steroids do not seem to be equal. Ciclesonide is another inhaled steroid which is readily available and recently investigated in humans. A review of the literature suggests that ciclesonide, as compared with fluticasone, achieves greater pulmonary deposition, deposits less biologically active drug in the systemic circulation, and has less potential for adrenal suppression (Kaliner 2006, Pandya et al. 2014). Moreover, a recent matched cohort analysis found that extrafine particles with ciclesonide had better impact on long term stabilization of severe asthma (Postma et al. 2017). Thus, it would be highly relevant to compare clinical benefit and biological impact on PAA of ciclesonide as single therapy in a group of dogs with EBP. Moreover, inhaled ciclosporine has been largely used in lung transplant patients and was more recently studied in COVID-19 infection (Ehtezazi 2021, Neurohr et al. 2022). Assessment of its clinical efficacy in dogs with EBP would be relevant. Unfortunately, such pressurised inhalers of ciclosporine are not currently available in Europe.

General conclusion

Firstly, definitive diagnosis of canine idiopathic EBP is straightforward and affordable. In European countries, validated diagnosis requires ruling out pulmonary angiostrongylosis as other parasites such as *Crenosoma vulpis*. The both parasitic infestations can perfectly mimic canine EBP. Faecal analysis is reported to be highly sensitive for *Crenosoma vulpis*. On the contrary, our results confirmed that qPCR on BALF is undeniably the only diagnostic tool which benefits from the best sensitivity to exclude pulmonary angiostrongylosis.

Secondly, our investigations failed to identify any bacteria specifically involved in the development or exacerbation of canine EBP. Nevertheless, our observation concerning *Bordetella bronchiseptica* invite to systematically search for this bacterium in dogs newly-diagnosed as having EBP, particularly in case of severe clinical presentation or acute exacerbation in previously diagnosed cases. Additional studies focused on other marginal mycoplasma species, *Mycoplasma*-specific antibodies, *Chlamydia* spp. and airway microbiome in dogs with EBP might be interesting perspectives.

Thirdly, our serological investigations of *A.fumigatus* suggest that dogs with EBP compared to dogs with chronic bronchitis may have multiple Ig G-mediated hypersensitivity reactions but cross-reactivity could not be ruled out and additional serological studies including other fungal recombinant antigens would be decisive.

Lastly, therapeutic management of dogs having EBP may be complex. Oral steroid treatment is particularly efficacious but long-term administration is associated to side effects; however, monotherapy by IST as currently used with fluticasone is suboptimal and may also have long-term impact on PAA.

Therefore, there is still a relevant challenge to search if particular allergens are involved in the development of canine idiopathic EBP in order to further assess potential benefit of desensitization protocols to overcome the need for long-term management with steroids.

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