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Clinicopathological findings, neutrophil extracellular traps, and lung
microbiota in dogs with bronchiectasis and/or dynamic bronchomalacia

Éléments clinicopathologiques, « neutrophil extracellular traps », et
microbiote pulmonaire chez les chiens atteints de bronchectasie et/ou de
bronchomalacie dynamique

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Abbreviations

ACT	Adenylyl cyclase toxin
ANC	Absolute neutrophil count
BALF	Bronchoalveolar lavage
BAR	Bronchial to artery ratio
BBP	Bacterial bronchopneumonia
BEBM	Combination of bronchiectasis and bronchomalacia
BE	Bronchiectasis
BM	Dynamic bronchomalacia
BTS	British Thoracic Society
cAMP	Cyclic adenosine monophosphate
CAP	Community-acquired pneumonia
CBC	Complete blood count
CBD	Chronic bronchial disease
CF	Cystic fibrosis
cfDNA	Cell-free DNA
CFU	Colony-forming unit
CIPF	Canine idiopathic pulmonary fibrosis
Cit	Citrullinated
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CT	Computed tomography
CT-BE	Computed tomography-based diagnosis of bronchiectasis
CT-BEBM	Computed tomography-based diagnosis of the combination of bronchiectasis and bronchomalacia
CT-BM	Computed tomography-based diagnosis of bronchomalacia
DAPI	4',6-diamidino-2-phenylindole
DCC	Differentiated cell count
DNA	Deoxyribonucleic acid
E-BE	Endoscopy-based diagnosis of bronchiectasis

E-BEBM	Endoscopy-based diagnosis of the combination of bronchiectasis and bronchomalacia
E-BM	Computed tomography-based diagnosis of bronchomalacia
EBP	Eosinophilic bronchopneumopathy
ELISA	Enzyme-linked immunosorbent assay
ERS	European Respiratory Society
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GERD	Gastroesophageal reflux disease
GMP	Granulocyte Monocyte Progenitor
ICAM	Intercellular adhesion molecule
IF	Immunofluorescence
IL	Interleukin
IMHA	Immune-mediated haemolytic anaemia
IPF	Idiopathic pulmonary fibrosis
JAM	Junctional adhesion molecules
LFA-1	Lymphocyte function-associated antigen-1
LM	Lung microbiota
mPA	Mean pulmonary artery
MPO	Myeloperoxidase
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NET	Neutrophil extracellular traps
NT-proBNP	N-terminal pro-brain natriuretic peptide
OTU	Operational taxonomic unit
PAD4	Peptidylarginine deiminase 4
PAMP	Pathogen-associated molecular patterns
PCD	Primary ciliary dyskinesia
PCR	Polymerase chain reaction
PH	Pulmonary hypertension
PSGL-1	P-selectin glycoprotein ligand-1

qPCR	Quantitative polymerase chain reaction
R-BE	Radiography-based diagnosis of bronchiectasis
R-BEBM	Radiography-based diagnosis of the combination of bronchiectasis and bronchomalacia
R-BM	Radiography-based diagnosis of bronchomalacia
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
ROS	Reactive oxygen species
SD	Standard deviation
SLE	Systemic lupus erythematosus
TCC	Total cell count
TNF	Tumour necrosis factor
VAP	Ventilator-associated pneumonia
WHWT	West Highland White Terrier
XR	Radiography

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Résumé - Abstract

Résumé

La toux chronique constitue l'un des motifs de consultation les plus fréquents en médecine interne des petits animaux et peut résulter d'un large éventail d'affections des voies respiratoires et du parenchyme pulmonaire. Parmi celles-ci, la bronchectasie (BE) et la bronchomalacie (BM) représentent deux conséquences irréversibles de lésions bronchiques chroniques, souvent observées chez le chien. Il n'est pas rare de constater la coexistence de BE et de BM chez un même patient lors d'une bronchoscopie. Bien que ces affections aient été décrites séparément, leur physiopathologie sous-jacente reste encore mal comprise. Cette thèse vise à combler partiellement cette lacune en caractérisant les aspects clinicopathologiques de ces maladies, en étudiant le rôle du microbiote pulmonaire (LM) et la présence de pièges extracellulaires de neutrophiles (NETs), et en proposant des stratégies diagnostiques et thérapeutiques.

La première étude a comparé rétrospectivement des chiens diagnostiqués avec BE, BM ou une combinaison des deux à l'aide de critères endoscopiques et d'imagerie. Malgré leurs définitions morphologiques distinctes, ces affections présentaient des profils cliniques et cytologiques similaires, caractérisés par une toux chronique, une inflammation bronchique neutrophilique et une colonisation bactérienne peu fréquente. Une concordance très limitée a été mise en évidence entre l'identification des lésions de BE et BM par la radiographie, la bronchoscopie et la tomодensitométrie, soulignant la difficulté d'identifier précisément les altérations dynamiques et structurelles des voies respiratoires. Ces résultats suggèrent que la BE et/ou la BM ne sont pas des maladies distinctes, mais plutôt des variantes phénotypiques d'un même processus inflammatoire chronique.

Sur base des informations récoltées lors de la première étude et face à la nécessité d'une approche thérapeutique standardisée, la deuxième étude a proposé un algorithme thérapeutique pragmatique distinguant les phénotypes infectieux et non infectieux à partir de la cytologie du lavage broncho-alvéolaire. La doxycycline est recommandée comme traitement de première intention lorsqu'une infection est confirmée cytologiquement, tandis que les corticostéroïdes inhalés sont proposés comme traitement de fond dans les formes inflammatoires non infectieuses. Cette approche favorise une utilisation raisonnée des antibiotiques, en accord avec les principes actuels de la médecine vétérinaire fondée sur la preuve, tout en limitant les perturbations inutiles du microbiote respiratoire.

Afin de mieux comprendre la pathophysiologie de la BE et/ou de la BM, la troisième étude a examiné le rôle des NETs en tant que mécanisme immunopathologique potentiel chez les chiens atteints de

maladies bronchiques chroniques, investiguant un lien éventuel entre l'inflammation, l'infection et le remodelage des voies respiratoires. Des taux élevés d'ADN libre circulant ont été détectés dans le liquide de lavage bronchoalvéolaire chez les chiens atteints de BE, de BM et de bronchopneumonie bactérienne en comparaison avec des chiens sains. Les complexes myéloperoxydase-ADN étaient également plus élevés chez les chiens présentant des maladies bronchiques chroniques par rapport aux chiens en bonne santé. Les chiens atteints de BE montraient l'augmentation la plus marquée par rapport aux chiens sains et aux chiens atteints de BM. Leur présence dans une maladie chronique non infectieuse indique que des stimuli inflammatoires stériles peuvent également induire la libération de NETs. Compte tenu de leurs propriétés pro-inflammatoires et délétères pour les tissus, les NETs pourraient être impliqués dans la pathogénie de la bronchectasie, représenter à la fois un biomarqueur de l'activité de la maladie et éventuellement une nouvelle cible thérapeutique.

Enfin, la quatrième étude a exploré le microbiote pulmonaire chez des chiens atteints de maladie bronchique chronique, comparés à des chiens sains. Le séquençage du gène 16S rRNA a révélé que les poumons sains abritent une communauté microbienne de faible biomasse mais diversifiée, dominée par *Pseudomonadota*, *Bacillota*, *Actinomycetota* et *Bacteroidota*. Chez les chiens malades, cette diversité était significativement réduite, avec une surreprésentation relative des Proteobacteria. Cette dysbiose reflète étroitement les modifications microbiennes rapportées chez l'humain atteint de BE. Ces résultats suggèrent une association entre l'inflammation bronchique chronique et des altérations du LM normal, indépendamment d'une infection bactérienne manifeste, bien qu'il demeure incertain si ces modifications microbiennes représentent une cause ou une conséquence de la maladie.

En conclusion, les résultats de cette thèse n'ont pas permis d'identifier de différences clinico-pathologiques, microbiologiques ou liées aux NET suffisamment nettes pour soutenir que le BE et la BM constituent des entités nosologiques distinctes nécessitant des approches diagnostiques ou thérapeutiques différentes. Ils suggèrent plutôt que ces deux affections pourraient être étroitement liées. Reste à déterminer si elles représentent différents stades d'un même processus pathologique ou des maladies distinctes partageant des caractéristiques communes.

Summary

Chronic cough is one of the most frequent reasons for referral in small animal internal medicine and may arise from a wide spectrum of airway and pulmonary disorders. Among these, bronchiectasis (BE) and bronchomalacia (BM) represent two irreversible outcomes of chronic airway injury, frequently observed in dogs. Moreover, a combination of both BE and BM (BEBM) can be observed in the same patient during bronchoscopy. While BE and/or BM have been well characterized individually, their underlying pathophysiology remains poorly understood. This thesis aims to address this gap by characterizing the clinicopathological features of these airway remodelling findings, investigating the role of the lung microbiota (LM) and the presence of neutrophil extracellular traps (NETs), and proposing evidence-based strategies for their diagnosis and management.

The first study retrospectively compared dogs diagnosed with BE, BM, or a combination of both using endoscopic and imaging criteria. Despite distinct morphological definitions, these airway abnormalities exhibited overlapping clinical and cytological profiles, characterized by chronic cough, neutrophilic airway inflammation, and infrequent bacterial colonization. A very limited agreement was demonstrated between the identification of BE and BM lesions by radiography, bronchoscopy, and computed tomography, highlighting the difficulty of accurately identifying dynamic and structural airway alterations. These results suggest that BE and/or BM are not separate conditions but rather phenotypic variations of a shared chronic inflammatory process.

Based on the information gathered during the first study and recognizing the need for standardized clinical management, the second study proposed a treatment algorithm distinguishing infectious from non-infectious phenotypes based on bronchoalveolar cytology. Doxycycline was recommended as first-line therapy when infection was cytologically confirmed, while inhaled corticosteroids were proposed as the mainstay of treatment in non-infectious inflammatory disease. This approach promotes rational antimicrobial use, aligning veterinary practice with current principles of antimicrobial stewardship and minimizing unnecessary disruption of the respiratory microbiota.

In order to better understand the pathophysiology of BE and/or BM, the third study investigated the role of NETs as a potential immunopathological mechanism in dogs with chronic bronchial diseases linking inflammation, infection, and airway remodelling. Elevated levels of cell-free DNA were

1 detected in bronchoalveolar lavage fluid from dogs with BE, BM, and bacterial bronchopneumonia
2 in comparison with healthy dogs. Elevated myeloperoxidase-DNA complexes were also higher in
3 dogs with chronic bronchial diseases compared to healthy dogs, with BE dogs showing the greatest
4 increase compared with healthy and BM dogs. Their presence in chronic non-infectious disease
5 indicates that sterile inflammatory stimuli can also drive NET release. Given their pro-inflammatory
6 and tissue-damaging properties, NETs could be involved in the pathogenesis of BE and may
7 represent both a biomarker of disease activity and a potential novel therapeutic target.

8
9 Lastly, the fourth study explored the role of LM in dogs with chronic bronchial disease compared to
10 healthy controls. 16S rRNA sequencing revealed that healthy lungs harbour a low-biomass but a
11 diverse microbial community dominated by *Pseudomonadota*, *Bacillota*, *Actinomycetota*, and
12 *Bacteroidota*. In diseased dogs, diversity was significantly reduced, with a relative
13 overrepresentation of Proteobacteria. This dysbiosis closely parallels microbial shifts reported in
14 human BE. These findings suggest an association between chronic bronchial inflammation and
15 alterations of the normal LM, independent of overt bacterial infection, although it remains unclear
16 whether these microbial changes represent a cause or a consequence of the disease.

17
18 In conclusion, the findings of this thesis did not identify clear clinicopathological, microbiological,
19 or NET-related differences that would support BE and BM as distinct disease entities requiring
20 different diagnostic or therapeutic approaches. Instead, they suggest that these conditions may be
21 interconnected. Whether they represent different stages of a common disease process or distinct
22 disorders with overlapping features remains to be determined.

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General preamble

4

1 The present work focuses on chronic bronchial diseases in dogs, particularly bronchiectasis (BE) and
2 bronchomalacia (BM), two irreversible outcomes of chronic airway injury. These airway remodelling
3 findings are increasingly recognized in canine medicine as important causes of chronic cough in
4 middle-aged to older dogs. BE is defined by progressive and irreversible dilation of the bronchial
5 wall, commonly associated with chronic inflammation and infection, while BM refers to a dynamic
6 collapse of the segmental and/or subsegmental bronchi. In some cases, both processes coexist
7 within the same patient, reflecting a complex and chronic remodelling of the lower airways.

8 Although BE and BM have been well described individually in dogs, their underlying pathophysiology
9 and clinical interrelationship remain poorly understood. In human medicine, BE is considered part
10 of a “vicious cycle” in which chronic neutrophilic inflammation, microbial imbalance, and airway
11 wall damage perpetuate each other. Similar mechanisms are suspected in dogs, but evidence has
12 so far been limited. This creates a challenge in the therapeutic standardization of these airway
13 remodelling findings. In current veterinary practice, therapeutic decisions often depend on
14 empirical use of antimicrobials and corticosteroids, with limited guidance from evidence-based
15 protocols. This variability highlights the need for rational algorithms integrating cytological,
16 microbiological, and clinical data to guide treatment decisions while promoting antimicrobial
17 stewardship. Furthermore, clinical observations suggest that BE and BM frequently coexist in canine
18 patients, yet the clinical and pathophysiological implications of this association remain largely
19 unexplored. Whether dogs presenting with combined BE and BM represent a distinct clinical
20 subgroup with specific inflammatory or microbiological characteristics is currently unknown. In
21 addition, the contribution of bacterial infection and host inflammatory responses to the
22 development and progression of these airway disorders remains poorly characterized. Emerging
23 areas of research in human respiratory medicine, including the study of the lung microbiota (LM)
24 and neutrophil extracellular traps (NETs), may provide valuable insights into host-pathogen
25 interactions and inflammatory mechanisms in chronic airway disease, but these aspects have not
26 yet been systematically investigated in dogs.

27 The present thesis addresses these knowledge gaps through 4 studies. The first study provides a
28 clinicopathological characterization of dogs with BE, BM, or both, emphasizing their overlapping
29 features and shared inflammatory profile. The second study proposes a therapeutic algorithm
30 distinguishing infectious from non-infectious phenotypes based on bronchoalveolar cytology. The
31 third study examines the presence of NETs in bronchoalveolar lavage fluid and their possible role in

- 1 airway injury and remodelling. The fourth investigates the LM in affected dogs compared with
- 2 healthy controls, using 16S rRNA sequencing to identify disease-associated dysbiosis.

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Introduction

1. Chronic cough

1.1. General

Cough is a vital physiological reflex that serves as a primary defence mechanism for maintaining respiratory health in both humans and small animals (Chang, 1992). It clears the airways of foreign material, pathogens, tissue debris, and excessive secretions, enhances mucociliary clearance, and helps prevent aspiration of noxious substances into the lower respiratory tract through the rapid expulsion of air (Hsieh and Beets, 2020).

In veterinary practice, cough is recognized as an important clinical sign that may indicate an underlying disease process. While it is essential in preserving airway hygiene, excessive or inappropriate coughing, especially when chronic and non-productive, can become detrimental, leading to mucosal damage, respiratory compromise, discomfort, and a reduced quality of life (Padrid, 1992).

Cough may present as a primary symptom of many respiratory conditions, yet it can also be associated with systemic or extrapulmonary disorders not directly related to the airways; conditions such as gastroesophageal reflux disease (GERD) for example, can trigger cough as a secondary manifestation (Grobman and Reiner, 2023).

Based on its duration, cough can be described as acute (less than 2 months) or chronic (more than 2 months) (Hsieh and Beets, 2020).

1.2. Pathophysiology

The cough reflex is characterized by a sequence of events, beginning with a deep inhalation followed by a rapid and forceful exhalation against a closed glottis. This is followed by the sudden opening of the glottis, closure of the nasopharynx, and a powerful expiration through the mouth, producing the characteristic cough sound through vibration of the vocal cords (Widdicombe and Fontana, 2006).

The cough reflex is mediated by a three-part reflex arc consisting of afferent, central, and efferent pathways (Polverino *et al.*, 2012). Mechanical, chemical, or inflammatory stimuli activate sensory receptors located primarily in the larynx, trachea, and bronchi, whereas stimulation of the bronchioles or alveoli does not elicit coughing (Fuller and Jackson, 1990). These receptors include rapidly adapting receptors, slowly adapting receptors, and C-fibers, whose distribution determines their sensitivity to different stimuli: upper airway receptors respond predominantly to mechanical irritation, whereas lower airway receptors are more sensitive to chemical and inflammatory mediators (Widdicombe, 1995; Lee and Undem, 1995). Afferent signals are transmitted via the vagal nerve to the cough centre in the pons and upper brainstem, where neurotransmitters such as

glutamate, substance P, and neurokinins initiate the cough response once the activation threshold is reached. The efferent pathway then transmits signals from the brainstem to the muscles involved in coughing, including the respiratory muscles and the larynx, resulting in the characteristic cough (Canning, 2009).

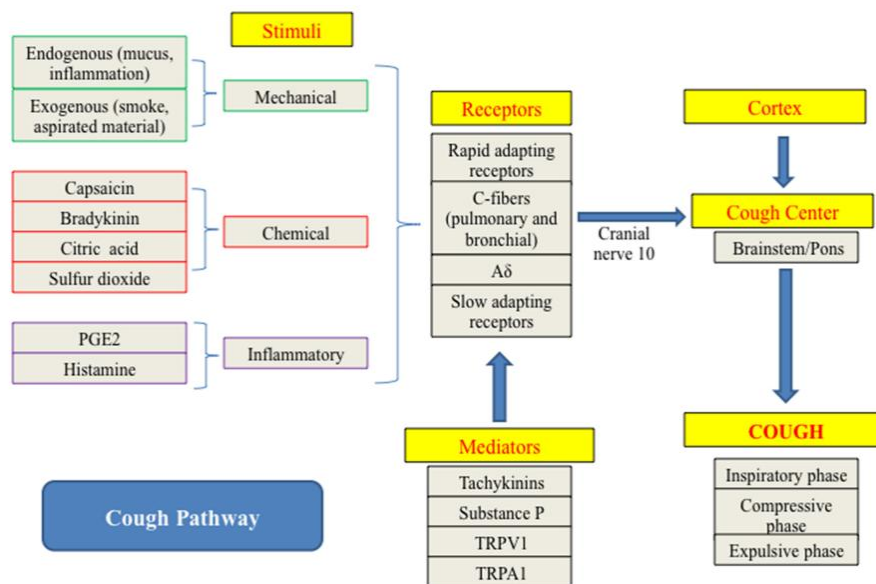


Figure 1: Mechanism of the cough reflex activation and signal transmission.

Abbreviations: PGE2: prostaglandin E2; TRPV1: transient receptor potential vanilloid 1 ; TRPA1 : transient receptor potential ankyrin 1. Figure adapted from Hsieh and Beets, 2020.

1.3. Aetiology

Cough can have many underlying causes. Common causes in dogs include inflammatory (laryngitis, tracheitis, bronchitis, bronchopneumonia,...), allergic (eosinophilic bronchopneumopathy (EBP)), degenerative (bronchiectasis, bronchomalacia, tracheal collapse, laryngeal paralysis), cardiovascular ((non-) cardiogenic oedema, cardiomegaly, pleural effusion, pericardial effusion, pulmonary thromboembolism), neoplastic, canine idiopathic pulmonary fibrosis (CIPF), gastrointestinal, infectious (parasitic, bacterial, fungal), and traumatic conditions (Rozanski, 2020). Within the broad differential diagnosis of cough, chronic respiratory diseases such as chronic bronchitis, bacterial bronchitis, EBP and CIPF represent common causes of persistent cough in dogs.

1.3.1. Chronic bronchitis

Chronic bronchitis is a common chronic respiratory disease in dogs and is defined as persistent bronchial inflammation characterized by a cough lasting at least two months in the absence of an identifiable underlying cause (Wheeldon *et al.*, 1974). This leads to inflammatory changes in the lower airways, typically including neutrophilic inflammation and increased mucus production (Wheeldon *et al.*, 1974). Ongoing airway inflammation and injury promote structural remodelling such as goblet cell hyperplasia, mucus hypersecretion, airway smooth muscle hypertrophy, impaired mucociliary clearance, lamina propria fibrosis, and small airway obstruction (Wheeldon *et al.*, 1974). Although the exact cause of chronic bronchitis is usually unknown, chronic or acute respiratory infections, allergies, and exposure to inhaled irritants such as tobacco smoke have been proposed as potential contributing factors, and affected dogs may present with concurrent respiratory abnormalities (McKiernan, 2000).

1.3.2. Bacterial bronchitis

Bronchial inflammation can be complicated by secondary bacterial infections. In dogs, *Bordetella bronchiseptica* is a well-recognized primary respiratory pathogen, while *Mycoplasma* spp., particularly *Mycoplasma cynos*, have also been associated with respiratory disease and may contribute to the development of chronic bronchitis or bronchopneumonia, although their pathogenic role remains less clearly defined (Lappin *et al.*, 2017). *Bordetella bronchiseptica* is a Gram-negative aerobic coccobacillus that can act either as a primary pathogen or as part of the canine infectious respiratory disease complex (Day *et al.*, 2020). After colonizing the respiratory tract, it expresses adhesins (e.g., filamentous haemagglutinin, pertactin, fimbriae) that facilitate attachment to respiratory epithelium and produces toxins that damage ciliated cells (Bock and Gross, 2001). This leads to ciliostasis and impaired mucociliary clearance, resulting in localized inflammation of the ciliated mucosa characterized by polymorphonuclear cell infiltration (Woolfrey and Moody, 1991). Although morbidity is high, mortality is typically low.

Unlike most bacteria, *Mycoplasma* spp. lack a cell wall. Several species, including *M. cynos*, have been implicated in canine respiratory disease; however, many *Mycoplasma* spp. are also part of the normal upper respiratory microbiota. Consequently, their detection alone does not establish causality, and interpretation should be based on the clinical context and supporting diagnostic findings (Chalker *et al.*, 2004; Day *et al.*, 2020).

1.3.3. Eosinophilic bronchopneumopathy

EBP is a chronic inflammatory respiratory disease in dogs characterized by marked eosinophilic infiltration of the bronchial mucosa and lung parenchyma (Clercx *et al.*, 2000). The underlying cause remains unclear, although a hypersensitivity reaction to inhaled allergens is suspected in many cases (Wessels *et al.*, 1973). Affected dogs typically present with persistent cough, dyspnoea, exercise intolerance, and sometimes nasal discharge, reflecting significant airway inflammation and mucus accumulation. Diagnosis relies on compatible clinical presentation together with evidence of eosinophilic inflammation in the lower airways, usually demonstrated on bronchoalveolar lavage cytology, and exclusion of other causes of pulmonary eosinophilia such as parasitic or infectious diseases (Clercx *et al.*, 2000).

1.3.4. Canine idiopathic pulmonary fibrosis

CIPF is a chronic, progressive interstitial lung disease primarily affecting West Highland White Terriers (WHWT) (Clercx *et al.*, 2018). It is characterized by progressive fibrosis of the pulmonary interstitium, leading to impaired gas exchange and gradually worsening respiratory function (Clercx *et al.*, 2018). Affected dogs are typically middle-aged to older and present with slowly progressive clinical signs such as exercise intolerance, chronic cough, tachypnoea, and restrictive dyspnoea (Roels *et al.*, 2019). Inspiratory crackles on lung auscultation are a characteristic clinical finding (Clercx *et al.*, 2018). The exact aetiology of CIPF remains unknown, although a strong breed predisposition suggests a genetic component, possibly interacting with environmental factors (Heikkila-Laurila and Rajamaki, 2014). Diagnosis is based on compatible clinical findings, thoracic imaging, particularly high-resolution computed tomography, and exclusion of other respiratory or cardiac diseases, while definitive confirmation requires histopathology (Clercx *et al.*, 2018). No curative treatment is currently available, and management is mainly supportive, focusing on controlling clinical signs, treating complications such as pulmonary hypertension or infections, and maintaining quality of life (Clercx *et al.*, 2018).

1.4. Clinical approach of chronic cough

The approach to a coughing dog should begin with a thorough history, including contact with other animals, use of medications, vaccination and deworming status and evolution of the clinical signs (Rozanski, 2020). Age and breed can offer diagnostic clues; chronic bronchitis and neoplasia for instance are more common in older dogs whilst infectious causes are more commonly seen in younger animals (Hsieh and Beets, 2020). Details about the nature of the cough, whether dry or productive, paroxysmal, intermittent or persistent, and its relation to activity, eating and vocalization can help in narrowing down possible causes (Rozanski, 2020).

During physical examination, respiratory pattern analysis and auscultation of the heart and the lungs is crucial. Wheezes, lung crackles or harsh lung sounds may suggest airway disease at different levels. For example, inspiratory crackles may indicate interstitial lung disease, while rhonchi are more consistent with bronchial involvement (Hsieh and Beets, 2020). Murmurs, arrhythmias, and respiratory sinus arrhythmia can provide further clues, though the presence of a murmur does not rule out pulmonary disease (Abbot, 1998). Jugular pulses, hepatomegaly, or ascites may suggest right-sided heart failure, a potential cause of cough via pulmonary congestion or tracheobronchial compression (Abbott, 1998). Additional findings like dullness on chest percussion may point to pleural effusion or masses causing mechanical compression and irritation (Martin and Pereira, 2013).

Overall, localizing the origin of the cough, whether upper or lower airway, pulmonary parenchyma, or cardiovascular system, is a critical first step in guiding diagnostic imaging and further testing.

1.5. Diagnosis

Following a thorough physical examination, a targeted diagnostic approach should be taken based on the suspected underlying cause of the cough. Importantly, the least invasive tests should be performed first, and patients experiencing respiratory distress must be stabilized before proceeding with further diagnostics (Rozanski, 2020).

Initial screening includes baseline laboratory tests, such as a complete blood count (CBC) and serum biochemistry (Rozanski, 2020). These help assessing overall health and detect systemic disease. Specific findings such as peripheral eosinophilia may point towards parasitic infections or allergic airway disease (Clercx *et al.*, 2000). Heartworm testing and faecal analysis (Baermann test) are also recommended, especially in endemic areas (Rozanski, 2020).

Thoracic imaging, including radiography (XR) or advanced imaging such as computed tomography (CT), is essential for evaluating the lungs, airways, and heart (Prather *et al.*, 2005). In cases where

cardiac disease is suspected, measuring N-terminal pro-brain natriuretic peptide (NT-proBNP) can be helpful. Elevated levels may indicate left atrial enlargement, congestive heart failure, or pulmonary hypertension (Oyama *et al.*, 2013).

Echocardiography might also be of interest to provide information about disease processes affecting the pericardial and pleural spaces (Abbott, 1998). On the other hand, echocardiography might also be useful to detect signs of pulmonary hypertension (PH) (Reinero *et al.*, 2020). Pulmonary hypertension is defined as an abnormal increase in pressure within the pulmonary vasculature and represents an important complication of chronic pulmonary diseases in human and veterinary medicine (Reinero *et al.*, 2020; Olsson *et al.*, 2023). The gold standard for diagnosis is direct measurement of pulmonary arterial pressure by right heart catheterization, although echocardiography is widely used in clinical practice as a non-invasive screening tool to identify echocardiographic signs suggestive of PH (Reinero *et al.*, 2020). In humans and dogs, PH commonly develops as a complication of chronic parenchymal lung diseases (Jaffey *et al.*, 2019; Reinero *et al.*, 2020; Olsson *et al.*, 2023). Chronic airway and parenchymal lung diseases may lead to persistent hypoxemia, pulmonary vasoconstriction, vascular remodelling, and eventual increases in pulmonary vascular resistance, ultimately resulting in PH (Reinero *et al.*, 2020). In canine medicine, PH has been described in association with several chronic respiratory disorders including chronic bronchitis, bronchiectasis, interstitial lung disease, and CIPF (Jaffey *et al.*, 2019; Roels *et al.*, 2021). If further investigation for chronic cough is needed, more advanced diagnostics such as, bronchoscopy, and bronchoalveolar lavage (BAL) may be performed (Hsieh and Beets, 2020). These allow direct visualization and assessment of the airways and help identify infectious or inflammatory processes.

Bronchoalveolar lavage is a diagnostic technique that allows sampling of the lower airways for cytologic evaluation, bacterial culture, and quantitative polymerase chain reaction (qPCR) testing (McCullough and Brinson, 1999). Interpretation of airway samples must consider the sampling technique and the anatomical site sampled, as these factors can influence cytologic composition and diagnostic interpretation (McCullough and Brinson, 1999). Cytologic analysis of BAL includes measurement of the total cell count (TCC) and differential cell count (DCC) (McCullough and Brinson, 1999). A normal BAL TCC is ≤ 600 cells/ μL (Canonne *et al.*, 2020). In healthy dogs, BAL fluid is composed predominantly of alveolar macrophages ($>80\%$), with only small numbers of lymphocytes ($<5\%$), neutrophils ($<12\%$), eosinophils ($<6\%$), and occasional epithelial cells ($>1\%$) (McCullough and Brinson, 1999). Acute neutrophilic inflammation is characterized by a predominance of neutrophils, which may be degenerative or non-degenerative, and can be further classified as septic or non-septic depending on the presence of intracellular bacteria (McCullough and Brinson, 1999). Chronic

inflammation typically shows increased activated or foamy macrophages with fewer neutrophils and may include reactive lymphocytes and plasma cells (McCullough and Brinson, 1999). Eosinophilic inflammation is identified by increased eosinophils and may be associated with mast cells; it is commonly linked to parasitic infections, allergic airway disease, EBP, or occasionally bacterial, fungal, or neoplastic conditions (McCullough and Brinson, 1999).

Additional diagnostic procedures include bronchial brushing, which samples ciliated bronchial epithelial cells and may increase the likelihood of detecting pathogens such as *Bordetella bronchiseptica*, as higher numbers of ciliated epithelial cells are obtained compared with BAL (Canonne *et al.*, 2016).

Microbiological interpretation requires integration of cytology and culture results, as low numbers of bacteria can be present in the lower airways without indicating true infection. A bacterial culture is typically considered clinically significant when $>1.7 \times 10^3$ colony-forming units (CFU)/mL are detected in BAL fluid (McCullough and Brinson, 1999; Peeters *et al.*, 2000). Molecular diagnostics such as qPCR can further assist in detecting respiratory pathogens, including *Mycoplasma spp.*, *Bordetella bronchiseptica*, and parasites such as *Angiostrongylus vasorum* or *Crenosoma vulpis*, particularly when organisms are difficult to culture using conventional techniques (McCullough and Brinson, 1999).

2. Bronchiectasis

2.1. General

Bronchiectasis (BE) is a common chronic lung disease in people and dogs, characterized by a progressive and irreversible dilation of the bronchial wall, commonly associated with chronic inflammation and infection (Hawkins *et al.*, 2003; Boyton and Altmann, 2016). The extent of BE can range from focal disease, limited to one segment or one lobe, to diffuse involving multiple lobes in both lungs (Cannon *et al.*, 2013; Flume *et al.*, 2018). In human medicine, BE is associated with significant morbidity, reduced quality of life, a high treatment burden and increased mortality rates (Polverino *et al.*, 2017).

2.2. Pathogenesis

Although the precise pathogenesis of BE remains unclear in both canine and human medicine, it is generally understood to involve a vicious cycle of impaired mucociliary clearance, persistent bacterial colonization, and chronic inflammation, leading to progressive tissue destruction (Figure 2) (Marolf *et al.*, 2007; Boyton and Altmann, 2016). Prolonged inflammation and/or infection overwhelms the pulmonary clearance system, leading to the accumulation of cellular debris, inflammatory cells and mucus in the airways. This process causes damage to the ciliary respiratory epithelium and submucosa, further impairing mucociliary clearance (Marolf and Blaik, 2006). The resulting mucus stagnation increases endoluminal pressure, creating an environment conducive to infection (Marolf and Blaik, 2006; Aller *et al.*, 2018; Flume *et al.*, 2018). Once infection establishes, bacteria and other pathogens persist in the bronchi for a prolonged period, triggering chronic inflammation of the bronchial walls (Marolf and Blaik, 2006). This self-perpetuating cycle of inflammation, infection, and clearance dysfunction leads to progressive bronchial and pulmonary damage, increasing susceptibility to further infections (Marolf and Blaik, 2006; Chandrasekaran *et al.*, 2018). Bronchial dilation occurs due to the destruction of elastic and muscular layers of the bronchial wall, while mucosal hypertrophy and hyperplasia contribute to bronchial wall thickening (Marolf and Blaik, 2006).

Not all individuals with chronic inflammation or infection develop BE, suggesting that preexisting abnormalities in immune function, inflammatory responses, or pulmonary clearance mechanisms contribute to its development (Marolf and Blaik, 2006; Boyton & Altmann, 2016).

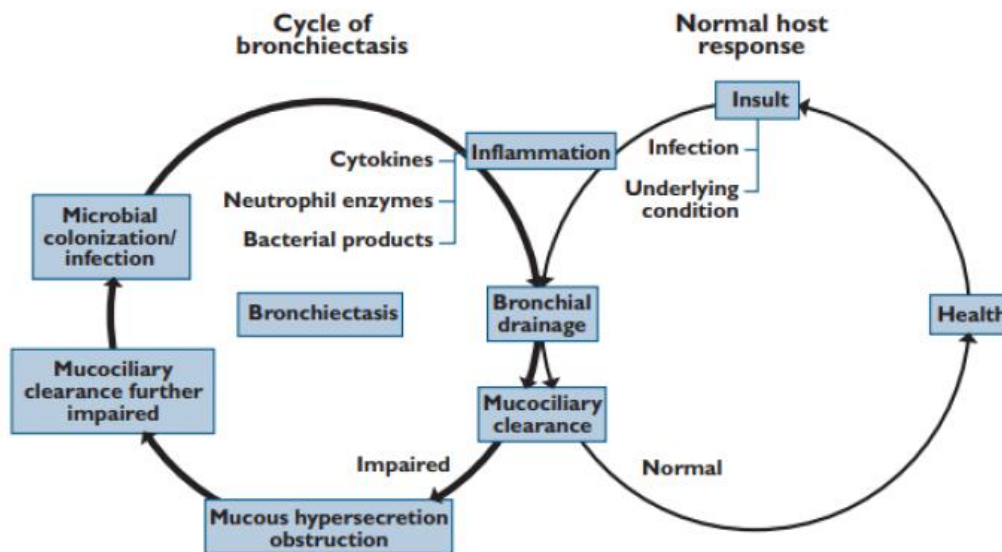


Figure 2: Pathogenesis of bronchiectasis versus a normal host response. Figure adapted from Marolf and Blaik, 2006.

2.3. Aetiology

BE can arise from multiple aetiologies, each contributing to different clinical symptoms and outcomes (Chandrasekaran *et al.*, 2018; Flume *et al.*, 2018; Bush and Floto, 2020). Most aetiologies share common pathophysiological features that result in airway remodelling and dilation (Flume *et al.*, 2018). In humans, BE may develop due to respiratory infections, airway obstructions that might be intraluminal, intrinsic or extrinsic, allergic airway disease, inhaled airway injuries or immunodeficiencies, including both systemic and pulmonary conditions such as cystic fibrosis (CF) and primary ciliary dyskinesia (PCD) (Chandrasekaran *et al.*, 2018; Flume *et al.*, 2018; Bush and Floto, 2020). Notably, BE is a common manifestation of various primary systemic immune deficiencies and immune regulation disorders, which may be congenital or acquired, classified based on the primary cells involved, with humoral immune deficiencies accounting for approximately 70% of all primary immune deficiencies (Flume *et al.*, 2018). Despite these known causes, nearly 50% of the BE cases in humans remains idiopathic (Chandrasekaran *et al.*, 2018; Bush and Floto, 2020). While BE itself is not regarded as a primary genetic disorder, associations with human leukocyte antigen polymorphisms and marked differences in disease prevalence across ethnic populations support a role for host immunogenetic susceptibility in determining the response to respiratory injury and infection (Boyton and Altmann, 2016). In dogs, the only reported congenital cause of BE is PCD, as CF has not been documented in this species (Spadafora *et al.*, 2010; Merveille *et al.*, 2014). Acquired causes of BE in dogs include aspiration-related BE, inhaled foreign bodies, chronic recurrent bacterial

infections, parasitic or fungal infections, chronic bronchitis, EBP, and CIPF (Hawkins *et al.*, 2003; Roels *et al.*, 2017; Mortier *et al.*, 2018; Nafe *et al.*, 2018; Johnson *et al.*, 2019; Viitanen *et al.*, 2019; Yang *et al.*, 2020). However, as with human BE cases, a significant proportion of canine BE cases remain idiopathic (Hawkins *et al.*, 2003).

2.4. Signalment and clinical signs

In humans, BE is more commonly observed in older individuals, likely due to several age-related physiological changes. With age, diaphragm strength decreases, and both breathing efficiency and vital capacity decline, while residual lung volume increases (Chandrasekaran *et al.*, 2018). These factors can impair effective airway clearance. In addition, diminished swallowing reflexes and a higher prevalence of GERD in the elderly may promote subclinical micro aspiration, including aspiration of nasopharyngeal microbiota, which can contribute to the development of BE (Chandrasekaran *et al.*, 2018). Ageing is also associated with changes in immune function and the microbiome, known as immunosenescence, which may influence susceptibility to chronic airway inflammation and infection, although the exact impact on BE remains poorly understood (Chandrasekaran *et al.*, 2018). BE in dogs is most commonly observed in those over 10 years of age, although cases have been reported in dogs ranging from 4 months to 18 years old, suggesting that while age is a significant factor, it is not the sole determinant (Hawkins *et al.*, 2003).

Dogs with BE can exhibit a wide range of clinical signs, from mild to severe respiratory issues (Hawkins *et al.*, 2003). The most common clinical sign is a persistent, productive cough, seen in almost 85% of dogs, followed by expiratory dyspnoea (Hawkins *et al.*, 2003; Marolf *et al.*, 2007). In human medicine, common additional symptoms include exacerbations, lethargy, chest pain, and haemoptysis (Rogers *et al.*, 2013; Aller *et al.*, 2018). Exacerbations are defined as the worsening of usual respiratory symptoms (Flume *et al.*, 2018). These episodes are significant events for BE patients, as research has shown that individuals experiencing three or more exacerbations per year tend to have a poorer health status, lower quality of life, a higher likelihood of hospitalization, and increased mortality (Richardson *et al.*, 2019). Interestingly, clinical symptoms at presentation do not always correlate with the long-term outcome of BE, as demonstrated in human studies (Flume *et al.*, 2018).

2.5. Diagnosis

BE diagnosis is typically made using thoracic imaging techniques, such as thoracic XR and thoracic CT, or bronchoscopy. In addition, it is important an echocardiography to identify underlying causes or associated conditions.

2.5.1. Blood examination

In humans with BE, blood tests are recommended to investigate potential underlying aetiologies, particularly immunodeficiencies and allergic conditions. These should include measurement of serum immunoglobulins (IgG, IgA, and IgM), serum protein electrophoresis, total serum IgE, *Aspergillus fumigatus*-specific IgE, and *Aspergillus* precipitins (Boyton and Altmann, 2016). To date, no studies demonstrated the clinical utility of a full blood examination in canine patients with BE.

2.5.2. Thoracic radiography

In veterinary medicine, thoracic XR is considered a first-line tool for detecting bronchial changes and diagnosing BE (Marolf and Blaik, 2006). Radiographic signs indicative of BE include thickening and dilation of the bronchial walls (Figure 3), which may appear as ring shadows ("doughnuts") or as tram track patterns when viewed end-on or longitudinally, respectively (Marolf and Blaik, 2006). A honeycombing pattern, representing the confluence of multiple thickened, dilated bronchi, may also be visible on thoracic XR (Marolf and Blaik, 2006). However, due to the relatively low sensitivity (47.7-87.7%) and specificity (74.4%) of thoracic XR compared to thoracic CT, the latter has largely replaced XR for diagnosing BE (Hawkins *et al.*, 2003).

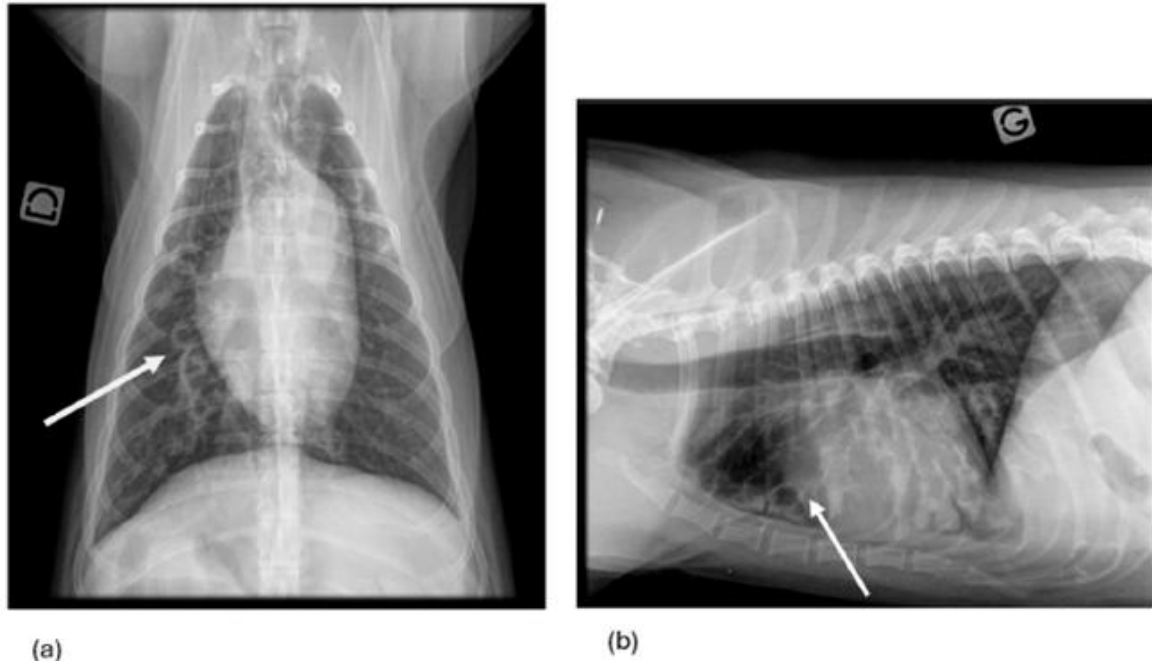


Figure 3: Ventrodorsal (a) and left-lateral (b) radiographs of a dog with bronchiectasis. Bilaterally (right > left), a severe saccular bronchiectasis can be observed (white arrow), as well as a diffuse bronchial pattern. The peripheral parts of the bronchi are dilated with a liquid content. Figure adapted from Lyssens *et al.*, 2025a.

2.5.3. Thoracic computed tomography

Primary CT features of BE observed in both humans and dogs include abnormal bronchial dilation and a lack of peripheral bronchial tapering (i.e. failure of an airway to gradually decrease in diameter while progressing toward the lung periphery) (Cannon *et al.*, 2013; Hill *et al.*, 2019). Secondary features include bronchial wall thickening (i.e. subjective comparison with the bronchial wall thickness in other unaffected lobes), mucus plugging within the bronchial lumen and peripheral air trapping as reflected by measurable reduced pulmonary density affected regions (Cannon *et al.*, 2013; Hill *et al.*, 2019). Bronchial dilation is assessed by relating the airway size to the accompanying pulmonary artery (bronchoarterial ratio; BAR) (Hill *et al.*, 2019). In dogs and humans, the BAR is nowadays considered as one of the most important criterion for diagnosing BE. In humans, a BAR of >1 to 1.5 is considered abnormal, whereas in dogs, a BAR of >2.0 during the expiratory phase is the threshold used to differentiate dogs with BE from those without BE (Cannon *et al.*, 2009). The study by Cannon *et al.* (2009) also demonstrated that the BAR in clinically normal dogs was consistent across individual dogs and across lung lobes in healthy dogs. However, a later study from Cannon *et al.* (2013) observed that a BAR of <2.0 could occasionally be observed during the expiratory phase in dogs with BE. While the BAR is an important diagnostic criterion for BE, it has certain limitations. In

humans, the BAR has been shown to increase with age and lung volume (Bush and Floto, 2019). Additionally, BAR measurements can be affected by measurement errors due to slice thickness artifacts or oblique orientation of bronchi or vessels (Cannon *et al.*, 2013). Furthermore, the calculation of the BAR assumes a normal pulmonary artery; however, in diseased lung lobes, decreased pulmonary arterial blood flow can lead to compensatory increased flow to unaffected lobes due to altered regional ventilation. This redistribution of blood flow could result in a higher BAR in diseased lobes and a lower BAR in unaffected lobes (Cannon *et al.*, 2013).

Based on thoracic XR and CT, different types of BE are described, namely cylindrical (compatible with dilation of the bronchi without tapering of the airways towards the periphery) and saccular (defined as airway dilatation that includes focal dilatation or cyst-like structures) (Figure 4) (Cannon *et al.*, 2013; Bush and Floto, 2019).



Figure 4: Transverse computed tomography image (lung window) in expiratory phase of a dog with severe bronchiectasis. Presence of an irregular dilation in longitudinal section of the stem bronchus of the right cranial lung lobe (white arrow). This dilation is associated with bronchial wall thickening along the entire length and takes on a saccular appearance toward the periphery. A heterogeneous increase in lung parenchymal attenuation is also present, taking on a ground-glass opacification. Figure adapted from Lyssens *et al.*, 2025a.

2.5.4. Bronchoscopy

In dogs, bronchoscopy provides a viable alternative to CT (Johnson *et al.*, 2016). Bronchoscopic diagnosis of BE is based on the observation of one or more dilated bronchi (Johnson *et al.*, 2016) (Figure 5). A study of Johnson *et al.* (2016) found that bronchoscopy accurately identified BE in 92% of examined dogs, compared to 100% with CT and 60% with XR. Bronchoscopy thus failed to diagnose BE in 8% of dogs, mainly due to the inability of the endoscope to access more peripheral airways or due to mucus plugging obstructing visualization (Johnson *et al.*, 2016). It is also important to note that age can influence bronchoscopy findings in apparently healthy dogs. For example, a study on healthy Beagle dogs revealed that older dogs showed more irregularities in the bronchial mucosa, bronchial dilation, and prominent mucosal vessels compared to younger dogs, potentially leading to bronchial wall remodelling, loss of mucosal elasticity, and, eventually, BE (Mercier *et al.*, 2011). In human medicine, bronchoscopy is not routinely used to diagnose BE. It is reserved for cases of single-lobe disease to rule out foreign bodies or proximal obstructions and in situations where sputum culture results are negative (Boyton and Altmann, 2016). For patients who do not expectorate, bronchial washing or aspiration targeted at the affected areas of BE is considered (Hill *et al.*, 2019).

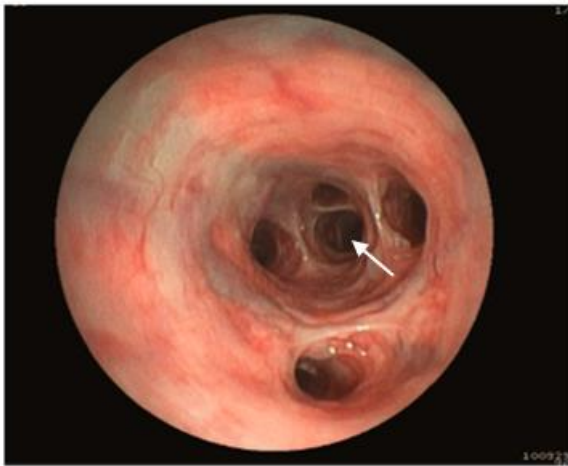


Figure 5: Example of bronchiectasis during bronchoscopy. An obvious macroscopic lack of tapering or increased diameter of the bronchial lumen is observed (indicated by the white arrow). Figure adapted from Lyssens *et al.*, 2025a.

2.5.5. Echocardiography

Bronchiectasis in humans is frequently associated with cardiopulmonary complications, including PH. A study conducted in 2010 reported that 67% of patients with BE had at least 1 echocardiographic abnormality, with PH being the most common finding and present in

approximately 33% of cases (Zeinaloo *et al.*, 2010). More recently, a study including 130 patients reported a suspicion of PH in 25% of individuals based on echocardiographic evaluation (Lee *et al.*, 2023). These findings indicate that PH is a relatively common complication of BE and may contribute to the development of cor pulmonale. The presence of PH in patients with structural lung disease has been associated with increased morbidity, exercise intolerance, and poorer clinical outcomes (Zeinaloo *et al.*, 2010). In canine medicine, PH has also been reported as a complication of chronic respiratory diseases, including BE. For example, Jaffey *et al.* (2019) described the occurrence of PH in dogs with respiratory disorders such as BE, highlighting that similar cardiopulmonary interactions may occur in canine patients with chronic airway disease.

3. Dynamic bronchomalacia

3.1. General

Besides BE, dynamic bronchomalacia (BM) is a common airway remodelling finding in dogs and has is defined as a regional to diffuse dynamic airway collapse of segmental bronchi, subsegmental bronchi or both (Adamama-Moraitou *et al.*, 2012). In contrast to BE, BM is rare in humans, primarily congenital, and thus more commonly observed in infants (Wallis *et al.*, 2019).

3.2. Pathogenesis

The exact underlying mechanisms of dynamic BM remain unclear, though infection, inflammation and degenerative changes are thought to contribute. In humans, idiopathic BM may have a genetic component, although current evidence remains limited and no specific genetic determinants have been identified (Wallis *et al.*, 2019). Similar to tracheal collapse in dogs, canine BM may be associated with a less homogenous cartilage matrix, reduced chondroitin sulphate and calcium levels, and fewer chondrocytes (Marolf *et al.*, 2007; Della Maggiore, 2020). Additionally, extrinsic compression, chronic inflammation, and alterations in elastic fibres are believed to play a role in its pathogenesis (Johnson and Pollard, 2010). BM has been reported in up to 83% of dogs with tracheal collapse, and 59% of dogs with airway collapse might have BM exclusively (Johnson and Pollard, 2010). BM can occur in any breed and at any age, though it is more commonly associated with Yorkshire Terriers and Poodles (Adamama-Moiratou *et al.*, 2012; Della Maggiore, 2020). Identified risk factors in dogs include infection, inflammation, degenerative changes, and obesity. Obesity may also contribute by impairing chest compliance, increasing airway resistance, and reducing tidal volume and expiratory reserve volume (Reinero and Masseau, 2021).

3.3. Clinical signs

The clinical signs of canine BM closely resemble those seen in paediatric cases, with cough being the predominant symptom (Reinero and Masseau, 2021). In severe cases, cyanosis, dyspnoea, syncope, and wheezing may occur (Adamama-Moiratou *et al.*, 2012). On physical examination, crackles during both inspiration and expiration may indicate mucus accumulation or small airway collapse (Della Maggiore, 2020).

3.4. Diagnosis

Diagnosing BM relies on thoracic imaging, including XR, fluoroscopy, CT with inspiratory and expiratory phases, and bronchoscopy, with bronchoscopy being the gold standard (Bottero *et al.*, 2013). To date, no studies demonstrated the clinical utility of a full blood examination in patients with BM.

3.4.1. Bronchoscopy

Bronchoscopy is considered the gold standard for diagnosing BM (Johnson and Pollard, 2010; Bottero *et al.*, 2013). However, it may underestimate the severity of BM or miss dynamic collapse in anesthetized patients with shallow breathing or apnoea, and it cannot visualize smaller, more distant subsegmental airways (Gamracy *et al.*, 2022; Gerhard *et al.*, 2025). In addition, there is currently no universally accepted system to classify BM severity based on bronchoscopy. The most recent human literature defines BM as an expiratory reduction of more than 50% in the cross-sectional luminal area during quiet respiration on bronchoscopy (Wallis *et al.*, 2019).

The first grading system for canine BM based on bronchoscopic findings was proposed by Bottero *et al.* (2013). More recently, Reinero and Masseau (2021) refined this approach using bronchoscopy images. Due to the lack of a clinically validated cut-off, they proposed classifying dogs with <25% airway collapse as grade 0, those with 25–50% reduction in airway diameter as grade I, those with 50–75% collapse as grade II, and those with >75% collapse as grade III (Figure 6).

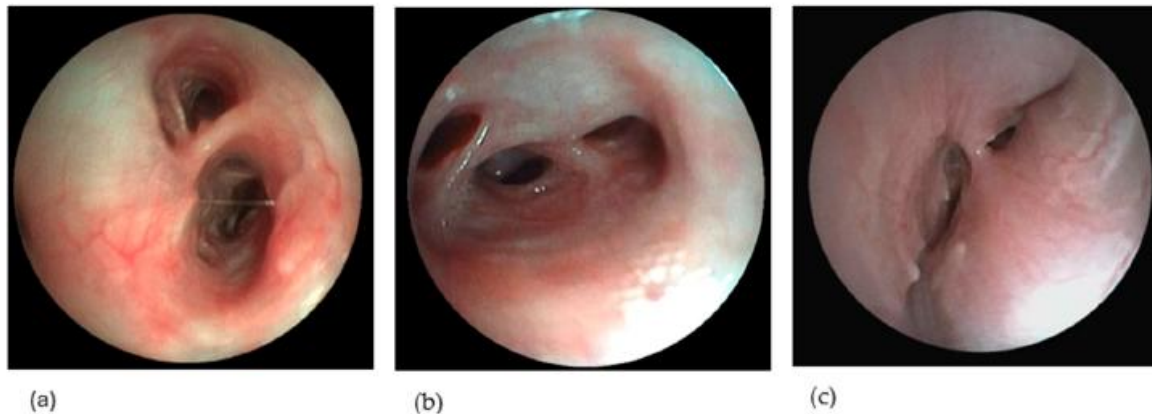


Figure 6: Examples of bronchomalacia during bronchoscopy. Collapse of the bronchi is observed. Based on the endoscopic grading system of [7], a subclassification of bronchomalacia was made.

(a) Subclassification (BE)BM-I, whereby a 25–50% reduction in airway diameter is observed.

(b) Subclassification (BE)BM-II, whereby a 50–75% reduction in airway diameter is observed.

(c) Subclassification (BE)BM-III, whereby a > 75% reduction in airway diameter is observed.

Figure adapted from Lyssens *et al.*, 2025a.

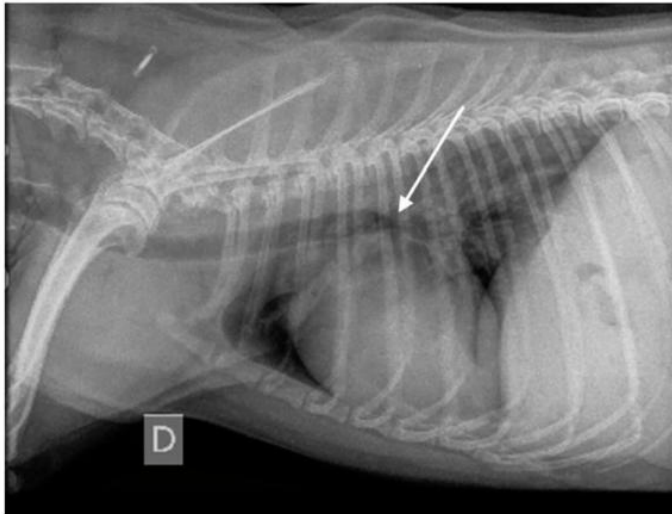
Common bronchoscopy findings in dogs with BM include evidence of airway inflammation, hyperaemia and mucus accumulation (Della Maggiore, 2020). Neutrophilic inflammation is detected in the bronchoalveolar lavage fluid (BALF) in almost 50% of examined dogs (Adamama-Moraitou *et al.*, 2012; Bottero *et al.*, 2013).

3.4.2. Thoracic radiography and fluoroscopy

Thoracic XR is rarely performed in human and veterinary medicine for detecting BM, as the latter techniques has a low sensitivity of 50–55% when using combined inspiratory and expiratory views (Marolf *et al.*, 2007; Johnson and Pollard, 2010; Wallis *et al.*, 2019). Radiographic criteria for BM are limited, though narrowing of the carina has been suggested as a possible indicator (Johnson *et al.*, 2015) (Figure 7).

Fluoroscopy, a rapid, non-invasive imaging technique requiring no sedation and minimal radiation, shows high specificity (93–100%) but low sensitivity (20–24%) for airway collapse (Wallis *et al.*, 2019). It detects more sites of collapse during respiration than radiography and more during coughing than bronchoscopy, with 100% correlation during cough (Johnson and Pollard, 2010; Singh *et al.*, 2012). Blank *et al.* (2024) reported that in healthy dogs, bronchi collapsed up to 15% during expiration and 35% during cough, whereas dogs with presumed BM exhibited significantly greater collapse. Cut-off

- 1 values of 30% for expiration and 60% for cough were proposed to distinguish affected dogs from
2 healthy controls using fluoroscopy (Blank *et al.*, 2024).



13 **Figure 7:** Right-lateral thoracic radiograph of a dog with bronchomalacia. A collapse of the carina
14 can be observed (white arrow). Figure adapted from Lyssens *et al.*, 2025a.

15

16 3.4.3. Thoracic computed tomography

17 As in human medicine, CT is preferred over thoracic XR and fluoroscopy due to its higher sensitivity
18 for detecting bronchial collapse, with reported sensitivities up to 96.6% (Johnson and Pollard, 2010;
19 Wallis *et al.*, 2019). However, the severity and location of BM identified during bronchoscopy may
20 not correspond to those detected on inspiratory–expiratory CT, reflecting the limited agreement
21 reported between CT and tracheobronchoscopy assessments (Levy *et al.*, 2022). Paired inspiratory
22 and expiratory breath-hold scans better capture dynamic collapse (Masseau and Reiner, 2019;
23 Bianco *et al.*, 2020) (Figure 8). A recent study by Gerhard *et al.* (2025) quantified pulmonary
24 parenchymal attenuation in 123 dogs with BM and 20 dogs without using automated 3-dimensional
25 software. Dogs with BM showed significantly larger differences between inspiratory and expiratory
26 scans, with higher overall lung attenuation, fewer low-attenuation areas, and more high-
27 attenuation areas compared to dogs without BM. These changes indicate impaired aeration of lung
28 tissue downstream of collapsed airways and demonstrate that quantitative CT analysis can provide
29 an objective method to evaluate the severity and impact of BM. It should also be noted that 50% of
30 bronchial collapse can sometimes be observed in healthy dogs during forced expiration (Oh *et al.*,
31 2018).

32

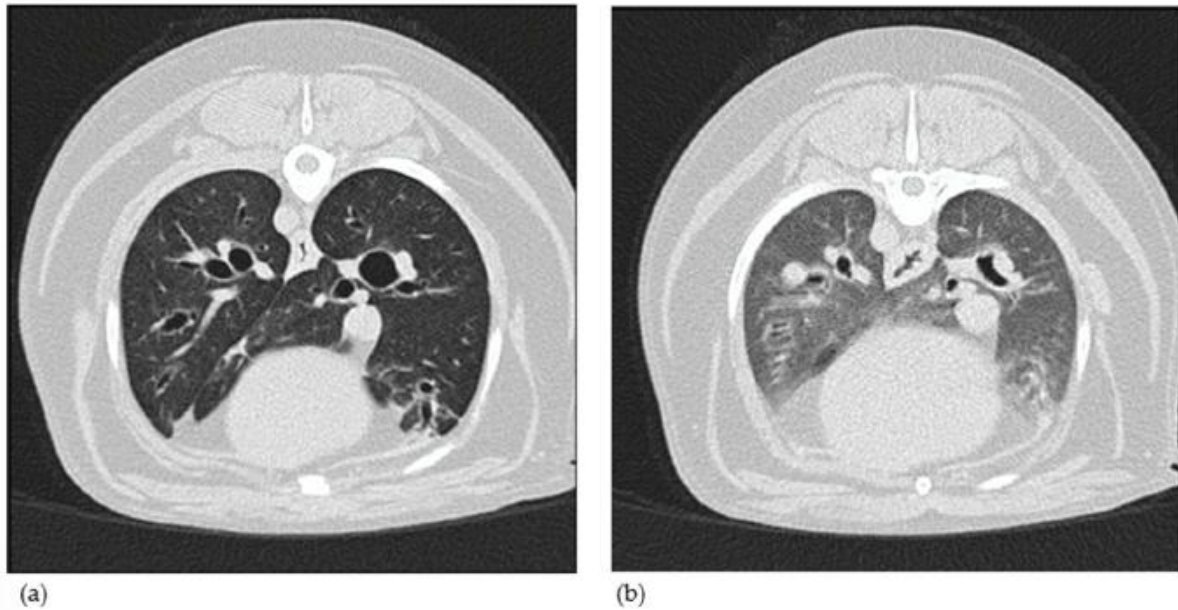


Figure 8: Computed tomography in inspiratory phase (a) and expiratory phase (b) of a dog with severe bronchomalacia (>75% on bronchoscopy). A dynamic bronchial collapse can be noted, especially at the caudal stem bronchi (clear reduction in diameter and flattening during expiration). The other bronchi also decrease in diameter during expiration, but less markedly. Figure adapted from Lyssens *et al.*, 2025a.

3.4.4. Echocardiography

In the context of BM, echocardiographic screening for PH is particularly important. A study by Gamracy *et al.* (2022) reported that 40% of dogs with BM had an intermediate or high probability of PH, compared to only 9% of dogs without BM. Although the sample size was limited and statistical significance could not be established, the notably higher prevalence of PH in dogs with BM supports the recommendation that echocardiography should be considered in all dogs with a strong clinical suspicion of BM.

4. Combination of bronchiectasis and bronchomalacia

In human medicine, BE and BM are frequently identified in the same patient. Although only sporadically reported in the literature (Chang *et al.*, 2002; Nishino *et al.*, 2006), bronchial collapse has been observed in up to 70% of patients with BE, suggesting that chronic airway inflammation and structural remodelling may promote bronchial wall weakness and dynamic airway collapse (Nishino *et al.*, 2006). Similarly, in dogs, concurrent airway collapse is common in dogs with BE. In the study by Johnson *et al.* (2016), 58% of dogs with BE had bronchoscopic evidence of airway collapse, and the author's experience indicates that both abnormalities are frequently identified during bronchoscopy. However, the prevalence, clinical significance, and relationship between these concurrent bronchoscopic and imaging findings have not been systematically investigated in dogs.

5. Role of bacterial infection in bronchiectasis and bronchomalacia

5.1. Human medicine

Chronic bacterial infection can occur in patients with both BE and BM. It is particularly common in individuals with BE, where it is considered a key driver of disease progression (Flume *et al.*, 2018). Effective management is crucial, as certain bacterial species have prognostic implications as demonstrated in studies in humans with BE (Rogers *et al.*, 2014; Flume *et al.*, 2018). Sputum cultures are the most frequently used method for bacterial detection in these patients (Flume *et al.*, 2018). Traditional culture methods indicate that nearly 80% of patients with BE consistently harbour pathogenic bacteria in their sputum samples (Flume *et al.*, 2018). Several bacterial species have been linked to lower airway infections in humans with BE through conventional culture-based diagnostics, including *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, and species of *Escherichia* and *Klebsiella* (Rogers *et al.*, 2013; Tunney *et al.*, 2013; Flume *et al.*, 2018; Niu *et al.*, 2024). Rogers *et al.* (2014) reported *H. influenzae* as the most prevalent species in sputum cultures, with *P. aeruginosa* detected in a smaller subset. In Niu *et al.* (2024), 37% of patients tested positive for *P. aeruginosa*, followed by *K. pneumoniae* (11.5%). Rarely, *Bordetella bronchiseptica* has also been identified as a pathogen in human non-CF BE (Karamooz *et al.*, 2018).

Certain bacterial species have been strongly correlated with clinical markers of disease severity in BE. *P. aeruginosa* is particularly associated with increased exacerbation frequency, greater sputum production, higher hospitalization rates, poorer clinical outcomes, and airway neutrophil-mediated

inflammation (Rogers *et al.*, 2013; Blanco-Aparicio *et al.*, 2019; Chai and Xu, 2020; Niu *et al.*, 2024). *P. aeruginosa* exhibits adaptive mechanisms that enhance its survival in the hostile environment of the airways. Its ability to form biofilms shields it from antibiotics and phagocytes, while the production of virulence factors enables it to evade immune responses, inhibit phagocyte killing, and reduce ciliary beat frequency, further promoting its persistence (Flume *et al.*, 2018). These adaptations make eradication particularly challenging (Blanco-Aparicio *et al.*, 2019). Meanwhile, *H. influenzae* is considered an opportunistic pathogen in susceptible individuals (Boyton and Altmann, 2016). It plays a potential role in driving neutrophilia in BE due to its ability to resist the host's innate oxidative stress responses. When neutrophils engulf *H. influenzae*, they undergo cell death, releasing neutrophil elastase and interleukin-8, which further amplifies neutrophil recruitment and inflammation (Boyton and Altmann, 2016). The role of other bacterial species in BE development remains unclear (Rogers *et al.*, 2014). In some cases, no recognized respiratory pathogen is isolated through conventional culture methods, despite the presence of elevated inflammatory markers (Rogers *et al.*, 2014). The mechanisms that determine which microorganisms dominate the airway microbiome are still unknown (Rogers *et al.*, 2014). Given the significant role of bacterial infection in sustaining chronic disease and triggering acute exacerbations, a deeper understanding of the relationship between airway bacterial communities and BE is essential (Rogers *et al.*, 2013).

5.2. Canine medicine

In dogs with BE, the role of bacteria in the development of BE remains unclear (Hawkins *et al.*, 2003; Johnson *et al.*, 2016). Bronchoalveolar lavage fluid (BALF) analysis in dogs with BE typically reveals suppurative inflammation, with or without bacterial growth on culture at the time of diagnosis (Hawkins *et al.*, 2003). However, data on classical bacterial culture of BALF in canine BE are limited, and the bacterial species identified are far more variable than those reported in human medicine (Hawkins *et al.*, 2003). In a study by Hawkins *et al.* (2003), BALF cytology was performed on 17 out of 27 dogs with endoscopically confirmed BE, revealing neutrophilic inflammation in all cases. Aerobic cultures were conducted in all 27 dogs, with variable results: a single bacterial species was isolated in 41% of cases, while multiple organisms were identified in the remaining 59%. A more recent study by Johnson *et al.* (2016) found bacterial isolates in only 28% of dogs, with *Streptococcus* spp., *Pasteurella* spp., enteric organisms, and *Stenotrophomonas* spp. being the most commonly cultured.

In dogs with BM, conflicting results exist regarding positive bacterial culture results on BALF. Some studies report a low (6-10%) while others note frequent (up to 56-62%) positive bacterial cultures (Johnson and Pollard, 2010; Adamama-Moraitou *et al.*, 2012).

6. Treatment of bronchiectasis and bronchomalacia

6.1. General

Since the pathogenesis of BE and BM, and the inflammatory, infective and molecular drivers of disease progression are not yet fully understood, establishing an optimal treatment protocol and developing novel therapeutic options remain challenging for both humans and dogs (Flume *et al.*, 2018; Richardson *et al.*, 2019; Wallis *et al.*, 2019; Reinero and Masseau, 2021). Given that BE is an increasingly recognized disease in humans worldwide, substantial research over the past two decades has focused on its treatment and management. However, there are currently no therapies approved by regulatory agencies globally. Clinical guidelines, such as those published by the European Respiratory Society (ERS) in 2017 and the British Thoracic Society (BTS) in 2019, provide recommendations for the management of BE. However, these guidelines are largely based on expert opinion and low-quality evidence derived from small randomized trials. They cover key aspects of treatment, including the management of acute exacerbations, eradication therapy for newly isolated pathogens, and the use of short- and long-term antibiotics. Additionally, they address the roles of anti-inflammatory agents, mucoactive therapies, and bronchodilators. No treatment guidelines exist to date for BE in dogs.

Given the rarity of BM in humans, treatment guidelines are also limited. In 2019, the ERS published guidelines on BM in children, which included a section on treatment options (Wallis *et al.*, 2019). Additionally, in 2021, Reinero and Masseau published a review on canine BM, which also addressed current treatment options.

The main goals of treating BE and BM in humans and in dogs should be to improve the clinical outcome, diminish the clinical signs, halt disease progression and improve the quality of life. Identifying and treating underlying causes while managing concurrent diseases is therefore highly important (Polverino *et al.*, 2017; Wallis *et al.*, 2019; Reinero and Masseau, 2021; Barbosa and Chalmers, 2023).

6.2. Antibiotics

6.2.1 Human medicine

Antibiotics are a cornerstone of BE treatment in humans due to the frequent presence of chronic bacterial infections. They are prescribed for acute exacerbations, eradication, and maintenance therapy (Polverino *et al.*, 2017; Hill *et al.*, 2019; Barbosa and Chalmers, 2023). Current guidelines recommend a 14-day antibiotic regimen for acute exacerbations, guided by bacterial culture and

sensitivity testing, though no specific antibiotic choices are outlined. However, shorter courses may be appropriate for mild exacerbations or when cultures identify pathogens with high antibiotic sensitivity, such as *Streptococcus pneumoniae* (Polverino *et al.*, 2017; Hill *et al.*, 2019). For *P. aeruginosa* infections, treatment recommendations include a susceptibility-guided oral fluoroquinolone or intravenous antipseudomonal antibiotics, followed by, or combined with inhaled antibiotics for 6 weeks to 3 months as part of an eradication regimen (Choi *et al.*, 2024). Long-term antibiotic therapy (≥ 3 months) is recommended for BE patients experiencing 3 or more exacerbations. For those with infections not caused by *P. aeruginosa*, first-line treatment includes macrolides such as azithromycin or erythromycin. If macrolides are not tolerated, doxycycline or inhaled gentamicin may be considered (Polverino *et al.*, 2017; Hill *et al.*, 2019). In cases of confirmed *P. aeruginosa* infection, recommended options include inhaled colistin, inhaled gentamicin, or macrolides (Polverino *et al.*, 2017; Hill *et al.*, 2019).

For patients with BM, ERS guidelines recommend prescribing antibiotics during acute exacerbations. Azithromycin is suggested, though its benefit is supported only by anecdotal evidence (Wallis *et al.*, 2019).

6.2.2 Canine medicine

Conflicting evidence exists regarding the presence and role of bacterial infection in canine BE and BM. Currently, antibiotics for dogs with BM should be prescribed based on the presence of airway cytology indicative of airway inflammation, in combination with a positive bacterial culture (Reinero and Masseau, 2021). While no specific guidelines for the type of antibiotic are currently available for dogs with BM, the choice of antibiotic should be guided by culture results and sensitivity testing.

6.3. Anti-inflammatory drugs

6.3.1. Human medicine

Glucocorticoids block phospholipase A, reducing the production of platelet-activating factor, prostaglandins, and other eicosanoids. This leads to decreased bronchoconstriction, airway oedema, and mucus production (Coutinho and Chapman, 2011). They also reduce eosinophil recruitment and adhesion, further limiting airway inflammation, which helps reduce coughing and improves exercise tolerance (Hsieh and Beets, 2020). The beneficial role of anti-inflammatory drugs in BE treatment remains however controversial in humans. Both ERS and BTS guidelines advise against long-term use of oral or inhaled corticosteroids unless another indication is present. No randomized controlled trials have evaluated oral glucocorticoids in BE, and long-term studies on inhaled corticosteroids have shown no significant improvement in lung function or exacerbation

rates, although they may reduce sputum volume. Similarly, evidence for inhaled non-steroidal anti-inflammatory drugs is limited (Pizzutto *et al.*, 2017). Current data are thus insufficient to support or refute their use in managing BE in either adults or children.

The ERS guidelines do not provide any evidence supporting the use of glucocorticoids, either inhaled or oral, for treating BM (Wallis *et al.*, 2019). Kamran and Jennings (2019), recommend against the use of corticosteroids as they may contribute to further cartilage degradation and the progression of BM.

6.3.2. Canine medicine

Currently, no specific recommendations exist regarding the use of anti-inflammatory drugs in canine BE. Glucocorticoids, while effective in comorbid conditions such as chronic bronchitis, do not have a well-defined role in the management of canine BM alone (Chan and Johnson, 2023).

Based on earlier literature, it remained unclear whether inhaled glucocorticoids should be preferred over oral formulations, as concerns existed regarding whether aerosolized drugs adequately reached the lower airways, particularly in dogs with airway collapse (Reinero and Masseau, 2021). However, more recent prospective evidence demonstrated that inhaled fluticasone significantly improved cough severity and quality of life in dogs with chronic inflammatory airway disease, including dogs with concurrent airway collapse, supporting its clinical efficacy as a long-term treatment option (Chan and Johnson, 2023).

6.4. Mucoactive treatments

6.4.1. Human medicine

Mucokinetic drugs are helpful for patients with thick or inspissated respiratory secretions that cannot be effectively cleared. These drugs reduce mucus viscosity, making it easier to mobilize and expel, thereby supporting a more effective cough (Balsamo *et al.*, 2010). N-acetylcysteine (NAC), a mucolytic with antioxidant and anti-inflammatory properties, breaks mucoprotein disulfide bonds to produce thinner mucus. It also supports glutathione production, reduces bacterial adhesion to airways, and lowers bacterial load (Balsamo *et al.*, 2010). It is typically nebulized but can irritate the airways and may cause bronchospasm, although less commonly seen in dogs (Balsamo *et al.*, 2010). The efficacy of NAC in patients with BE is not well established (Polverino *et al.*, 2017, Hill *et al.*, 2019; Barbosa and Chalmers, 2023). ERS guidelines recommend the use of mucoactive drugs, tailored to each individual patient. However, if prescribed, bronchodilator pre-treatment is advised, particularly for patients at risk of bronchoconstriction (Hill *et al.*, 2019). In patients with BM, the use of mucoactive agents has not been shown to be effective (Wallis *et al.*, 2019).

6.4.2. Canine medicine

No recommendations exist to date regarding the use of mucoactive agents in dogs with BM or BE.

6.5. Bronchodilators

6.5.1. Human medicine

Bronchodilators fall into 2 main classes: beta-2 agonists such as terbutaline or albuterol, and derivatives of methylxanthines such as theophylline (Cazzola *et al.*, 2012). Beta-2 agonists work by increasing intracellular cyclic adenosine monophosphate (cAMP), leading to smooth muscle relaxation, reduced airway resistance, and enhanced mucociliary clearance. They also stabilize mast cells and reduce inflammation (Cazzola *et al.*, 2012). Methylxanthines inhibit phosphodiesterase, preventing cAMP breakdown and promoting bronchodilation. They may also improve diaphragmatic function, stabilize mast cells, enhance mucociliary clearance, and reduce vascular leakage (Cazzola *et al.*, 2012).

There is limited evidence supporting the use of bronchodilators in BE (Polverino *et al.*, 2017; Hill *et al.*, 2019; Choi *et al.*, 2024). Therefore, their use is currently recommended only for patients experiencing severe breathlessness. Treatment should be stopped if no improvement is seen (Polverino *et al.*, 2017). In humans with BM, bronchodilator use remains controversial and is generally not recommended, as reducing airway smooth muscle tone may exacerbate airway obstruction (Wallis *et al.*, 2019).

6.5.2. Canine medicine

In dogs, no prospective studies have confirmed the efficacy of bronchodilators for managing BE and/or BM. Jeung *et al.* (2019) investigated theophylline in dogs with tracheal collapse, reporting clinical improvement in 33% of cases, though side effects were common. Bronchodilators might be of use in case of bronchospasm. However, the underlying pathophysiology of BM does not involve bronchospasm but rather airway size reduction due to structural changes, and bronchial hyperreactivity is considered uncommon in dogs compared to cats (Grotheer *et al.*, 2019).

6.6. Cough suppressants

6.6.1. Human medicine

The goal of antitussive treatment is to decrease the frequency and intensity of coughing, thereby protecting the mucociliary defences (Bolser, 2011). Persistent coughing can damage and irritate the airways, which in turn triggers further coughing. This creates a self-perpetuating cycle of cough and inflammation that antitussive therapy aims to interrupt (Bolser, 2011).

The most commonly used cough suppressants are opioids. Opioids suppress coughing by reducing the sensitivity of the cough center and decreasing the perception of airway irritation, primarily via μ -opioid receptors. They may act both centrally, in the brainstem, and peripherally, on sensory nerve endings. Additionally, they might reduce mucus production or enhance mucociliary clearance (Bolser, 2011). Due to the lack of randomized controlled trials, the use of cough suppressants in human patients with BE is currently not recommended (Hill *et al.*, 2019). No specific recommendations exist regarding the use of cough suppressants in humans with BM.

6.6.2. Canine medicine

In veterinary medicine, hydrocodone is the most commonly used opioid, offering greater potency and less sedation than codeine (Hsieh and Beets, 2020). Because repetitive coughing may contribute to dynamic airway collapse by increasing intrathoracic pressure and mechanical stress on weakened bronchi, antitussive therapy can be proposed as a potentially beneficial adjunct in selected dogs with severe, non-productive cough, although this hypothesis has not yet been scientifically validated. In contrast, cough suppression may be less appropriate in dogs with BE, in which cough likely plays an important role in facilitating mucus clearance from permanently dilated airways.

6.7. Others

Several anti-inflammatory and immunomodulatory therapies have been investigated in human BE in an attempt to reduce neutrophilic airway inflammation. However, the prolonged use of statins, methylxanthines, CXCR2 antagonists, neutrophil elastase inhibitors, phosphodiesterase type 4 inhibitors, and leukotriene receptor antagonists is currently not recommended, as studies have not demonstrated significant clinical benefits (Polverino *et al.*, 2017; Hill *et al.*, 2019).

7. Neutrophil extracellular traps

7.1. Neutrophils

Neutrophils are innate immune cells characterized by a multilobed nucleus and are primarily produced in the bone marrow from granulocyte-monocyte progenitors (GMPs), under the regulation of transcription factors and stimulated by granulocyte colony-stimulating factor (G-CSF) (Summers *et al.*, 2010). Neutrophils play a critical frontline role in the innate immune system. They neutralize pathogens through several mechanisms, including phagocytosis, the release of cytotoxic granules, the generation of reactive oxygen species (ROS), and the formation of NETs (Hidalgo *et al.*, 2019; Kobayashi *et al.*, 2023). Beyond pathogen defence, neutrophils also contribute positively to physiological processes such as angiogenesis during sterile injury, tissue repair, and the resolution of inflammation (Hidalgo *et al.*, 2019).

7.2. Neutrophil extracellular traps

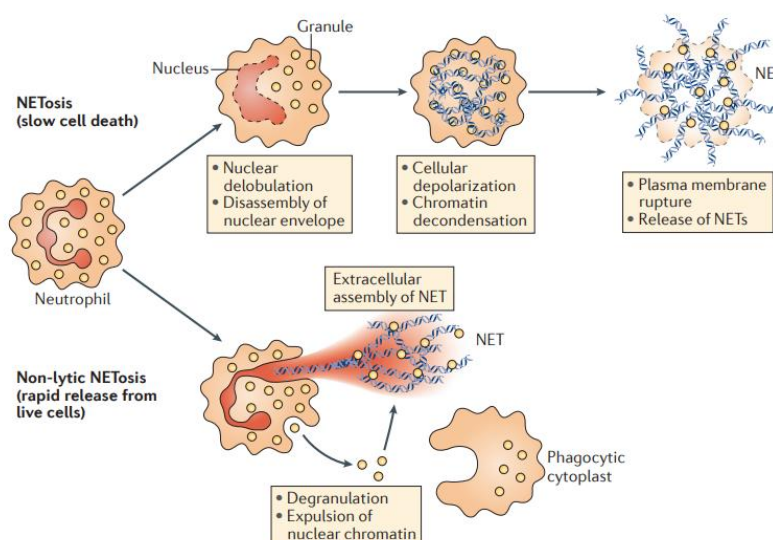
7.2.1. General

NETs were first described by Brinkmann *et al.* (2004). They consist of extracellular web-like fibers, composed of DNA, citrullinated histones (Cit-H3; H1, H2A, H2B, H3, H4), and granule-derived antimicrobial proteins such as neutrophil elastase (NE), myeloperoxidase (MPO), cathepsin G, defensins, lactoferrin, gelatinase, LL37, and proteinase 3 (Brinkmann *et al.*, 2004; Yang *et al.*, 2016). The exact composition of NETs can vary depending on the triggering stimulus, and the functional implications of this variability are still under investigation (Yang *et al.*, 2016; Papayannopoulos, 2018). NETs play a critical role in innate immunity by concentrating antimicrobial molecules at sites of infection (Brinkmann *et al.*, 2004; Yang *et al.*, 2016; Keir and Chalmers, 2022). They create a meshwork that captures and immobilizes pathogens, preventing their dissemination, and neutralizing virulence factors (Yang *et al.*, 2016). The formation of NETs results in the localized exposure of pathogens to high concentrations of effector molecules, contributing thus to microbial killing and containment (Yang *et al.*, 2016).

7.2.2. NET formation

The term NETosis was initially used to describe NET formation, viewed as a unique form of cell death (Yang *et al.*, 2016). However, it is now recognized that NET formation can occur with or without cell lysis, prompting a shift toward the broader term NET formation (Yang *et al.*, 2016; Papayannopoulos, 2018). This multifactorial process is typically ROS-dependent and involves nicotinamide adenine

1 dinucleotide phosphate (NADPH) oxidase and MPO activity, though ROS-independent pathways
 2 have also been documented (Yang *et al.*, 2016).
 3 NET formation starts with intracellular calcium release and protein kinase C activation, leading to
 4 NADPH oxidase assembly and ROS production (Yang *et al.*, 2016). ROS trigger granule rupture and
 5 the release of NE and MPO into the cytoplasm (Yang *et al.*, 2016). MPO, which is normally stored in
 6 the azurophilic primary granules of neutrophils, contributes to microbial killing by generating HOCl
 7 and promoting chromatin decondensation in synergy with NE. It modifies histones and supports NE
 8 nuclear translocation (Papayannopoulos *et al.*, 2010). NE is a serine protease that degrades bacterial
 9 components and histones, facilitating chromatin decondensation (Papayannopoulos *et al.*, 2010;
 10 Hahn *et al.*, 2019). Its activity is tightly regulated by antiproteases, such as alpha1-antitrypsin, and is
 11 modulated by DNA concentrations during NET formation (Papayannopoulos *et al.*, 2010; Hahn *et al.*,
 12 2019). In parallel to NE and MPO release, peptidylarginine deiminase 4 (PAD4) catalyses histone
 13 citrullination (Yang *et al.*, 2016). Citrullination of histones causes a reduction of their positive charge
 14 and weakens DNA interactions. This modification is essential for further chromatin decondensation
 15 (Yang *et al.*, 2016). Eventually, the nuclear and plasma membranes rupture, releasing chromatin into
 16 the extracellular space as a trap (Figure 9) (Yang *et al.*, 2016; Papayannopoulos, 2018).
 17 An alternative form, known as vital NETosis allows neutrophils to remain viable. Here, chromatin is
 18 extruded via vesicles without disrupting the plasma membrane (Keir and Chalmers, 2022). While
 19 vital NETosis is well described in humans, its existence in veterinary species remains unconfirmed.
 20



21
 22 **Figure 9:** NET formation via 2 different pathways. The first pathway is the cell death pathway, called
 23 NETosis. The second pathway is the non-lytic form of NETosis, called vital NETosis. Figure adapted
 24 from Papayannopoulos, 2018

NETs can form rapidly, within 10 minutes of neutrophil activation, and are usually completed within 1–4 hours (Keir and Chalmers, 2022). Their formation is influenced by species, type of stimulus, context, and microbial size. Smaller pathogens, such as small yeast and single bacteria, are more efficiently eliminated by phagocytosis, whereas larger pathogens such as fungi (for example *Candida albicans*) and bacteria that tend to form large aggregates (such as *Mycobacterium* species) tend to trigger NET formation as an alternative defence mechanism (Branzk, *et al.*, 2014; Wang *et al.*, 2024). Although the size of invading pathogens influences NET formation, recent evidence suggests that the intracellular load of phagocytosed particles may be an even more important determinant of NET release (Wang *et al.*, 2024). Other NET-inducing agents include other bacteria (e.g., *S. aureus*, *S. pneumoniae*, *M. tuberculosis*), viruses, cytokines (IL-1, IL-8, TNF- α) and immune complexes (Brinkmann *et al.*, 2004; Branzk *et al.*, 2014; Keir and Chalmers, 2022; Wang *et al.*, 2024).

7.2.3. Regulation and clearance of NETs

While NETs are vital for host defence, their components can be toxic to host tissues (Papayannopoulos, 2018). Excessive or uncontrolled NET release is associated with chronic inflammation, tissue damage, impaired mucociliary function, and autoimmune diseases like vasculitis (Branzk *et al.*, 2014).

NETs are cleared by macrophages once infection resolves, mainly via the complement system; C1q can opsonize NETs, facilitating non-inflammatory phagocytosis (Farrera and Fadeel, 2013). Enzymatic degradation, particularly by DNase I, also aids in NET clearance (Toussaint *et al.*, 2017). Failure in the elimination process of NETs increases the risk of autoimmunity and tissue injury, especially in the lungs, where NETs can accumulate in alveoli and damage epithelial and endothelial cells (Nery Porto and Stein, 2016).

7.2.4. Pathogen-specific NET interactions

Several bacterial species have evolved mechanisms to evade or inhibit NETs, thereby enhancing their survival and pathogenicity. These strategies include the inhibition of host inflammatory responses, degradation of antimicrobial molecules through catalytic enzymes, and resistance to host proteases (Figure 10) (Storisteanu *et al.*, 2016).

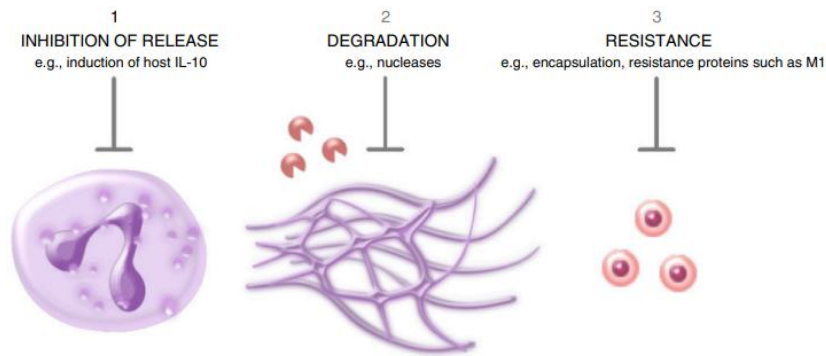


Figure 10: The three main NET evasion strategies. Figure adapted from Storisteanu *et al.*, 2016.

A common evasion tactic of NETs involves the production of nucleases that degrade the DNA backbone of NETs, effectively dismantling the traps before they can exert their antimicrobial effects (Storisteanu *et al.*, 2017). Notable examples of such NET-degrading pathogens include *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, *Vibrio cholerae*, *Yersinia*, *Streptococcus agalactiae*, *Streptococcus suis*, *Staphylococcus aureus*, and *Aeromonas hydrophila* (Storisteanu *et al.*, 2017). By neutralizing NETs in this way, bacteria reduce their vulnerability to innate immune responses and promote persistent infections (Storisteanu *et al.*, 2017).

While many bacteria share the ability to dismantle NETs through nucleases, others employ more specialized mechanisms.

Bordetella pertussis for example, inhibits NET formation by suppressing the oxidative burst necessary for NETosis (Eby *et al.*, 2014). This is achieved through the secretion of adenylyl cyclase toxin (ACT), which increases intracellular cAMP levels, inhibits extracellular signal-regulated kinase (ERK) phosphorylation, and ultimately blocks ROS generation (Eby *et al.*, 2014). *Staphylococcus aureus* not only degrades NETs but also generates toxic byproducts that are harmful to macrophages (Thammavongsa *et al.*, 2013). This impairs macrophage infiltration into abscesses and reduces their antimicrobial effectiveness (Thammavongsa *et al.*, 2013). *Streptococcus pneumoniae* escapes NET entrapment through multiple mechanisms (Beiter *et al.*, 2006). It can degrade NETs enzymatically and resist adhesion by modifying its cell surface charge or by forming a polysaccharide capsule, both of which interfere with NET binding (Beiter *et al.*, 2006; Wartha *et al.*, 2007). *P. aeruginosa* shows both resistance to NET-mediated killing and the ability to inhibit NET formation (Storisteanu *et al.*, 2017). For example, *P. aeruginosa* reduces NET formation by displaying surface sialic acids that activate Siglec-9 on neutrophils, suppressing oxidative burst and NETosis (Khatua *et al.*, 2012). Removing these sialic acids increases NET production on the other hand (Khatua *et al.*, 2012; Storisteanu *et al.*, 2017). *Haemophilus influenzae* employs a combination of

antioxidant production and biofilm formation to resist the antimicrobial action of NETs, allowing it to survive and persist in hostile environments (Hong *et al.*, 2009).

Mycoplasma species, at last, can induce NET formation but they also can simultaneously secrete nucleases capable of degrading the DNA scaffold of NETs (Figure 11) (Cacciotto and Alberti, 2022). This not only enables them to escape immune entrapment but also provides access to extracellular nucleotides, which they require for survival and replication (Cacciotto and Alberti, 2022).

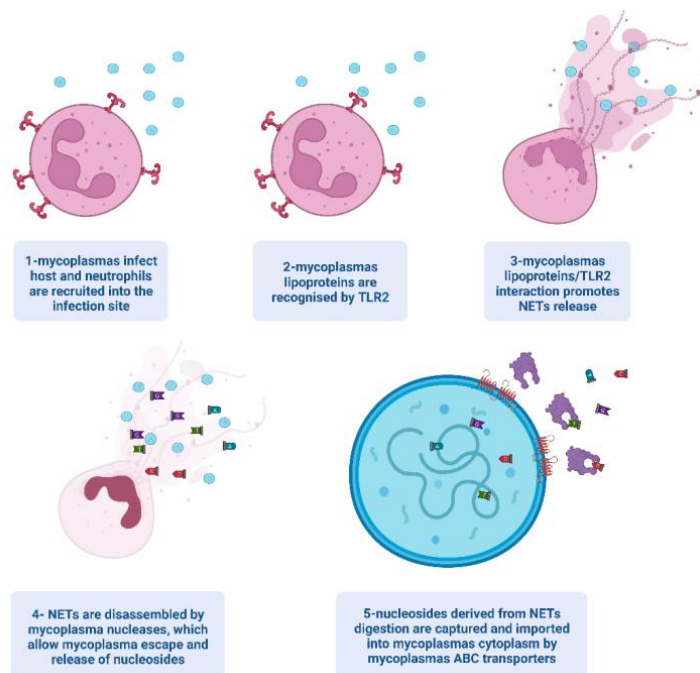


Figure 11: Mechanisms of NET induction and disassembling by *Mycoplasma* species. Adapted from Cacciotto and Alberti, 2022

7.3. Detection of NETs

Several methods have been developed to quantify NETs in biological samples, including cell cultures and tissue sections from mice, humans, and horses (Toussaint *et al.*, 2017; Radermecker *et al.*, 2021; Janssen *et al.*, 2022). Although many candidate biomarkers have been investigated for identifying and quantifying NETosis, no gold standard currently exists.

7.3.1. Detection of NETs via measurement of cell-free DNA

Cell-free DNA (cfDNA) refers broadly to extracellular DNA present in plasma, serum, and various other bodily fluids of vertebrates (Hui *et al.*, 2015; Kowarsky *et al.*, 2017). cfDNA consists of both nuclear and mitochondrial DNA derived from the host, along with DNA originating from external sources such as bacteria or viruses (Kowarsky *et al.*, 2017). Most cfDNA exists as double-stranded DNA (Stroun *et al.*, 1987). Evidence suggests that NET formation can affect circulating cfDNA

concentrations, as the rapid release of substantial amounts of DNA into tissues or the bloodstream may elevate these levels (Grabuschnig *et al.*, 2020). Studies across various medical conditions have shown that cfDNA can be increased in affected individuals (Pisetsky, 2012). Impaired degradation of NETs also contributes to this rise, namely when the normal breakdown mechanisms fail, several diseases can cause incomplete clearance of NETs, resulting in persistently higher cfDNA levels (Hakim *et al.*, 2010; Leffler *et al.*, 2012; Yangsheng and Kaihong, 2013). Measuring the amount of double-stranded cfDNA present in samples, might thus serve as an indirect indicator of NET formation or cell death (Grilz *et al.*, 2019; Grabuschnig *et al.*, 2020; Janssen *et al.*, 2022).

The most widely used technique for measurement of cfDNA is by using PicoGreen (van Breda *et al.*, 2019). PicoGreen Assays involve staining cell-free supernatants with PicoGreen, a fluorescent dye that selectively binds double-stranded DNA, enabling sensitive quantification using a fluorescence microplate reader (van Breda *et al.*, 2019) (Figure 12).

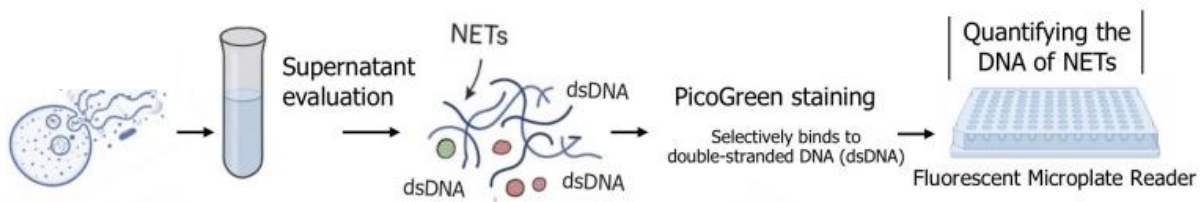


Figure 12: PicoGreen Assays involve staining cell-free supernatants with PicoGreen, a fluorescent dye that selectively binds double-stranded DNA (dsDNA), enabling sensitive quantification using a fluorescence microplate reader. Figure adapted from Zambrano *et al.* (2025).

cfDNA measurement is highly sensitive and capable of detecting very low concentrations of double-stranded DNA, even at nanogram levels (Volik *et al.*, 2016). It is relatively specific for double-stranded DNA, which helps to minimize interference from RNA or single-stranded DNA (Volik *et al.*, 2016). Moreover, quantitative results are provided, allowing for accurate measurement of DNA concentration (Volik *et al.*, 2016; van Breda *et al.*, 2019). It is fast, simple, and non-destructive, meaning the sample remains available for additional analyses (Volik *et al.*, 2016; van Breda *et al.*, 2019). Furthermore, the method is well-suited for high-throughput applications, allowing many samples to be processed at once (Grilz *et al.*, 2020). However, the method has some disadvantages. It cannot distinguish the source of the DNA, meaning it is not possible to tell whether the DNA originates from neutrophil extracellular traps, apoptotic cells, necrotic cells, or other sources (Masuda *et al.*, 2016; van Breda *et al.*, 2019). If cells are not completely removed from the sample, intracellular DNA may contaminate the results (Polatoglou *et al.*, 2022). Additionally, the method

provides no information about the structure, integrity, or fragmentation of the DNA (van Breda *et al.*, 2019; Grabuschnig *et al.*, 2020). Finally, dilution steps required to bring the sample within the detection range may introduce variability or errors (Bronkhorst *et al.*, 2015; Pedini *et al.*, 2021).

7.3.2. Detection of NETs via MPO-DNA complexes detection by ELISA

A sandwich ELISA can be used to detect MPO-DNA complexes, which serve as more specific markers of NETs (Kano *et al.*, 2017; Islam *et al.*, 2023). The method works by capturing the MPO-DNA complexes through their MPO component. DNA is then identified using anti-DNA antibodies. Signal amplification is achieved through the use of secondary antibodies and enzymatic reactions. A resulting colour change is measured to quantify the amount of MPO-DNA complexes in the sample (Kano *et al.*, 2017) (Figure 13).

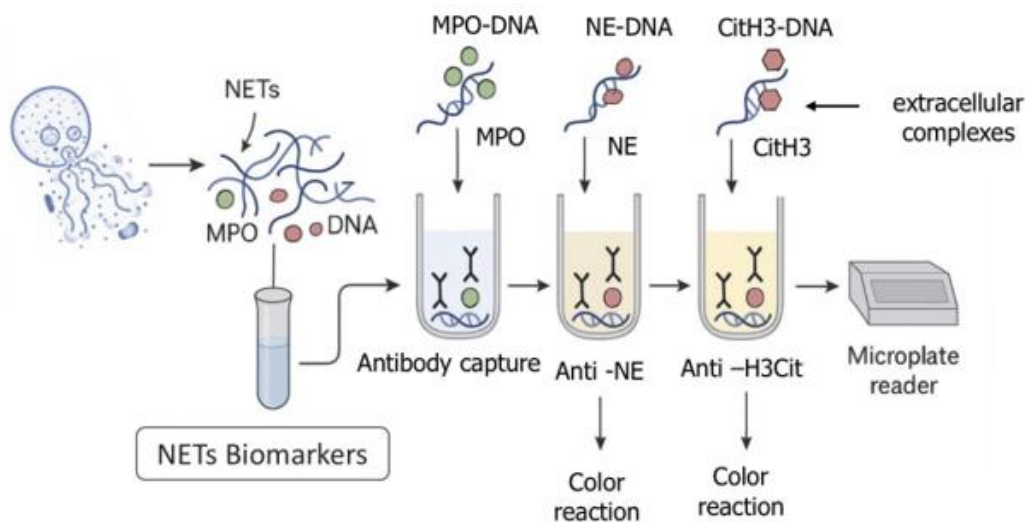


Figure 13: ELISA-based methods measure NET-related components present in biological samples, including MPO–DNA, NE–DNA, and CitH3–DNA complexes. In these assays, target molecules are bound by specific capture antibodies coated onto microplate wells, and subsequently detected using HRP-linked anti-DNA antibodies. The resulting colorimetric signal corresponds to the amount of NET material in the sample. Figure adapted from Zambrano *et al.* (2025).

MPO-DNA complexes are considered more specific indicators of NETosis than either DNA or MPO alone (Kano *et al.*, 2017; Islam *et al.*, 2023). While DNA can originate from any dying cell, including those undergoing apoptosis or necrosis, MPO is present in neutrophils regardless of whether NETosis has occurred (Kano *et al.*, 2017; Islam *et al.*, 2023). The simultaneous presence of MPO and DNA within a complex strongly suggests active NET formation (Masuda *et al.*, 2016; Kano *et al.*, 2017; Islam *et al.*, 2023). This technique offers several advantages. It is highly specific for NET-associated

structures, allows for quantitative analysis, is reproducible, and is compatible with high-throughput applications (Masuda *et al.*, 2016). Additionally, it is relatively simple to perform in a standard laboratory setting (Kano *et al.*, 2017). However, there are also some limitations. The method does not allow for the direct visualization of NETs (Masuda *et al.*, 2016). DNase treatment may introduce variability depending on the degree of digestion (Masuda *et al.*, 2016; Hayden *et al.*, 2021).

7.3.3. Detection of NETs via immunofluorescence

Microscopy-based methods play a key role in identifying and characterizing NETs, offering valuable information about their structure and the dynamics of NET formation (Zambrano *et al.*, 2025). Immunofluorescence microscopy is widely considered the benchmark technique (Zambrano *et al.*, 2025). It combines nuclear stains such as 4',6-diamidino-2-phenylindole (DAPI) with antibodies directed against NET-related proteins, such as MPO, NE, and Cit-H3, to reveal chromatin relaxation and the spatial overlap of NET components (Radermecker *et al.*, 2020; Stoimenou *et al.*, 2022; Schöenfeld *et al.*, 2023). The co-localization of extracellular Cit-H3 and MPO structures is considered strong evidence of NET formation and this allows differentiating NETosis from other types of cell death (Radermecker *et al.*, 2021) (Figure 14).

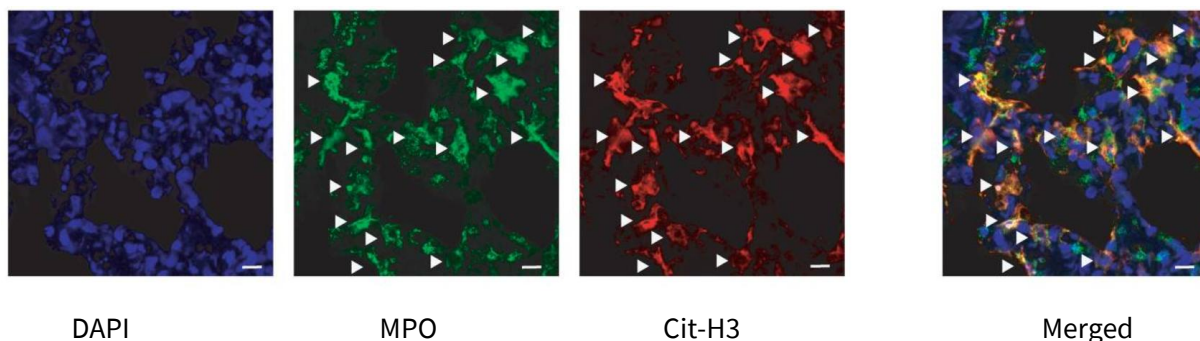


Figure 14: Immunofluorescence visualization of NETs, highlighted as extracellular strands displaying overlapping signals for MPO and Cit-H3, indicated by the white arrows. Figure adapted from Radermecker *et al.* 2021.

Quantification is possible through digital image analysis programs, which enables semi-quantitative measurement of NETs (van Breda *et al.*, 2019). This method also offers high specificity when using controls, as the inclusion of negative controls and assessment of colocalization reduce false positives and confirm that the structures identified are truly NETs (van Breda *et al.*, 2019). Lastly, immunofluorescence is applicable to small samples, making it a useful technique for analysing limited-volume clinical specimens (van Breda *et al.*, 2019).

A key disadvantage is that the method is labour-intensive and time-consuming, as it involves multiple steps, including fixation, permeabilization, staining, imaging, and analysis, that can take several hours to days to complete (Gupta *et al.*, 2018; Radermecker *et al.*, 2021). The method also requires specialized equipment, such as high-resolution confocal microscopy, and it demands proper training, making it a resource-intensive approach. It also has low throughput, meaning that only a few slides or fields can be analysed at a time, which makes it unsuitable for large sample sets (Radermecker *et al.*, 2021). It also remains important to note that due to the different stimulation of neutrophils, histone H3 may not be citrullinated during the NET formation, so the use of Cit-H3 as a marker for NETs remains controversial (Li *et al.*, 2022).

Another limitation is that quantification is relative, since the technique does not provide absolute counts of NETs but only gives relative measurements based on colocalization area or signal intensity (Radermecker *et al.*, 2021). Lastly, sample quality is critical, because techniques like cytopspin and proper cell preservation are essential. Poorly prepared slides can reduce the reliability and reproducibility of results (van Breda *et al.*, 2019; Tan *et al.*, 2021).

7.4. Role of NETs in respiratory diseases in human medicine

Neutrophilic inflammation plays a central role in the pathophysiology of multiple chronic and acute lung diseases. In conditions such as asthma, COPD, CF, BBP, and BE, the formation of NETs has been strongly associated with increased disease severity, more frequent exacerbations, impaired lung function, and reduced quality of life (Keir and Chalmers, 2022).

7.4.1. COPD and asthma

In patients with COPD, levels of cfDNA and NET-associated antimicrobial proteins are significantly elevated in sputum samples compared to healthy controls (Wright *et al.*, 2016). These markers are particularly high in neutrophilic phenotypes of COPD, suggesting a direct link between NET formation and neutrophilic airway inflammation (Wright *et al.*, 2016). Furthermore, elevated cfDNA levels have been associated with more severe forms of asthma, heightened innate immune responses, and increased expression of pro-inflammatory genes (Lachowicz-Scroggins *et al.*, 2019).

7.4.2. Bacterial bronchopneumonia

In community-acquired pneumonia (CAP), typically caused by *Streptococcus pneumoniae* or *Haemophilus influenzae*, neutrophils are massively recruited to the lungs, where they release NETs (Mikacenic *et al.*, 2018; Sanders *et al.*, 2022). Although intended as a defence mechanism, NETs in this context often fail to exhibit bactericidal activity and instead contribute to collateral lung

1 damage. Elevated levels of Cit-H3 have been found in BALF from mice with diverse bacterial
2 pneumonias (Sanders *et al.*, 2022). These levels also correlated with neutrophil infiltration and cell
3 death in the lungs (Sanders *et al.*, 2022). A study of Mikacenic *et al.* (2018) included BALF samples of
4 humans with clinically suspected ventilator-associated pneumonia (VAP). High alveolar
5 concentrations of MPO-DNA were found in patients with VAP. Moreover, NET concentrations were
6 associated with increased bacterial burden, local alveolar inflammation, and markers such as IL-8
7 (Mikacenic *et al.*, 2018).

8 9 7.4.3. Bronchiectasis

10 Studies have shown that increased bacterial load directly correlates with neutrophil activation and
11 elevated airway concentrations of inflammatory mediators, including NE, MPO, TNF- α , IL-6, and
12 CXCL-8 (Angrill *et al.*, 2001 Chalmers *et al.*, 2018). NETs and their associated proteins, such as
13 lactoferrin and LL-37, were elevated in the sputum of patients with more severe disease (Angrill *et*
14 *al.*, 2001). High levels of NETs and NE were also associated with reduced lung function, increased
15 exacerbation frequency, *P. aeruginosa* colonization, and shorter time to the next exacerbation
16 (Chalmers *et al.*, 2017; Keir *et al.*, 2021).

17 Treatment with macrolides and antibiotics has been shown to reduce NET levels and upregulate
18 anti-protease defences, highlighting their potential immunomodulatory effects (Keir *et al.*, 2021).
19 Several studies, including those by Keir *et al.* (2021) and Chalmers *et al.* (2017), have confirmed that
20 sputum NE activity serves as a biomarker for disease severity, exacerbation risk, and overall
21 prognosis in BE.

22 23 7.4.4. Bronchomalacia

24 No studies have investigated the role of NETs in human BM.

25 26 **7.5. Role of NETs in respiratory diseases in veterinary medicine**

27 No studies have investigated the role of NETs in canine BE and BM.

28 29 **7.6. Unexplored fields**

30 Despite the growing body of literature on NETs in various infectious and inflammatory conditions in
31 dogs, significant knowledge gaps persist regarding their role in chronic respiratory disorders,
32 particularly BE and BM. These diseases are characterized by chronic neutrophilic inflammation,
33 mucus accumulation, and recurrent infections, conditions that are conducive to NET release.
34 However, no studies have systematically examined NET biomarkers (e.g., cfDNA, Cit-H3, NE, MPO) in

1 respiratory secretions of affected dogs. In human medicine, elevated NET-associated markers in
2 sputum or BALF have been linked to disease severity, frequency of exacerbations, and lung function
3 decline. Whether similar correlations exist in dogs is not yet investigated. The potential utility of NET-
4 related biomarkers for diagnosis, prognosis, or monitoring treatment response in canine BE or BM
5 warrants exploration.

8. Lung microbiota

8.1. General

The LM corresponds to the community of bacteria residing in the lower respiratory tract. Key terms commonly used in microbiota research, including those relevant to this study, are summarized in Table 1.

Table 1: Key terms in microbiota research

Microbiota	The whole collection of microorganisms within a specific location such as the lung and the gut, which included bacteria, fungi and viruses
Microbiome	The genetic information (genomes) and inferred physico-chemical properties of the gene products of a microbiota
16S ribosomal RNA gene	Component of the 30S small subunit of prokaryotic ribosomes that is unique to bacteria and that comports variable and common regions between each bacterium
Amplicon	An amplified fragment of DNA from a region of a marker gene (such as 16S rDNA) that is generated by PCR
OTU	Operational Taxonomic Unit. Clusters of similar 16S rRNA gene sequences. Each OTU represents a taxonomic unit of a bacteria family or genus depending on the sequence similarity threshold (typical threshold is 97%)
Richness	Number of different species within a sample
Evenness	Equality of the relative abundances among different taxa
Alpha diversity	Refers to the diversity of species within a single sample
Beta diversity	Refers to the diversity of species among sampling units
Ecological parameters	Refers to the richness, evenness and diversity
Dysbiosis	A condition in which the normal structure of the microbiome is disturbed, often through external pressures

Abbreviations: DNA: deoxyribonucleic acid; OTU: operational taxonomic unit; PCR: polymerase chain reaction; RNA: ribonucleic acid.

Table adapted from Faner *et al.* (2017) and Richardson *et al.* (2019).

For many years, healthy lungs were believed to be sterile, largely because standard culture-based microbiological methods consistently failed to detect microbial life (Faner *et al.*, 2017; Richardson *et al.*, 2019). Indeed, some are unculturable, or require specific growth conditions (culture media, incubation conditions, *etc.*) that have not or cannot be reproduced experimentally and some are rare, slow growing and may be missed (Streit and Schmitz, 2004; Woo *et al.*, 2008). Culture-based techniques also tend to favour the growth of known, predominantly aerobic respiratory pathogens, thereby underrepresenting the full microbial diversity present in the lower airways (Faner *et al.*, 2017). However, this view has been overturned in the past decade with the advent of culture-independent molecular techniques, such as 16S ribosomal RNA (rRNA) sequencing. These approaches have revealed that the lungs, even in healthy individuals, host rich and diverse communities of microorganisms, including bacteria, fungi, and viruses, collectively referred to as the LM (Faner *et al.*, 2017). It is now understood that traditional culture methods detect only a small fraction of microbial life, with estimates suggesting that as little as 1% of all bacterial species can be grown in the laboratory (Stewart, 2012). Consequently, the absence of cultivable bacteria does not necessarily imply sterility.

The first report about the presence of LM in healthy dogs was published in 2016 by Ericsson *et al.* Their research showed that while the microbial communities vary between the nasal passages, oropharynx, and lungs, the LM, though low in abundance, tends to have a fairly consistent composition across individuals.

8.2. 16S rRNA amplicon sequencing

Culture-independent methods to detect for example bacteria, are based on the direct analysis of bacterial deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), without any cultural step (Ames *et al.*, 2017).

The 16S rRNA sequencing technique is the most commonly used method to study bacterial communities and offers a simple yet effective alternative to traditional microbial culture methods (Ames *et al.*, 2017). The 16S rRNA gene encodes a component of the bacterial ribosome, a ribosomal subunit, that is highly conserved across bacterial species but also contains hypervariable regions interspersed among these conserved sequences (Clarridge, 2004). These hypervariable regions are species-specific, enabling bacterial identification and classification. Meanwhile, the conserved regions serve as binding sites for universal primers, which target sequences common to most bacteria. Since there are nine hypervariable regions, selecting the appropriate target region, and consequently the correct primers, is a crucial aspect of study design, as different regions provide varying resolution for bacterial characterization (Ghyselinck *et al.*, 2013; Mizrahi-Man *et al.*, 2013).

For instance, the V1-V3 region is often favoured in research focusing on the oral and lung microbiome (Kim *et al.*, 2010). The sequencing data are ultimately grouped into operational taxonomic units (OTUs), which correspond to bacterial groups typically identified at the genus or species level (Dickson *et al.*, 2016). The 16S rRNA amplicon sequencing method offers several benefits, such as being relatively inexpensive and highly sensitive, enabling species-level bacterial identification in approximately 62 to 92% of samples. Additionally, it provides rapid results and allows bacteria to be grouped in ways that closely reflect their phylogenetic relationships (Woo *et al.*, 2008; Faner *et al.*, 2017).

8.3. Limitations of lung microbiota studies

While 16S rRNA sequencing has revolutionized the study of microbial communities, it comes with several important limitations. One major limitation is the lack of functional insight: sequencing reveals which microbes are present but does not provide information about their activity, such as gene expression, metabolic functions, or virulence. Additionally, standard 16S rRNA sequencing cannot distinguish between live and dead bacteria, as it detects bacterial DNA fragments regardless of viability (Doggett *et al.*, 2016). This limitation is critical because without viable isolates, antimicrobial susceptibility testing cannot be performed, which is an essential step in managing active infections.

Another challenge arises from biases in polymerase chain reaction (PCR) amplification (Marsh *et al.*, 2018). The technique relies on primers that bind to conserved regions flanking hypervariable sections of the 16S gene. However, not all bacteria share identical conserved regions. Primers may preferentially bind to some taxa while missing others with unusual sequences, causing some bacteria to be overrepresented and others underrepresented in the sequencing data. This bias can skew the perceived composition of the microbial community.

Contamination poses an additional problem, especially in samples with low bacterial biomass, such as those from the lungs. In such cases, even small amounts of contaminant DNA from laboratory reagents, air, or sampling equipment can overwhelm the true microbial signal (Marsh *et al.*, 2018). This can lead to false positives. To minimize this, strict laboratory controls and the inclusion of negative controls are essential. Procedures like collecting pre-sampling controls and careful bronchoscope handling help reduce contamination, though some minor contamination during sample collection may be unavoidable.

Practical limitations also affect the use of 16S sequencing. The process can be costly, and the necessary technology is not always readily available (Faner *et al.*, 2017). Additionally, variability in the number of 16S gene copies per bacterium can affect quantification accuracy. Analysing and

interpreting sequencing data require specialized expertise, which limits widespread clinical application, as few clinicians are trained in this method (Faner *et al.*, 2017).

Finally, results can differ depending on the sequencing platform used (e.g., 454, Illumina HiSeq, MiSeq), due in part to differences in targeted 16S regions, primers, and amplicon lengths. These technical variations contribute to discrepancies between studies and highlight the need for standardized protocols (Faner *et al.*, 2017).

8.4. Lung microbiota in healthy individuals

8.4.1. Origin of the lung microbiota

The LM is nowadays well recognized as a dynamic ecosystem shaped by a balance of 3 key processes: microbial immigration, microbial elimination and regional growth conditions as shown in Figure 15 (Dickson *et al.*, 2014). In healthy lungs, microbial immigration and elimination dominate, while in disease states, the composition is more heavily influenced by regional growth conditions (Yagi *et al.*, 2021).

Microbial immigration refers to the entry of microbes into the lower airways (Yagi *et al.*, 2021). This occurs primarily via micro aspiration of oropharyngeal secretions, inhalation of airborne microorganisms and potentially direct dispersion along mucosal surface, though the latter remains a theoretical pathway (Yagi *et al.*, 2021). Micro aspiration is ubiquitous and non-pathologic in healthy humans and animals, and is considered the main route of microbial entry (Dickson *et al.*, 2017). Studies in humans and dogs have demonstrated strong similarities between the oral and lower airway microbiota, reinforcing the role of the oropharynx as a primary microbial source (Ericsson *et al.*, 2016; Dickson *et al.*, 2017). Inhaled air also contains approximately 10^4 – 10^6 bacteria per mm^3 , which may contribute to microbial immigration (Dickson *et al.*, 2014).

Elimination is governed by mechanical, immune, and microbial factors (Yagi *et al.*, 2021). The mucociliary escalator plays a central role by transporting inhaled particles and microbes upward to be either swallowed or expectorated. Additionally, coughing enhances this mechanical clearance (Yagi *et al.*, 2021). The lungs also host a robust immune defence system, including both innate and adaptive responses, capable of recognizing, neutralizing, and clearing microbes (Yagi *et al.*, 2021).

While immigration and elimination primarily determine microbial presence in health, local growth conditions become more influential in disease (Yagi *et al.*, 2021). Factors such as oxygen tension, pH, temperature, blood perfusion, ventilation, nutrient availability, and host immune activity create regional microenvironments that affect microbial replication (Segal *et al.*, 2014).

In diseased lungs, altered microenvironments, due to factors such as inflammation, epithelial damage, or gastroesophageal reflux, can disrupt the ecological balance, promoting the growth of

more pathogenic or resilient microbes (Yagi *et al.*, 2021). This process, dysbiosis, has been linked to both acute and chronic respiratory diseases. However, the exact mechanisms behind the establishment and progression of the LM in disease remain incompletely understood (Yagi *et al.*, 2021).

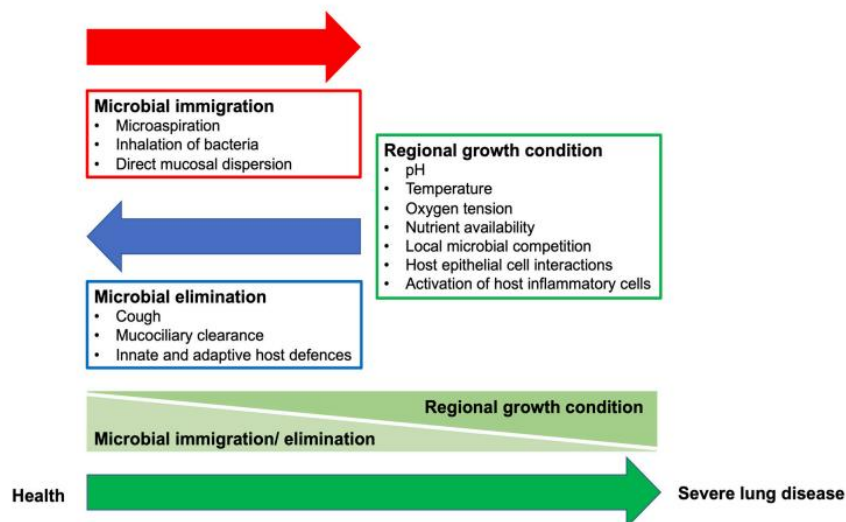


Figure 15: Ecological factors determining the lung microbiota: microbial immigration, microbial elimination and regional growth conditions. Figure adapted from Dickson *et al.*, 2014

8.4.2. Lung microbiota composition in healthy dogs

In healthy dogs, the LM shares similarities with that of humans, but shows also some distinct features. Research based primarily on BALF samples from experimental Beagle dogs has shown that the microbiome is dominated by the phylum *Pseudomonadota*, which accounts for more than 80% of the bacterial community (Ericsson *et al.*, 2016). Alongside *Proteobacteria*, 3 other major phyla are consistently reported: *Actinomycetota*, *Bacillota*, and *Bacteroidota* (Fastrès *et al.*, 2019, Fastrès *et al.*, 2020a, Fastrès *et al.*, 2020b). Although these phyla are also present in the human LM, the relative abundance differs: in dogs, *Pseudomonadota* are predominant, while in humans, *Bacteroidota* and *Bacillota* typically take the lead (Dickson *et al.*, 2016). At the genus level, several taxa are recurrently identified across studies. These include *Cutibacterium*, *Streptococcus*, *Staphylococcus*, *Pseudomonas*, *Corynebacterium_1*, *Acinetobacter*, *Pasteurellaceae* genus, *Conchiformibius*, *Flavobacterium*, and *Porphyromonas* (Fastrès *et al.*, 2020a). Among these, the genera *Cutibacterium*, *Streptococcus*, *Acinetobacter*, and *Pseudomonas* are considered to form the core LM in healthy dogs (Ericsson *et al.*, 2016; Fastrès *et al.*, 2019). Notably, *Cutibacterium* and *Streptococcus* comprise Gram-positive bacteria, the former being anaerobic and the latter facultatively anaerobic (Fastrès *et al.*, 2019).

1 Interestingly, as in humans, the LM in dogs appears to be more similar to the oral microbiota than to
2 the nasal or faecal microbiota, supporting the idea that microaspiration from the oropharynx
3 contributes significantly to lung colonization (Ericsson *et al.*, 2016). However, the LM in dogs tends
4 to be more uniform across individuals compared to that of humans (Ericsson *et al.*, 2016). This
5 homogeneity may be attributed to the controlled environments in which experimental dogs were
6 kept in this study, including standardized diets, shared housing, and consistent handling (Ericsson
7 *et al.*, 2016).

8 Living conditions seem to play an important role. A study of Fastrès *et al.* (2020a) showed that
9 experimental dogs, kept in uniform conditions, demonstrate greater microbial richness and
10 different diversity patterns compared to domestic dogs (Fastrès *et al.*, 2020a). In contrast, breed
11 does not appear to significantly influence the composition of the LM in healthy animals, except for
12 WHWT dogs (Fastrès *et al.*, 2020a).

13 Uptake of antimicrobials might influence the LM in healthy dogs. Fastrès *et al.* (2019) examined the
14 effects of a 10-day course of oral amoxicillin-clavulanate on the LM using BALF samples collected at
15 baseline (D0), 10 days (D10), and 26 days (D26) after stopping treatment. While bacterial load,
16 richness, and alpha diversity remained unchanged, beta diversity was altered at D10, indicating a
17 temporary shift in community composition that largely normalized by D26. At the phylum level,
18 *Pseudomonadota* and *Bacteroidota* increased at D10, largely due to *Pseudomonas spp.*, while
19 *Bacillota* declined. By D26, *Bacillota* showed partial recovery, and *Actinomycetota* had increased.
20 Despite these shifts, no significant changes were observed at lower taxonomic levels. These findings
21 suggest that short-term treatment with antimicrobials induces transient, moderate changes in the
22 LM of healthy dogs, which resolve within 2 weeks post-treatment.

24 8.5. Lung microbiota in human bronchiectasis

25 The LM has been extensively described in chronic lung diseases including asthma, chronic
26 obstructive pulmonary disease (COPD), CF, BE and IPF. In the majority of chronic lung diseases, a
27 shift in the microbiota composition from *Bacteroidota* phylum towards *Pseudomonadota* phylum
28 has been described together with a decrease in diversity and an increase in richness and bacterial
29 burden (Dickson and Huffnagle, 2015).

30 Chronic bacterial infection is a hallmark of non-CF BE and is found in 45–72% of BE patients, most
31 commonly involving *Pseudomonas aeruginosa* and *Haemophilus influenzae*, followed by
32 *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Moraxella catarrhalis*, based on culture-
33 based techniques (Rogers *et al.*, 2013; Rogers *et al.*, 2014; Flume *et al.*, 2018). However, culture-
34 independent sequencing methods have uncovered a far more complex and diverse polymicrobial

environment, often revealing pathogens undetectable through standard culture (Richardson *et al.*, 2019; Azoicai *et al.*, 2024). For instance, Tunney *et al.* (2013) and Cox *et al.* (2017) demonstrated that sequencing often detects pathogens missed by culture. For *P. aeruginosa*, culture showed only 52% sensitivity; for *H. influenzae*, sensitivity dropped to 18%; *S. aureus* exhibited a high false-positive rate, while cultures for *Streptococcus* had very poor agreement with sequencing (only 4% sensitivity). These discrepancies highlight the inability of culture to detect low-abundance organisms and the dominance of fast-growing species like *Pseudomonas* or *S. aureus* in culture media.

The sputum bacteriome in BE is often stable over time and dominated by the phyla *Pseudomonadota* and *Bacillota*, with genera such as *Pseudomonas*, *Haemophilus*, *Streptococcus*, *Veillonella*, *Prevotella*, *Stenotrophomonas*, and *Neisseria* (Rogers *et al.*, 2013; Woo *et al.*, 2019). Less abundant taxa include *Rothia*, *Staphylococcus*, *Enterobacteriaceae*, and *Leptotrichia* (Rogers *et al.*, 2013). Patients with more severe disease often display a dysbiotic microbiome dominated by one or a few pathogenic taxa, especially *Pseudomonas*, *Streptococcus*, or *Haemophilus* (Mac Aogian *et al.*, 2024). In BE patients, polymicrobial communities are frequently observed. Studies like those by Tunney *et al.* (2013) and Cox *et al.* (2017) revealed the presence of both aerobic (*Achromobacter*, *Stenotrophomonas*, *Streptococcus*) and anaerobic (*Prevotella*, *Veillonella*, *Actinomyces*) genera. These microbial communities were highly individualized with generally low diversity, frequently harbouring multiple pathogens (Tunney *et al.*, 2013; Cox *et al.*, 2017).

Regarding anatomical niches, a studies comparing the upper and lower airway microbiota have shown some differences (Rogers *et al.*, 2013; Byun *et al.*, 2017; Shteinberg *et al.*, 2024). For instance, nasopharyngeal swabs were enriched in *Corynebacterium* and *Dolosigranulum*, while sputum had higher proportions of *Bacillota* and *Streptococcus* (Shteinberg *et al.*, 2024). Nevertheless, studies using BALF found minimal differences between sputum and BALF bacteriomes, suggesting that sputum samples may adequately reflect lower airway communities (Rogers *et al.*, 2013; Byun *et al.*, 2017).

Acute exacerbations remain partly unexplained by changes in LM. Studies from Tunney *et al.* (2013) and Woo *et al.* (2019) found no significant change in microbial composition during exacerbations, though patient responses varied. This suggests the existence of distinct exacerbation subtypes, some of which may involve undetected microbiome shifts.

Antimicrobial therapy has a transient effect on the LM. While antibiotics may reduce microbial diversity temporarily, the pre-treatment community structure generally recovers. Rogers *et al.* (2014) showed that long-term macrolide use (e.g., erythromycin) increased microbial richness overall but had limited impact on *Pseudomonas*-dominated microbiomes. In patients without *P.*

aeruginosa infection, erythromycin use led to a decline in *H. influenzae* and a relative increase in macrolide-tolerant pathogens, including *Pseudomonas*. These findings raise concerns about the potential for antibiotic-driven dysbiosis. Similarly, Woo *et al.* (2019) reported that fluoroquinolones reduced observed OTU richness and altered the relative abundance of several genera. While *Escherichia* and *Moraxella* increased, others such as *Veillonella*, *Prevotella*, and *Haemophilus* declined, again reflecting the selective pressures of antibiotic therapy.

8.6. Lung microbiota in human bronchomalacia

No studies have investigated the role of LM in humans with BM.

8.7. Lung microbiota in canine respiratory disease

The LM in dogs remains a largely unexplored area of research. However, recent studies have begun to shed light on its composition and the impact of respiratory diseases such as bacterial bronchopneumonia, *Bordetella bronchiseptica* infection, and chronic bronchitis. No studies to date have been conducted on canine BE and BM.

8.7.1. Bacterial bronchopneumonia

In a study by Vientos-Plotts *et al.* (2019), the LM of 15 dogs with bacterial bronchopneumonia (BBP) was analysed, revealing significant alterations compared to healthy controls. The relative abundance of bacterial taxa typically associated with health was markedly reduced in diseased animals, indicating the presence of respiratory dysbiosis. Notably, the dominant bacterial taxa identified by culture methods did not always match those identified by 16S rRNA gene sequencing. In 9 of 20 samples analysed, *Acinetobacter*, *Brevundimonas*, and members of the *Pseudomonadaceae* family were present at relative abundances between 10% and 50%. These findings contrast with those of Ericsson *et al.* (2016), who reported that in healthy dogs, *Acinetobacter johnsonii*, *Brevundimonas diminuta*, and *Pseudomonadaceae* were among the most abundant taxa, with mean relative abundances of 19.81%, 22.48%, and 29.6%, respectively.

8.7.2. *Bordetella bronchiseptica*

Bordetella bronchiseptica is a common and clinically significant respiratory pathogen in dogs, part of the canine infectious respiratory disease complex, while also existing as a commensal organism in healthy, asymptomatic individuals (Fastrès *et al.*, 2020a). Interestingly, in the study by Vientos-Plotts *et al.* (2019), *B. bronchiseptica* was not detected via 16S rRNA gene sequencing, which may be

1 due to the high sequence homology between *Bordetella spp.* and *Achromobacter spp.*, such as *A.*
2 *xylosoxidans*, which was identified in both samples.

3 In a study by Fastres *et al.* (2020a), dogs with *B. bronchiseptica* infection showed clear evidence of
4 lung dysbiosis, characterized by reduced bacterial richness and alpha diversity, increased bacterial
5 load, and altered beta diversity when compared to healthy dogs. The *Alcaligenaceae family* and the
6 *Bordetella* genus were significantly overrepresented in infected animals.

8 8.7.3. Idiopathic pulmonary fibrosis

9 Fastrès *et al.* (2020a) investigated the lower airway microbiota in WHWT with IPF. This study
10 compared dogs diagnosed with IPF to healthy pet dogs of various breeds (including other WHWTs)
11 and healthy research dogs. While the overall relative abundance of specific bacterial genera was low,
12 certain genera such as *Limnohabitans*, *Rhodoluna*, *Curvibacter*, *Pseudarcicella*, and *Sporochthyaceae*
13 were found exclusively in healthy WHWTs, and *Brochothrix* was identified only in WHWTs and healthy
14 research dogs. Interestingly, significant differences in beta diversity were detected when comparing
15 WHWTs with IPF to healthy non-WHWT dogs, but not when comparing healthy and diseased WHWTs.
16 These findings suggest that breed-specific microbial patterns may obscure disease-related
17 differences and underscore the need for breed-matched controls in microbiome studies.

19 8.7.4. Chronic bronchitis

20 In a study by Ericsson *et al.* (2020), the LM of dogs with chronic bronchitis was investigated using
21 BALF samples. Compared to healthy dogs, those with chronic bronchitis exhibited a decreased
22 relative abundance of *Pseudomonadaceae*, along with reduced bacterial richness and alpha
23 diversity, indicating a disrupted and less diverse microbial community. Notably, the microbial
24 composition was highly variable within the chronic bronchitis group, both overall and when
25 analysing the most discriminatory OTU. Dogs with chronic bronchitis also showed an increased
26 relative abundance of *Pseudomonas* species, particularly in samples with higher microbial biomass.
27 Intragroup variability was greater among dogs with respiratory disease (both chronic bronchitis and
28 without chronic bronchitis) than among healthy research dogs, with some affected individuals
29 harbouring elevated levels of *Agrobacterium*, *Stenotrophomonas*, and *Bradyrhizobium*. Hierarchical
30 clustering further revealed that Gram-negative *Proteobacteria*, including potential pathogens such
31 as *Enterobacter hormaechei*, *Roseomonas*, and *Stenotrophomonas spp.*, were major contributors to
32 the differences observed between healthy and diseased dogs.

8.8. Unexplored fields

Given the potential role of bacterial infections in chronic respiratory diseases and their potential impact on treatment strategies, studies have increasingly focused on the LM in both humans and dogs. However, while LM dysbiosis has been described in chronic respiratory diseases such as chronic bronchitis, bacterial pneumonia, and IPF, it has yet to be studied specifically in BE or BM. Further investigation into the LM of patients with BE, BM and BEBM may offer valuable insights into the microbial changes that contribute to disease development and progression.

1

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Objectives

Although BE and BM are typically described separately in the literature, the author frequently observes both conditions occurring concurrently in the same patient during bronchoscopy. To the authors' knowledge, while this coexistence has been noted clinically, it has not yet been systematically investigated. The primary aim of this study was to retrospectively identify cases of BE and/or dynamic generalized BM, based on endoscopic criteria, and to compare the clinicopathological characteristics across the three groups. The goal was to assess whether these airway remodelling findings should be regarded as distinct conditions, each requiring different management strategies based on their underlying pathophysiological mechanisms. This involved determining whether BE and BM, occurring independently or together, exhibit unique clinical signs. By analysing clinicopathological characteristics, the study aimed to clarify whether their BE and BM should be classified as separate conditions or whether their coexistence suggests a broader spectrum of airway pathology. If significant differences were identified, this would support the need for condition-specific diagnostic, therapeutic, and prognostic approaches rather than a generalized management protocol. During the same retrospective study, we compared imaging findings, including thoracic XR and/or CT scans, with bronchoscopy results to evaluate the diagnostic accuracy and complementarity of these modalities in detecting airway abnormalities associated with BE, BM, or both. Understanding these relationships could help refine non-invasive diagnostic protocols, guiding clinicians in selecting the most effective imaging modality for evaluating affected dogs and optimizing diagnostic workflows.

Given the absence of a standardized treatment protocol for dogs diagnosed with chronic bronchitis having BE, BM, or both, the second aim of this study was to design a standardized treatment protocol by synthesizing established diagnostic and therapeutic guidelines while incorporating insights from both veterinary and human medical literature. This involved reviewing current treatment strategies for BE and BM in both species, identifying areas where management principles overlap, and adapting relevant human medical strategies to veterinary practice. The proposed treatment protocol was structured to address pharmacological interventions with a specific focus given to antimicrobial therapy and anti-inflammatory therapy. By establishing a standardized protocol, the goal was to enhance clinical decision-making, improve patient outcomes, and provide a foundation for future prospective studies evaluating the efficacy of different treatment approaches in dogs with chronic airway disease.

The third objective of this study aimed to improve the understanding of the pathophysiology of chronic airway remodelling findings such as BE and BM by investigating the potential role of NETs.

1 In human medicine, excessive NET formation has been associated with several chronic airway
2 diseases, including cystic fibrosis and BE, where NETs contribute to persistent neutrophilic
3 inflammation, mucus accumulation, and airway damage. These findings suggest that NETosis may
4 play a key role in the progression of chronic inflammatory airway disorders.

5 To explore whether similar mechanisms may be involved in dogs, this study investigated the
6 presence and extent of NET formation in BALF samples from dogs with BE and/or BM. Dogs with BBP
7 were included as a comparison group representing an acute infectious inflammatory condition
8 known to induce strong neutrophil activation, while healthy dogs served as negative controls to
9 establish baseline NET levels. By comparing NET levels across these groups, the study aimed to
10 determine whether NETosis is involved in the pathogenesis of chronic airway remodelling
11 conditions in dogs and whether it differs between chronic structural airway disease and acute
12 infectious inflammation. Demonstrating increased NET formation in BE and BM could suggest that
13 NET-driven inflammation contributes to disease progression and may represent a potential target
14 for future therapeutic strategies in veterinary medicine.

15
16 Finally, the fourth study aimed to analyse the LM composition in BALF of dogs diagnosed with
17 chronic cough and airway alterations such as in BE, BM or BEBM, compared to healthy dogs. The LM
18 plays a crucial role in maintaining respiratory health, influencing local immune responses, and
19 modulating inflammation. In chronic airway diseases, airway remodelling, mucus accumulation,
20 and impaired mucociliary clearance can disrupt the normal microbial balance, leading to dysbiosis.
21 This disruption may contribute to persistent inflammation, increased susceptibility to secondary
22 infections, and disease progression. By characterizing the LM composition in affected dogs, this
23 study aimed to identify microbial shifts associated with BE, BM, and BEBM, including differences in
24 alpha diversity, beta diversity and distribution of bacterial relative abundance at phylum level and
25 at genus level. A key aspect of this analysis was to determine whether each disease phenotype
26 harbours a distinct microbial community or whether BE, BM, and BEBM share common dysbiotic
27 patterns. If significant differences were observed, this would support the hypothesis that these
28 conditions represent distinct disease entities with unique microbial ecosystems, which could
29 influence diagnostic, therapeutic, and preventative strategies. Additionally, understanding LM
30 alterations could provide insights into the potential role of microbiota-targeted therapies in
31 managing chronic respiratory diseases in dogs.

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Experimental section

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Experimental section

Study 1 :

Clinical Relevance of Distinguishing Between Three Endoscopy-Based
Conditions, Bronchiectasis, Bronchomalacia, and their combination in
Dogs: A Retrospective Study

Preface

Bronchiectasis (BE) and bronchomalacia (BM) are recognized causes of chronic cough in dogs. BE is characterized by irreversible dilation of the bronchi associated with chronic airway inflammation, whereas BM refers to a dynamic collapse of the bronchial lumen during respiration. Although these conditions are generally discussed separately, clinical observations during bronchoscopy suggest that they may frequently coexist in the same patient. Despite this apparent association, the clinical and pathophysiological relationship between BE and BM has not been systematically investigated in dogs. In particular, it remains unclear whether these conditions represent distinct disease entities with different clinicopathological characteristics or whether they may reflect different manifestations within a broader spectrum of chronic airway disease. Additionally, the diagnostic agreement between imaging modalities such as thoracic radiography or computed tomography and bronchoscopic findings remains poorly characterized in this context. This study therefore aimed to retrospectively evaluate dogs diagnosed with BE, BM, or both conditions based on bronchoscopic findings, to compare their clinicopathological characteristics, and to assess the concordance between imaging findings and endoscopic diagnoses.

This retrospective study included 65 dogs diagnosed with BE and/or BM. Clinicopathological characteristics and bronchoalveolar lavage findings were compared among the three groups, and the agreement between thoracic imaging modalities and bronchoscopy was evaluated. The results showed minimal clinicopathological differences between the groups and limited diagnostic agreement between imaging findings and bronchoscopic diagnoses.

These findings suggest that BE and BM frequently coexist in dogs and may represent overlapping manifestations of chronic airway disease rather than clearly distinct clinical entities. These observations call into question the clinical relevance of distinguishing among these diagnoses.

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Experimental section

Study 1 :

Clinical Relevance of Distinguishing Between Three Endoscopy-Based Conditions, Bronchiectasis, Bronchomalacia, and their combination in Dogs: A Retrospective Study

<i>Veterinary Sciences</i> 12(5):487

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Abstract

Bronchiectasis (BE) and bronchomalacia (BM) are chronic respiratory diseases in dogs, yet their combined occurrence (BEBM) is not well studied. This retrospective study analyzed 65 dogs diagnosed via endoscopy with BE, BM, or BEBM (E-BE, E-BM, E-BEBM) to identify clinical and pathological differences and assess how imaging results (radiography and computed tomography (CT)) align with endoscopic findings. Clinical signs like coughing, dyspnoea, and exercise intolerance were similar across all groups, except lung crackles, which were more common in E-BEBM. Inflammation seen during bronchoscopy and bronchoalveolar lavage fluid results, including neutrophil counts, showed no significant differences between groups. Bacterial infections were present in 15% of dogs with no difference among groups. Diagnostic agreement between radiography and endoscopy was low: 18.1% for E-BE, 10.5% for E-BM, and 38.4% for E-BEBM. CT results matched endoscopic findings in all E-BE cases but only in half of E-BM and 40% of E-BEBM cases. The bronchial-to-arterial ratio, a benchmark for BE diagnosis, did not align with CT findings. Overall, the study found limited clinical or pathological differences between BE, BM, and BEBM and limited concordance between imaging and endoscopic findings, emphasizing the need for further research to clarify potential implications for treatment strategies.

1 Introduction

2 Bronchiectasis (BE) is a common chronic lung disease in people and dogs, which is characterized by
3 a progressive and irreversible dilatation of the bronchial wall and associated with chronic
4 inflammation and infection (Hawkins *et al.*, 2003; Marolf *et al.*, 2007; Boyton and Altmann, 2016; Bush
5 and Floto, 2019). Similar to human medicine, the pathogenesis of BE in dogs is unclear (Johnson *et al.*,
6 2016). It is believed that BE involves a complex interplay of chronic infection, inflammation, and
7 impaired mucociliary clearance (Boyton and Altmann, 2016; Chandrasekaran *et al.*, 2018). Dynamic
8 bronchomalacia (BM) is also a common airway disease in dogs and has recently been redefined as a
9 regional to diffuse dynamic airway collapse of segmental bronchi, subsegmental bronchi, or both
10 (Reinero and Masseau, 2021). As for BE, the exact underlying mechanism of canine BM is still
11 unknown, but infection and inflammation could possibly play a role, as well as degenerative
12 changes (Marolf *et al.*, 2007; Della Maggiore, 2020). In human medicine, a combination of BE and BM
13 (BEBM) has been reported sporadically in which airway inflammation in BE is believed to potentiate
14 airway collapse due to bronchial wall weakness (Chang *et al.*, 2002; Nishino *et al.*, 2006). In dogs,
15 concurrent airway collapse has been described in dogs with BE, and the authors' experience
16 indicates that a combination of BE and BM is often observed in the same patient during
17 bronchoscopy (Johnson *et al.*, 2016). However, a combined BEBM entity has not been well
18 documented.

19 In dogs, a diagnosis of BE and BM involves thoracic imaging and bronchoscopy (Hawkins *et al.*, 2003;
20 Reinero and Masseau, 2021). For diagnosing BE, computed tomography (CT) and bronchoscopy are
21 generally considered as the preferred diagnostic methods (Hawkins *et al.*, 2003; Cannon *et al.*, 2009).
22 Using CT, measurement of the bronchial-to-arterial ratio (BAR) is the most widely used criterion for
23 detecting bronchial dilatation, with BAR values of ≥ 2.0 during the expiratory phase suggesting
24 evidence of bronchiectasis (Cannon *et al.*, 2009). Bronchoscopic diagnosis of BE is based on the
25 observation of one or more dilated bronchi (Mercier *et al.*, 2011; Johnson *et al.*, 2016). For diagnosing
26 BM, bronchoscopy is considered the gold standard, showing flattening and collapse of the airway
27 lumen (Bottero *et al.*, 2013; Della Maggiore, 2020; Reinero and Masseau, 2021). Other common
28 bronchoscopy findings in dogs with BM include airway inflammation and mucus accumulation
29 (Della Maggiore, 2020). CT is also useful to diagnose BM, but both the inspiratory and expiratory
30 phases need to be explored to highlight dynamic collapse (Reinero and Masseau, 2021). Since the
31 pathogenesis of BE and the inflammatory, infective, and molecular drivers of disease progression
32 are not yet fully understood in both human and veterinary medicine, development of an ideal

1 treatment protocol or novel treatment options is challenging (Johnson *et al.*, 2016; Flume *et al.*,
2 2018). Current human treatment strategies for BE focus on airway clearance and anti-inflammatory
3 medications, including oral or inhaled corticosteroids, and antibiotics (Henkle *et al.*, 2022). However,
4 no guidelines exist for the ideal treatment protocol for neither dogs with BE nor for dogs with BM.
5 Despite conflicting evidence regarding the presence and role of bacterial infection in the
6 pathogenesis of both BE and BM, antibiotics are frequently administered empirically to dogs with
7 these conditions (Hawkins *et al.*, 2003; Johnson *et al.*, 2016; Reinero and Masseau, 2021). This
8 contradicts the One Health approach and the need for reduced antibiotic use. To better guide
9 clinicians in treating BE and BM, more knowledge and insight into the pathogenesis, the role of
10 bacterial and inflammation in both conditions are needed.

11 Dogs with BE, dynamic generalized BM, and BEBM, based on bronchoscopic criteria, were
12 retrospectively included in this study. The primary objective was to describe the clinicopathological
13 characteristics across the three groups to assess whether these conditions should be considered
14 distinct entities, warranting different management strategies in the future. A secondary aim was to
15 compare the imaging findings of the affected dogs with the corresponding bronchoscopic results.

1 Material and methods

2 *Case selection and Data collection*

3 Medical records from client-owned dogs, presented to the Small Animal Teaching Hospital, Liège,
4 Belgium, between November 2014 and November 2021 that underwent bronchoscopy with a
5 bronchoalveolar lavage and for which videos during bronchoscopy were obtained were
6 retrospectively reviewed. Data collected included age, sex, breed, body weight, duration, and
7 severity (mild, moderate, or severe) of clinical signs (chronic cough, exercise intolerance, dyspnoea),
8 abnormalities during physical examination (increased lung sound and crackles on lung
9 auscultation), any previous treatment, ongoing treatment, results of the blood examination
10 (complete blood count, biochemistry, electrolytes, and C-reactive protein (CRP)), results of
11 Baermann analysis, complete description of bronchoscopy and bronchoalveolar lavage fluid
12 analysis (including bacterial culture and quantitative polymerase chain reaction (qPCR) analysis),
13 results of thoracic radiography (RX), CT, and echocardiographic findings.

14

15 *Bronchoscopy*

16 All videos made during bronchoscopy were double-blind reviewed by 2 independent diplomates of
17 the European College of Veterinary Internal Medicine. Only dogs with video recordings of sufficient
18 quality were considered. Each dog was classified into 3 different groups based on endoscopy: E-BE
19 in the presence of BE only, E-BM in the presence of BM only, and E-BEBM in the presence of both BE
20 and BM. E-BE was defined as an obvious macroscopic lack of tapering or increased diameter of the
21 bronchial lumen when compared to adjacent bronchi (Figure 1).



22

23 **Figure 1.** Example of bronchiectasis during bronchoscopy. An obvious macroscopic lack of tapering
24 or increased diameter of the bronchial lumen is observed.

25 E-BM was defined as dynamic airway collapse of the bronchi whereby changes in luminal diameter
26 were observed during respiration (Figure 2a–c). E-BE and E-BM were considered diffuse if observed

in minimum two lobes. Dogs with localized E-BE or E-BM (i.e., in only 1 lobe), dogs with E-BM with less than 25% reduction in bronchial lumen diameter, dogs with static E-BM (defined as persistent reduction in luminal diameter during all phases of respiration) such as dogs with brachycephalic obstructive airway syndrome, and lastly, dogs diagnosed with a grade III or IV tracheal collapse were all excluded from the study. Dogs diagnosed with E-BM were subclassified into 3 subcategories based on a previously described grading system [7]: E-(BE)BM-I (25–50% reduction in airway diameter; Figure 2a), E-(BE)BM-II (50–75% reduction in airway diameter; Figure 2b) and E-(BE)BM-III (>75% reduction in airway diameter; Figure 2c).

The presence and severity of bronchial inflammation (mild to moderate versus severe) was noted. Mild to moderate bronchial inflammation was defined as the presence of erythema of the bronchial mucosa (Figure 3a). Severe bronchial inflammation was defined as the presence of a thickened, oedematous, and erythematous bronchial mucosa (Figure 3b).

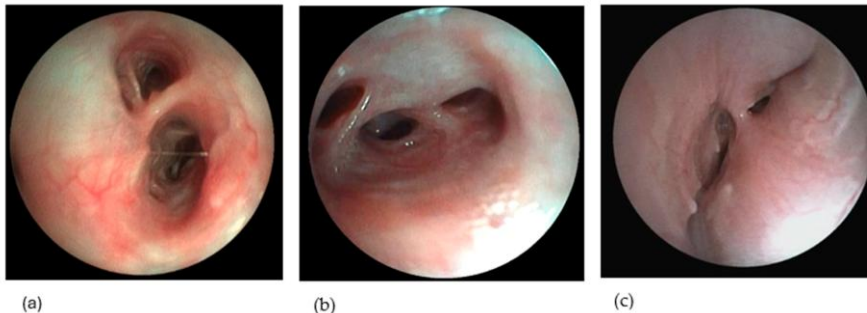


Figure 2. Examples of bronchomalacia during bronchoscopy. Collapse of the bronchi is observed. Based on the endoscopic grading system of [7], a subclassification of bronchomalacia was made. (a) Subclassification (BE)BM-I, whereby a 25–50% reduction in airway diameter is observed. (b) Subclassification (BE)BM-II, whereby a 50–75% reduction in airway diameter is observed. (c) Subclassification (BE)BM-III, whereby a > 75% reduction in airway diameter is observed.

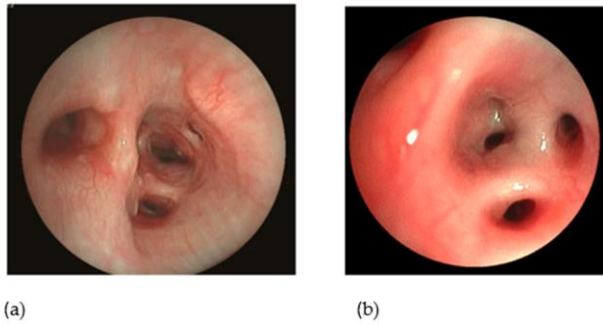


Figure 3. Evaluation of bronchial inflammation based on bronchoscopy.

(a) Mild to moderate bronchial inflammation was defined as the presence of erythema of the bronchi.

(b) Severe bronchial inflammation was defined as the presence of a thickened, oedematous, and erythematous bronchial mucosa.

Bronchoalveolar Lavage Fluid Analysis

Bronchoalveolar fluid (BALF) analysis results were retrospectively reviewed and included cytology, total cell count (TCC), differentiated cell count (DCC), bacterial culture, and qPCR results. Total cell count was calculated using a haemocytometer at 2 occasions and averaged. Normal TCC was considered equal to or less than 600 cells per microliter (Canonne *et al.*, 2020). Cytospin preparations, prepared by cytocentrifugation (centrifugation at 197×g for 4 min at 20°C, Thermo Scientific Cytospin 4 (Waltham, MA, USA), were stained with Diff Quick® to assess cytology and DCC. DCC was performed by counting 100–300 nucleated cells with an oil-immersion objective lens at 100× magnification. Slides were examined for presence of signs of inflammation and intra- and extracellular bacteria. Neutrophilic inflammation was concluded if >12% of neutrophils were observed (Canonne *et al.*, 2020). TCC and DCC were established by 1 experienced veterinarian and 1 independent lab technician. DCC and cytospin slides were not re-evaluated in this retrospective study. Semi-quantitative aerobic culture testing was performed by a commercial veterinary diagnostic laboratory (Antech, Liège, Belgium). Finally, qPCR testing for *Bordetella bronchiseptica*, *Mycoplasma cynos*, *Angiostrongylus vasorum*, and *Crenosoma vulpis* was performed at the clinical pathology laboratory (Department of Veterinary Pathology, Liège, Belgium). For the purpose of the study, infection was defined as an increased TCC, neutrophilic inflammation, presence of intra- or extracellular bacteria and/or positive culture ($>1.7 \times 10^3$ colony-forming units (CFU) per milliliter of BALF) and/or positive qPCR results (cycle threshold <34) (Peeters *et al.*, 2000).

Diagnostic Imaging

All RX and thoracic CT were systematically reviewed by a board-certified radiologist unaware of the dog's group. Right lateral and ventrodorsal RX, not standardized for the respiratory phase, were reviewed for indications of BE and BM. BE was diagnosed based on radiography (R-BE) in presence of non-tapering airways and further defined as saccular or cylindrical BE (Figure 4) (Bush and Floto, 2019). Saccular BE referred to dilatation of the bronchi with focal saccular dilatations (Bush and Floto, 2019). Cylindrical BE referred to dilatation of the bronchi without tapering toward the periphery (Bush and Floto, 2019).

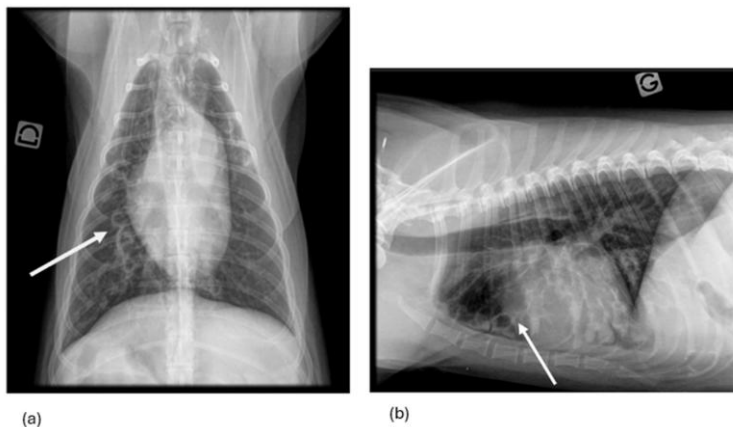


Figure 4. Ventrodorsal (a) and left-lateral (b) radiographs of a dog with bronchiectasis. Bilaterally (right > left), a severe saccular bronchiectasis can be observed (white arrow), as well as a diffuse bronchial pattern. The peripheral parts of the bronchi are dilated with a liquid content. Other abnormalities on these radiographs include an alveolar pattern cranioventrally in the right middle lung lobe, mild tracheal flaccidity, and presence of air in the esophagus. D means “right”.

Since sparse information exists regarding radiographic criteria for diagnosing BM, R-BM was diagnosed on RX when narrowing of the carina was present (Johnson *et al.*, 2015) (Figure 5). Collapse of the carina was identified as either absent or present. Other recorded radiographic findings included pulmonary consolidation, bronchial wall thickening, and bronchial occlusion.

Thoracic CTs, using a two-phased breath-hold technique (during inspiration with airway pressure maintained at 15 to 20 cm of water and during a respiratory pause), were evaluated for the presence of BE and BM (CT-BE and CT-BM). Dogs were sedated with butorphanol, induced with propofol, and anaesthesia was maintained with isoflurane gas and 100% oxygen. For acquisition of the thoracic CT, the dogs were placed in sternal recumbency, under general anaesthesia after a transient period of hyperventilation to induce apnoea and to avoid motion artifacts. Two different scanners were

used: a 16- and a 64-multislice scanner before and after April 2019, respectively. Acquisition parameters used were as follows for the 16-multislice CT scanner: tube voltage 120 kV, reference tube current 130 mA, and pitch 0.7–1.15. Scan tube current was modulated by automatic exposure control (Care Dose, Siemens Medical Solutions, International). Image data sets were reconstructed using parameters of 200–300 mm field of view, 512×512 matrix, 1 mm slice thickness, and B60f reconstruction algorithm (window level –500 and window width of 1500) with filter back projection. Acquisition parameters for the 64-multislice CT scanner were as follows: tube voltage 120 kV, reference tube current 170 mA, and pitch 0.8 to 1.2. Scan tube current was modulated by automatic exposure control (Care Dose, Siemens Medical Solutions, International). Image data sets were reconstructed using parameters of 300 mm field of view, 512×512 matrix, 1 mm slice thickness, and Br59 reconstruction algorithm (window level –500 and window width of 1500) with iterative reconstruction.

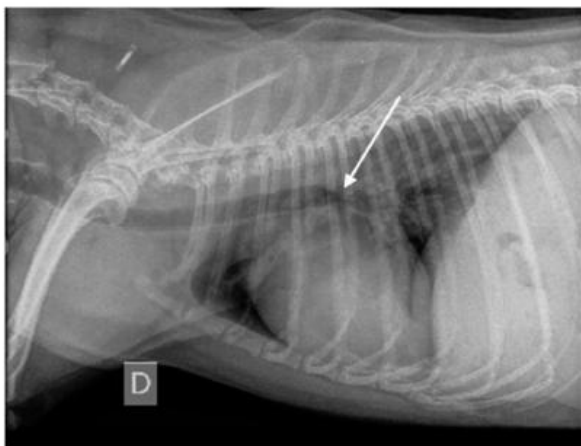


Figure 5. Right-lateral thoracic radiograph of a dog with bronchomalacia. A collapse of the carina can be observed (white arrow). D means “right”.

CT-BE was defined by the presence of non-tapering airways with either saccular or cylindrical BE (Figure 6) (Cannon *et al.*, 2013).



Figure 6. Transverse computed tomography image (lung window) in expiratory phase of a dog with severe bronchiectasis. Presence of an irregular dilation in longitudinal section of the stem bronchus of the right cranial lung lobe (white arrow). This dilation is associated with bronchial wall thickening along the entire length and takes on a saccular appearance toward the periphery. A heterogeneous increase in lung parenchymal attenuation is also present, taking on a ground-glass opacification.

Additionally, but not for diagnostic purpose, BAR was measured as described in the article of Cannon *et al.* (2009) for dogs with an endoscopic diagnosis of BE and BEBM. Measurements of the BAR for the right and the left cranial lobar bronchi were evaluated at the level of the 4th rib on cross-section images and compared to its corresponding artery. The BARs for the right middle lung lobe and the caudal part of the left cranial lung lobe were measured at the level where the bronchus was at its largest diameter, on long-axis orientation, and compared to its corresponding artery. The BAR for the right accessory lung lobe was measured at the level of the bifurcation of the right caudal lobar bronchus and the accessory lobar bronchus and compared to its corresponding artery. The bronchial diameter was measured at the inner part of the bronchus during inspiration and expiration. The BAR for the right and left caudal lobar bronchi was not assessed in this study. CT-BM was defined by the presence of airway collapse of the main bronchi and segmental bronchi (Oh *et al.*, 2019) (Figure 7).

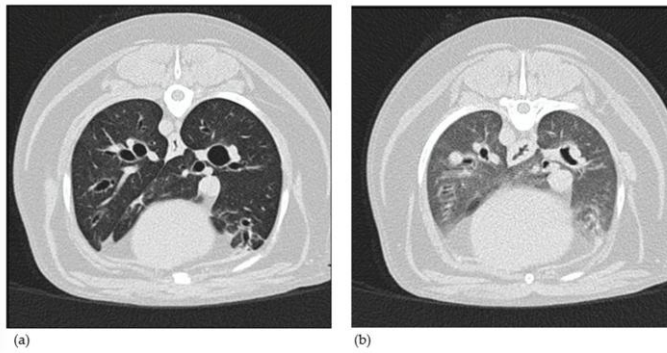


Figure 7. Computed tomography in inspiratory phase (a) and expiratory phase (b) of a dog with severe bronchomalacia (>75% on bronchoscopy). A dynamic bronchial collapse can be noted, especially at caudal stem bronchi (clear reduction in diameter and flattening during expiration). The other bronchi also decrease in diameter during expiration, but less markedly. A heterogeneous increase in lung parenchymal attenuation is also present, taking on a ground-glass opacification.

Other recorded CT findings included pulmonary consolidation, bronchial wall thickening, bronchial occlusion, and ground-glass opacification.

Final Aetiology

Based on all available data, a final diagnosis was attributed to each dog. Dogs were diagnosed with eosinophilic bronchopneumopathy if they exhibited a moderate to severe bronchointerstitial pattern, abundant yellow-green mucopurulent material on bronchoscopy, and more than 50% eosinophils in the BALF (Johnson *et al.* 2019). A diagnosis of parasitic bronchitis was made when parasites were observed either during bronchoscopy, identified in BALF analysis by qPCR, or detected through a Baermann test. Dogs were suspected to have idiopathic pulmonary fibrosis based on the signalment, clinical signs at presentation, compatible CT findings, exclusion of other cardiopulmonary diseases, and histopathology of the lungs, if available (Clercx *et al.*, 2018). Lastly, dogs were diagnosed with chronic bronchitis if they were coughing for more than 2 months and for which no underlying specific cause could be identified (Rozanski, 2020).

Statistical Analysis

Statistical analyses were conducted using SAS (version 9.4) software. Categorical variables were summarized using frequency tables, presenting both the number and per centage of occurrences. Continuous variables were described using the mean and standard deviation ($\pm$ SD), the median and first and third quartiles [Q1; Q3], and the range of extreme values (Min; Max). The 3 groups (BE, BM, and BEBM) were described and compared for the signalment of the dogs and their clinical signs

(cough severity, presence of dyspnoea, presence of crackles on lung auscultation), and the remaining study variables were performed. A Chi-square test was used for categorical variables, while a non-parametric Kruskal-Wallis test followed by Dunn's post hoc tests with Bonferroni correction were applied for continuous and ordinal variables. In order to examine the relationship between the continuous variables (neutrophil percentage or TCC) and a categorical variable (degree of inflammation for example), a non-parametric Kruskal-Wallis test was applied. The relationship between qualitative variables was analyzed using the Chi-square test to measure the association between them (presence of positive bacterial culture and the degree of inflammation). A paired Student's t-test was used to assess significant differences in BAR measurements between the inspiratory and expiratory phases. If no statistical differences were noted between the three groups regarding their clinicopathological findings and BAR measurements, no additional details were provided about the subclassification within each BM group (BM-I, BM-II, BM-III, BEBM-I, BEBM-II, and BEBM-III). The results were considered significant at the 5% threshold ($p < 0.05$).

Results

Description of the Clinicopathological Findings

Group Allocation Based on Bronchoscopy

Based on the medical reviews, 97 dogs were initially included. Twenty dogs were excluded, since only images of bronchoscopy were available instead of videos. Nine dogs were excluded since they had a reduction in airway diameter of $<25\%$. Three dogs were excluded after being diagnosed with a grade III tracheal collapse. After double-blinded review of the bronchoscopy videos, a total of 65 dogs met the inclusion criteria. In 16 of those 65 dogs, there was an interobserver disagreement. In 5/16 dogs, this interobserver disagreement was linked to the classification in either E-BM-I or E-BM-II in 2 dogs and in either E-BM-II or E-BM-III in 3 dogs. In the remaining 11 cases, the disagreement was related to the presence or absence of BE. All the bronchoscopy videos were reviewed once more by both observers together, and a consensual appraisal was performed blindly from previous assessments. Of the 65 dogs, 16 dogs were classified in the E-BE group, 31 in the E-BM group (10, 11, and 10 in BM-I, BM-II, and BM-III, respectively), and 18 in the E-BEBM group (5, 11, and 2 in BEBM-I, BEBM-II, and BEBM-III, respectively) (Table 1).

1 **Table 1.** Signalment, imaging findings, and final diagnosis for each dog.

Endoscopy Diagnosis	Dog	Age (year)	Sex	Breed	RX Findings	CT Findings	RX Diagnosis/CT Diagnosis	Final aetiology
E-BE	1	7	FN	Beagle	NTA+, S+ C+, CMB-, PC-, BWTh+, BWO-	N/A	R-BE	PB
	2	5	FN	Bouvier des Flandres	NTA+, S+ C+, CMB+, PC+, BWTh+, BWO+	N/A	R-BEBM	CB
	3	12	MN	Crossbreed	N/A	N/A	/	EBP
	4	13	MN	Crossbreed	NTA+, S- C+, CMB+, PC-, BWTh+, BWO-	N/A	R-BEBM	CB
	5	11	MN	Crossbreed	N/A	N/A	/	CB
	6 [§]	10	FN	Crossbreed	NTA+, S+ C-, CMB+, PC+, BWTh+, BWO-	N/A	R-BEBM	CB
	7	12	FN	Dachshund	NTA-, S- C-, CMB+, PC-, BWTh+, BWO+	N/A	R-BM	CB
	8	6	FN	Dachshund	N/A	N/A	/	CB
	9	5	ME	Fox Terrier	N/A	N/A	/	CB
	10	15	FN	Jack Russel Terrier	NTA+, S- C+, CMB-, PC-, BWTh+, BWO-	N/A	R-BE	EBP
	11	10	MN	Malinois	NTA+, S+ C+, CMB+, PC+, BWTh+, BWO-	NTA+, S+ C-, CMB-, PC+, BWTh+, BWO+, GGO+	R-BEBM/CT-BE	CB
	12	9	FN	Maltese	NTA-, S- C-, CMB-, PC-, BWTh+, BWO-	N/A	-/-	CB
	13	8	ME	West Highland White Terrier	N/A	NTA+, S- C+, CMB-, PC-, BWTh+, BWO+, GGO+	CT-BE	CB
	14 [§]	3	ME	Whippet	NTA-, S- C-, CMB-, PC-, BWTh+, BWO-	N/A	-/-	EBP

	15	4	MN	Whippet	NTA-, S- C-, CMB-, PC-, BWTh+, BWO-	N/A	-/-	EBP
	16	9	FN	Yorkshire Terrier	NTA+, S- C+, CMB+, PC-, BWTh-, BWO-	N/A	R-BEBM	CB
E-BM 25- 50%	17	8	FN	Cavalier King Charles Spaniel	NTA-, S- C-, CMB-, PC-, BWTh-, BWO-	N/A	-/-	CB
	18	12	FE	Crossbreed	NTA-, S- C-, CMB-, PC-, BWTh+, BWO-	N/A	-/-	CB
	19	12	ME	Crossbreed	NTA+, S- C+, CMB-, PC+, BWTh+, BWO+	N/A	R-BE	CB
	20	13	MN	Crossbreed	NTA+, S+ C+, CMB-, PC-, BWTh+, BWO-	N/A	R-BE	CB
	21 *	11	FN	Griffon Bruxellois	NTA+, S+ C+, CMB+, PC+, BWTh+, BWO-	NTA+, S+ C-, CMB+, PC-, BWTh+, BWO+, GGO+	R-BEBM/CT- BEBM	CB
	22	4	FN	Pomerian	NTA-, S- C-, CMB-, PC-, BWTh+, BWO-	N/A	-/-	CB
	23	9	FN	Sheltie	NTA+, S- C+, CMB+, PC-, BWTh+, BWO-	N/A	R-BEBM	CB
	24	7	MN	Shih Tzu	NTA+, S- C+, CMB-, PC-, BWTh+, BWO-	NTA-, S- C-, CMB+, PC-, BWTh-, BWO-, GGO-	R-BE/CT-BM	CB
	25	7	FN	Whippet	NTA+, S- C+, CMB+, PC-, BWTh+, BWO-	N/A	R-BEBM	CB
	26	7	MN	Yorkshire Terrier	NTA-, S- C-, CMB-, PC-, BWTh+, BWO-	N/A	-/-	CB
E-BM 50- 75%	27	8	FN	Crossbreed	NTA-, S- C-, CMB-, PC-, BWTh+, BWO-	N/A	-/-	CB

	28	13	MN	Labrador Retriever	N/A	N/A	-/-	CB
	29	13	MN	Maltese	N/A	N/A	-/-	CB
	30	11	MN	Poodle	NTA-, S- C-, CMB-, PC-, BWTh+, BWO+	N/A	-/-	CB
	31	12	ME	Poodle	NTA+, S+ C+, CMB-, PC-, BWTh+, BWO-	N/A	R-BE	CB
	32 ^{\$}	8	FN	Sheltie	NTA+, S+ C-, CMB-, PC+, BWTh+, BWO-	NTA-, S- C-, CMB+, PC+, BWTh+, BWO-, GGO+	R-BE/CT-BM	CB
	33	8	FN	West Highland White Terrier	N/A	N/A	/	IPF
	34 ^{\$}	13	FN	Yorkshire Terrier	N/A	N/A	/	CB
	35 ^{\$}	7	MN	Yorkshire Terrier	N/A	N/A	/	CB
	36	11	MN	Yorkshire Terrier	NTA-, S- C-, CMB+, PC-, BWTh+, BWO-	N/A	R-BM	CB
	37	13	ME	Yorkshire Terrier	N/A	N/A	/	CB
E-BM > 75%	38	2	FN	Cavalier King Charles Spaniel)	NTA-, S- C-, CMB+, PC+, BWTh-, BWO-	NTA-, S- C-, CMB+, PC+, BWTh+, BWO-, GGO+	R-BM/CT-BM	CB
	39 ^{\$}	8	FN	Crossbreed	N/A	NTA+, S+ C-, CMB+, PC-, BWTh+, BWO-, GGO+	CT-BEBM	CB
	40 ^{\$}	10	FN	Jack Russel Terrier	N/A	N/A	/	CB
	41 ^{\$}	10	FN	Jack Russel Terrier	NTA+, S- C+, CMB+, PC+, BWTh+, BWO-	NTA-, S- C-, CMB+, PC-, BWTh+, BWO-, GGO+	R-BEBM/CT-BM	CB
	42	12	FN	Maltese	N/A	N/A	/	CB
	43	17	ME	Maltese	N/A	N/A	/	CB

	44 ^{\$}	7	ME	Pomerian	NTA+, S+ C-, CMB+, PC+, BWTh+, BWO-	N/A	R-BEBM	CB
	45 ^{\$}	9	MN	Shih Tzu	NTA+, S+ C-, CMB+, PC-, BWTh+, BWO-	N/A	R-BEBM	CB
	46	11	FN	Yorkshire Terrier	N/A	N/A	/	CB
	47	14	MN	Yorkshire Terrier	N/A	N/A	/	CB
E-BEBM 25- 50%	48	7	ME	Leonberger	NTA+, S+ C+, CMB-, PC+, BWTh+, BWO-	N/A	R-BE	CB
	49	9	FE	Münsterländer	NTA+, S+ C+, CMB-, PC-, BWTh+, BWO-	N/A	R-BE	CB
	50	8	FN	Samoyed	NTA+, S- C+, CMB+, PC+, BWTh+, BWO-	N/A	R-BEBM	EBP
	51	11	ME	West Highland White Terrier	N/A	NTA-, S- C-, CMB+, PC+, BWTh+, BWO-, GGO+	CT-BM	IPF
	52	7	ME	Whippet	N/A	N/A	/	CB
E-BEBM 50- 75%	53 ^{\$}	11	FN	Basset Fauve de Bretagne	NTA-, S- C-, CMB+, PC+, BWTh+, BWO-	N/A	R-BM	CB
	54	13	ME	Beagle	NTA+, S- C+, CMB-, PC-, BWTh+, BWO-	N/A	R-BE	CB
	55	8	MN	Chihuahua	NTA-, S- C-, CMB-, PC-, BWTh-, BWO-	N/A	-/-	CB
	56	13	MN	Crossbreed	NTA+, S+ C-, CMB+, PC+, BWTh+, BWO-	N/A	R-BEBM	CB
	57	6	FN	Crossbreed	NTA+, S+ C+, CMB-, PC+, BWTh+, BWO+	N/A	R-BEBM	CB
	58	9	ME	Sheltie	N/A	NTA-, S- C-, CMB+, PC-, BWTh+, BWO-, GGO+	CT-BM	CB

	59 [§]	13	MN	Shih Tzu	NTA+, S- C+, CMB+, PC+, BWTh+, BWO+	N/A	R-BEBM	CB
	60	10	FN	Shih Tzu	NTA-, S- C-, CMB+, PC-, BWTh+, BWO-	N/A	R-BM	CB
	61	14	MN	Tibetan Terrier	NTA+, S+ C-, CMB-, PC-, BWTh+, BWO-	NTA+, S+ C+, CMB+, PC+, BWTh+, BWO-, GGO+	R-BE/CT-BEBM	CB
	62	13	FN	West Highland White Terrier	N/A	NTA-, S- C-, CMB+, PC-, BWTh+, BWO-, GGO+	CT-BM	IPF
	63	10	FN	West Highland White Terrier	N/A	NTA+, S+ C-, CMB+, PC+, BWTh+, BWO-, GGO+	CT-BEBM	IPF
E-BEBM > 75%	64	10	ME	Shih Tzu	NTA-, S- C-, CMB+, PC-, BWTh-, BWO-	N/A	R-BM	CB
	65 [§]	8	MN	Yorkshire Terrier	NTA+, S- C+, CMB+, PC-, BWTh+, BWO+	N/A	R-BEBM	CB

1
2 Abbreviations: BALF = bronchoalveolar lavage fluid; FN = female neutered; MN = male neutered; ME
3 = male intact; FE = female intact; E-BE = endoscopic bronchiectasis, E-BM = endoscopic
4 bronchomalacia; E-BEBM = endoscopic bronchiectasis + bronchomalacia; + = present; – = absent; *
5 indicates that the dog received antimicrobial treatment at the moment of presentation; § indicates
6 that the dog received corticosteroids at anti-inflammatory dosage within 2 weeks prior to
7 presentation; RX = radiography; CT = computed tomography; NTA = non-tapering airways; S =
8 saccular bronchiectasis; C = cylindrical bronchiectasis; CMB = collapse of the main bronchi; PC =
9 pulmonary consolidation; BWTh = bronchial wall thickening; BWO = bronchial wall occlusion; GGO
10 = ground-glass opacification; R-BE = radiographic diagnosis of bronchiectasis; R-BM = radiographic
11 diagnosis of bronchomalacia; R-BEBM = radiographic diagnosis of bronchiectasis + bronchomalacia;
12 CT-BE = computed tomography diagnosis of bronchiectasis; CT-BM = computed tomography
13 diagnosis of bronchomalacia; CT-BEBM = computed tomography diagnosis of bronchiectasis +

bronchomalacia; PB = parasitic bronchitis; CB = chronic bronchitis; EBP = eosinophilic bronchopneumopathy; IPF = idiopathic pulmonary fibrosis.

Study Population

The mean age of dogs for all groups was 9.6 years $\pm$ 3.1. No significant statistical difference in the mean age among the three groups (8.7 years $\pm$ 3.5 in E-BE, 9.9 years $\pm$ 3.1 in E-BM, and 10 years $\pm$ 2.4 in E-BEBM) was present ($p = 0.44$). There were 31 female dogs (2 intact and 29 neutered) and 34 male dogs (14 intact and 20 neutered). Overall, a total of 24 breeds was represented in this study (Table 1). Two dogs (dog 21 and dog 23) received an antibiotic treatment within 2 weeks prior to presentation, and only one dog (dog 21) was still receiving treatment at the time of presentation. Thirteen dogs (dog 6, 14, 32, 34, 35, 39, 40, 41, 44, 45, 53, 59, and 65) received an anti-inflammatory dosage of steroidal anti-inflammatory drugs (prednisolone or dexamethasone) within 2 weeks prior to presentation and all were still on this medication at the time of evaluation.

Clinical Signs

Clinical signs for each group are reported in Table 2. The duration of the clinical signs varied from 1 month to 48 months (mean 10.3 months). Cough, severe (50.8%) or moderate (49.2%), was observed in all dogs. Exercise intolerance was also observed in 47.7% of dogs, while expiratory dyspnoea was a clinical complaint in 30.8% of the cases. On pulmonary auscultation, increased lung sounds were present in 13.8% of dogs, and lung crackles were auscultated in 27.7% of dogs. The only significant difference between the three groups was the presence of lung crackles ($p = 0.017$), which was more frequently auscultated in E-BEBM (50%) compared to E-BM (25.8%) and E-BE (6.3%). No significant differences were found regarding the severity of cough ($p = 0.57$), presence of dyspnoea ($p = 0.13$), exercise intolerance ($p = 0.091$), or increased lung sounds ($p = 0.097$) between the three groups.

1 **Table 2.** Clinical signs of the dogs.

Clinical Sign	E-BE (n = 16)	E-BM (n = 31)	E-BEBM (n = 18)	p-Value
	n (%)	n (%)	n (%)	
Cough				
Mild to moderate	9 (13.85%)	16 (24.62%)	7 (10.77%)	0.57
Severe	7 (10.77%)	15 (23.08%)	11 (16.92%)	
Exercise intolerance	4 (25%)	16 (51.6%)	11 (61.1%)	0.091
Dyspnoea	2 (12.5%)	10 (32.3%)	8 (44.4%)	0.13
Increased lung sounds	/	7 (22.6%)	2 (11.1%)	0.097
Lung crackles	1 (6.3%)	8 (25.8%)	9 (50%)	0.017 *

2

3 The results are presented as number (%) and statistical differences are indicated by an asterisk (*).

4 Abbreviations: E-BE = endoscopy-based diagnosis of bronchiectasis, E-BM = endoscopy-based
5 diagnosis of BM; E-BEBM = endoscopy-based diagnosis of bronchiectasis/bronchomalacia.

6

7 Blood and Fecal Analysis

8 Complete blood count was normal in 90.7% of dogs. Mild leukocytosis with neutrophilia was present
9 in seven cases, and mild eosinophilia was present in two cases. Serum biochemistry revealed no
10 abnormalities in 93.8% of cases. In the remaining dogs, alkaline phosphatase values were increased
11 from two up to five times the upper reference limit. All these dogs had a history of recent
12 prednisolone treatment. CRP was measured in only four dogs, with increased CRP values in three
13 dogs (dog 21, dog 48, and dog 57) ranging from 15 mg/L to 26.4 mg/L (upper reference limit: 9 mg/L).
14 The Baermann test was performed in 15.4% of dogs and was positive once for *Crenosoma vulpis* in
15 dog 1.

16

17 Bronchoscopy Findings

18 Mild to moderate macroscopic bronchial inflammation was observed in 34 dogs, including 19 dogs
19 with E-BM, 9 dogs with E-BE, and 6 dogs with E-BEBM. Severe inflammation was noted in 11 dogs,
20 including 2 dogs with E-BE, 5 dogs in the E-BEBM group, and 4 with E-BM. However, no significant
21 difference was noted in the degree of macroscopic inflammatory aspect of the bronchial mucosa
22 (mild/moderate and severe) between the 3 groups ($p = 0.18$). Even after excluding dogs that had
23 received prednisolone prior to presentation, the severity of the macroscopic inflammatory aspect of
24 the bronchial mucosa on bronchoscopy remained comparable across the three groups ($p = 0.27$).

BALF Cytological and Microbiological Analysis

The median TCC was 1100.0 cells/ μ L [720.0; 2000.0] for all three groups and 1012.0 cells/ μ L [752.5-1560.0], 1160 cells/ μ L [720.0-2015.0], and 1220.0 cells/ μ L for E-BE, E-BM, and E-BEBM, respectively. No significant difference was noted in the median TCC between any group ($p = 0.89$). The median percentage of neutrophils was 20% [10.0-50.0] for all three groups and 20% [10.0-65.0], 15% [7.0-30.0], and 39.5% [13.0-75.0] for E-BE, E-BM, and E-BEBM, respectively, but those percentages did not differ significantly between groups ($p = 0.087$). Even after excluding dogs receiving antibiotics at presentation and/or receiving prednisolone upon presentation, no significant differences in the median neutrophil percentage in BALF were observed between the three groups ($p = 0.088$ and $p = 0.084$, respectively). The median percentage of neutrophils varied significantly according to the degree of macroscopic inflammation of the bronchial mucosa ($p = 0.0053$). In contrast, no significant association was found between the TCC and the degree of inflammation ($p = 0.72$). An increase in percentage of eosinophils ($>10\%$) was only seen in dogs diagnosed with eosinophilic bronchopneumopathy and in the dog with parasitic bronchitis.

Ten dogs were diagnosed with a bacterial infection based on culture (eight dogs) and qPCR (two dogs). In all eight cases, culture demonstrated the presence of a single bacterium. Organisms identified in each group are summarized in Table 3. There was no significant difference in the presence of concomitant bacterial infection among the three groups, whether including dogs receiving antimicrobials at presentation ($p = 0.081$) or excluding them ($p = 0.10$). Additionally, no significant association was observed between the presence of bacterial infection in BALF and the degree of inflammation observed during endoscopy ($p = 0.29$), even after excluding dogs receiving antibiotics at presentation ($p = 0.32$).

Table 3. Distribution of bacteria retrieved from cultures or PCR of bronchoalveolar lavage fluid in dogs with bronchiectasis, bronchomalacia, and bronchiectasis/bronchomalacia.

Bacteria	E-BE (n = 16)	E-BM (n = 31)	E-BEBM (n = 18)	Total (n = 65)
<i>Escherichia</i> sp.	2 (dogs 7 & 9)	0	0	2
<i>Pseudomonas</i> sp.	2 (dogs 2 & 16)	0	1 (dog 60)	3
<i>Serratia</i> sp.	0	2 (dogs 19 and 26)	0	2
<i>Streptococcus</i> sp.	0	0	1 (dog 48)	1
<i>Bordetella bronchiseptica</i> (qPCR)	0	0	1 (dog 64)	1
<i>Mycoplasma cynos</i> (qPCR)	1 (dog 14)	0	0	1
Total number of dogs	5	2	3	10

Abbreviations: E-BE = endoscopy-based diagnosis of bronchiectasis, E-BM = endoscopy-based diagnosis of BM; E-BEBM = endoscopy-based diagnosis of bronchiectasis/bronchomalacia; qPCR = quantitative polymerase chain reaction; sp. = species.

Thoracic Radiography Findings

RXs were available for review in 43/65 dogs. In the remaining 22 dogs, RXs were taken by the referring veterinarian and not available for review. A summary of the RX findings is available in Table 1. Based on the RXs, a diagnosis of R-BE, R-BM, and R-BEBM was made in 11, 6, and 16 dogs, respectively. In 10 dogs, a diagnosis of neither R-BE, R-BM, nor R-BEBM was made based on the available RXs. Pulmonary consolidation was observed in three dogs with E-BE, six with E-BM, and six with E-BEBM. Bronchial wall thickening was noted in 10 dogs with E-BE, 17 with E-BM, and 11 with E-BEBM. Bronchial occlusion was observed in two dogs with E-BE, two with E-BM, and three with E-BEBM.

Computed Tomography Findings

A thoracic CT with inspiratory and expiratory breath holds was performed in 13/65 dogs. The other dogs had either no CT performed (46 dogs), a CT performed only under sedation (1 dog), or with only one respiratory phase available (5 dogs). A summary of the CT findings is available in Table 1. Based on the CTs, a diagnosis of CT-BE, CT-BM, and CT-BEBM was made in two, seven, and four dogs, respectively. Pulmonary consolidation was observed in one dog with E-BE, two with E-BM, and three with E-BEBM. Bronchial wall thickening was noted in two dogs with E-BE, five with E-BM, and five with E-BEBM. Bronchial occlusion was observed in two dogs with E-BE, one with E-BM, and no dog with E-BEBM.

Sixty BAR measurements were calculated from various locations (i.e., five lobes) during both the inspiratory and expiratory phases from all six dogs with a CT diagnosis of BE and BEBM (Table 4). The difference in mean BAR measurements between the inspiratory and expiratory phases was evaluated in dogs with CT-BE and CT-BEBM. In dogs with CT-BE and CT-BEBM, no statistically significant differences were observed in the mean BAR measurements across both respiratory phases for all five lobes. However, when considering all dogs together (CT-BE and CT-BEBM), significant differences in the mean BAR measurements between the inspiratory and expiratory phases were observed in the left cranial lung lobe ($p = 0.015$), right cranial lung lobe ($p = 0.032$), and right middle lung lobe ($p = 0.047$).

Table 4. Description of the bronchial-to-arterial ratio of cross-sectional CT images of the inspiration and expiration phases in dogs diagnosed with BE or BEBM based on CT. The results are presented as median with the interquartile range [Q1; Q3].

Location	Median BAR [Interquartile Range Q1; Q3] (Inspiratory)	Median BAR [Interquartile Range Q1; Q3] (Expiratory)
R cranial lobe 4th rib		
CT-BE	2.4 [2.3; 2.6]	2.2 [1.9; 2.5]
CT-BEBM	2.1 [1.8; 2.3]	1.5 [1.0; 1.6]
<i>p</i> -value	0.56	0.081
L cranial lobe 4th rib		
CT-BE	2.4 [2.3; 2.5]	2.0 [1.8; 2.2]
CT-BEBM	1.6 [1.1; 1.65]	0.8 [0.7; 0.8]
<i>p</i> -value	0.081	0.081
R middle lobe		
CT-BE	2.2 [1.4; 2.9]	1.9 [1.4; 2.3]
CT-BEBM	1.6 [1.4; 2.5]	1.3 [1.2; 1.5]
<i>p</i> -value	0.85	0.33
L cranial (caudal part)		
CT-BE	1.2 [1.2; 1.3]	1.5 [1.4; 1.7]
CT-BEBM	1.0 [1.0; 1.0]	0.8 [0.8; 1.1]
<i>p</i> -value	0.18	0.081
R accessory lobe		
CT-BE	1.5 [1.3; 1.7]	1.4 [1.3; 1.5]
CT-BEBM	1.4 [0.9; 1.5]	1.2 [1.1; 1.2]
<i>p</i> -value	0.85	0.081

Abbreviations: BAR = bronchial-to-arterial ratio; SD = standard deviation; R = right; L = left; CT-BE = computed tomography-based diagnosis of bronchiectasis; CT-BEBM = computed tomography-based diagnosis of bronchiectasis/bronchomalacia.

Final aetiology

Overall, chronic bronchitis was the most common final diagnosis (55/65 dogs) in all three groups. Eosinophilic bronchopneumopathy (n = 5), idiopathic pulmonary fibrosis (n = 4), and parasitic bronchitis (n = 1) constituted the other final diagnoses (Table 1). Histopathology was performed in only one dog and confirmed idiopathic pulmonary fibrosis.

Comparison of the Imaging-Based Diagnosis with the Endoscopy-Based Diagnosis

Thoracic Radiography Versus Endoscopy

Among the 11 dogs with E-BE, only 2 were also diagnosed with R-BE (18.1%). Similarly, of the 19 dogs with E-BM, 2 had a corresponding diagnosis of R-BM (10.5%). Finally, among the 13 dogs with E-BEBM, 5 were identified as R-BEBM (38.4%). No significant associations were observed for any of the RX findings (non-tapering airways and carina collapse) and the endoscopic diagnoses ($p = 0.81$ and $p = 0.73$, respectively).

Thoracic CT Versus Endoscopy

Thoracic CTs were available in two dogs with E-BE, six dogs with E-BM, and five dogs with E-BEBM. Both dogs with E-BE were also diagnosed with CT-BE. Among the six dogs with E-BM, three were diagnosed with CT-BM (50%). For the five dogs with E-BEBM, only two were diagnosed as CT-BEBM (40%). A significant association was found between carina collapse on thoracic CT and the diagnosis of E-BM and E-BEBM ($p = 0.0015$). While all E-BE dogs in this small sample also showed carina collapse, the limited number (n = 2) likely prevented statistical significance for this group. In contrast, no such association was observed between non-tapering airways on thoracic CT and any of the diagnostic groups ($p = 0.25$). Although all E-BE patients exhibited non-tapering airways, this was not consistent in E-BM and E-BEBM groups, and the small sample size further limits interpretability.

Discussion

The present study reports the clinicopathological and imaging features of 65 dogs with endoscopically diagnosed BE, BM, and BEBM. Across the three groups, clinicopathological differences were minimal. No significant differences in the TCC or neutrophil percentages among the groups was present, but an association was found between the neutrophil count in BALF and the macroscopic inflammatory aspect of the bronchial mucosa. Concomitant bacterial infections were

identified in a minority of cases, with no significant differences in their prevalence between the groups. The clinical relevance of differentiating E-BE, E-BM, and E-BEBM remains thus questionable. Furthermore, the diagnostic association between imaging modalities and bronchoscopy was limited.

Most dogs included in this retrospective study were small breed adult or elderly dogs. The mean age of dogs with E-BE, E-BM, and E-BEBM aligns with earlier reports of BE and BM in dogs, although no significant age differences were found between the three groups (Hawkins *et al.*, 2003; Marolf *et al.*, 2007; Della Maggiore, 2020). Among the clinical signs evaluated, only the presence of lung crackles on auscultation showed a significant difference between the three groups, being more commonly heard in dogs with bronchial collapse (E-BEBM and E-BM) compared to E-BE. This is not surprising, as lung crackles are well documented in dogs with BM and are attributed to the sudden reopening of collapsed small airways as air rushes in during inspiration (Adamama-Moraitou *et al.*, 2012). The low number of lung crackles in dogs with E-BE is quite surprising. In humans with BE, lung crackles are frequently auscultated, primarily due to retained secretions in damaged and dilated bronchi (Nate *et al.*, 1980; Sarkar *et al.*, 2015). Given this well-documented correlation in human patients, a higher occurrence of lung crackles in dogs with E-BE might have been anticipated. Among the 16 dogs diagnosed with E-BE, only 4 showed evidence of mucus accumulation in the bronchi during bronchoscopy, suggesting that secretion retention was not as prominent as typically seen in human cases. Interestingly, of these four dogs, only one also displayed mucus accumulation on CT, indicating lack of concordance between imaging and endoscopic findings. Conversely, another dog exhibited mucus accumulation on CT but not on bronchoscopy, highlighting potential differences in sensitivity between these diagnostic modalities. These findings suggest that either mucus retention is less common in canine E-BE than in human bronchiectasis or that current diagnostic techniques may not be equally effective in detecting secretions in dogs. Interestingly, no significant differences were observed among the three groups in the presence of increased lung sounds, despite such sounds being commonly associated with collapsed airways due to airflow limitation (Sarkar *et al.*, 2015; Wallis *et al.*, 2019). It is worth noting that both human and canine medicine have documented cases of bronchial airflow limitation without the presence of wheezes (Johnson and Pollard, 2010; Sarkar *et al.*, 2015). Finally, as expected, no significant differences were noted between the groups in terms of dyspnoea, which is a common feature in both canine BE and BM (Marolf *et al.*, 2007; Reinero and Masseau, 2021).

In this study, 69.2% of dogs showed macroscopic evidence of airway inflammation during bronchoscopy, but no statistically significant differences in the degree of inflammation were observed between the groups. Similarly, at the microscopic level, BALF analysis revealed increased

TCC in all dogs with no significant differences in median TCC between groups. The more abundant inflammatory cells in BALF across the three groups were neutrophils. This finding aligns with prior studies, such as the one from Bottero *et al.* (2013), who reported a predominance of neutrophilic inflammation in 59 dogs with BM diagnosed by bronchoscopy, and all of them exhibited some degree of macroscopic airway inflammation. Similarly, studies by Hawkins *et al.* (2003) and by Johnson and Pollard (2010) observed that dogs with BE primarily exhibited neutrophilic inflammation, reinforcing the understanding that chronic inflammation is central to the pathogenesis of BE (Boyton and Altmann, 2016). Despite this, the neutrophil counts did not differ significantly between groups, highlighting that macroscopic and microscopic findings alone cannot distinguish BE, BM, and BEBM. Our study also found that neutrophil percentage in BALF was associated with the degree of macroscopic inflammation observed during bronchoscopy. Dogs with more severe macroscopic inflammation exhibited higher neutrophil percentages, confirming that neutrophils play a key role in the inflammation seen in chronic airway diseases (Johnson and Pollard, 2010; Boyton and Altmann, 2016).

Twenty-one dogs did not show any signs of inflammation on bronchoscopy. Of these, one dog received antibiotics at the time of sample collection, while four dogs had been treated with prednisolone within 2 weeks prior to procedure. The absence of inflammation in these dogs may be attributed to the effects of these medications administered before bronchoscopy. Moreover, even after excluding dogs that had received prednisolone or antibiotics prior to presentation, inflammation and BALF neutrophil percentages remained comparable across groups. It is also worth noting that in older dogs with severely affected airways, the macroscopic appearance of the bronchi may not accurately reflect the degree of inflammation, as the tissue may already be damaged or fibrosed. This could lead to a less pronounced inflammatory appearance compared to younger dogs (Mercier *et al.*, 2011). However, the mean age of dogs without bronchial inflammation (8.9 years) was similar to the study population's mean age.

Standard bacterial culture of BALF in dogs with BE and BM has been reported in only a limited number of publications, making the role of bacteria in their development unclear (Hawkins *et al.*, 2003; Johnson and Pollard, 2010; Adamama-Moraitou *et al.*, 2012; Johnson *et al.*, 2016). In this study, 10/65 dogs were diagnosed with concomitant bacterial infection. Human studies have linked species like *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* with BE (Rogers *et al.*, 2014; Flume *et al.*, 2018). Understanding the specific bacterial species involved is crucial for effective management, as organisms like *P. aeruginosa* have significant prognostic implications in human medicine. These infections are linked to more frequent exacerbations, increased sputum production, higher hospitalization rates, and poorer outcomes in humans (Rogers

et al., 2014; Flume et al., 2018). In this study, three dogs tested positive for *P. aeruginosa* (two with BE and one with BEBM), with moderate to severe clinical signs. Follow-up showed one dog being euthanized due to respiratory deterioration, while another showed improvement with antibiotics, but it relapsed after discontinuation. Overall, no significant differences in bacterial infection prevalence were found between the groups, nor was there an association between infection and inflammation severity. The use of antibiotics prior to or at the time of enrolment did not affect results. Considering the low frequency of bacterial infections found in this study, it is more probable that these infections are a result of complications or consequences of BE and/or BM, rather than being the primary cause of these conditions.

This study highlights the diagnostic challenges and limitations of thoracic imaging, particularly radiography, in accurately diagnosing BE, BM, and BEBM in dogs. RXs showed poor diagnostic association with endoscopic findings. Additionally, no significant associations were found between radiographic features (e.g., non-tapering airways and carina collapse) and endoscopic diagnoses. The low detection rate of E-BM as R-BM aligns with earlier research, which reports the sensitivity of RX in identifying bronchial collapse to be markedly lower (50–55%) than that of bronchoscopy or CT (70% and 96.6%, respectively) (Johnson and Pollard, 2010; Johnson et al., 2015). Interestingly, severe cases of BM (classified as BM-III or BEBM-III) consistently exhibited carina collapse on radiographs, suggesting that advanced disease stages may be more readily detectable radiographically. However, the limitations of RX as a primary diagnostic tool for these conditions are increasingly being recognized. Its two-dimensional nature, along with anatomical superimposition, restricts the evaluation of smaller airways, making it less reliable for accurate diagnosis (Hawkins et al., 2003; Reinero and Masseau, 2021). This is particularly evident in dyspneic dogs, where obtaining both inspiratory and expiratory radiographs for comparison is often not feasible, further reducing their diagnostic value. Thoracic CT images showed varying levels of diagnostic association with endoscopy-based diagnoses. Interestingly, CT images demonstrated a significant association with endoscopy in detecting main bronchi collapse, suggesting it may be a more reliable tool for detecting such specific structural bronchial changes compared to radiography. However, no significant association was found between the presence of non-tapering airways on CT and the endoscopic diagnoses. Due to the limited number of dogs undergoing CT in this study, these findings should be interpreted with caution. The comparison between CT and bronchoscopy in diagnosing BE, BM, and BEBM warrants further investigation. Notably, sparse information exists in the veterinary literature regarding imaging criteria (either radiographic or CT-based) for diagnosing BM. In this retrospective study, the presence or absence of BM on CT for example was based solely on the aspect of the main bronchi, excluding the smaller bronchi, which were not assessed by CT. This contrasts

with endoscopic criteria, where both the main and smaller bronchi are considered. Therefore, so far, CT and bronchoscopy should be considered as examinations that complement each other. The BAR is considered a key criterion for diagnosing BE on CT, with a suggested threshold of >2.0 during the expiratory phase (Cannon *et al.*, 2009). In our study, focusing on the expiratory phase alone, a BAR >2.0 was observed in only 30% of measurements in dogs with BE. This contrasts with the findings of Cannon *et al.* (2009), although a later study of Cannon *et al.* (2013) reported that a BAR <2.0 could occasionally be observed during the expiratory phase in dogs with BE. Despite the limited number of dogs that underwent CT in this study, it remains important to consider the classical criteria, such as the presence of non-tapering airways and bronchial wall thickening, in order to make a diagnosis of BE (or BEBM), since a BAR of <2.0 does not necessarily exclude a diagnosis of BE, particularly when other imaging and clinical findings support the diagnosis. In the BEBM group, the median BAR did not exceed 1.5 in any lobe. This may be attributed to the presence of bronchomalacia in this group, which causes greater bronchial collapse during expiration compared to dogs with BE alone. Such collapse may reduce the apparent bronchial diameter, thus lowering the BAR values in dogs with BEBM. However, no significant associations were found between BAR measurements and CT diagnoses of BE or BEBM in this study. When combining the CT-BE and CT-BEBM groups, significant differences in the mean BAR values between inspiratory and expiratory phases were noted in the cranial lung lobes and the right middle lung lobe, suggesting that dynamic airway changes can be detected in these regions. However, no significant differences in BAR measurements across lung lobes were detected within the CT-BE or CT-BEBM groups independently. These findings align with Cannon *et al.* (2009) and Cannon *et al.* (2013), though it is important to note that those studies focused solely on dogs without clinical pulmonary disease and those with BE. Overall, these results suggest that respiratory phase variations may influence BAR measurements and highlight the importance of obtaining paired inspiratory and expiratory CT. The dynamic nature of airway collapse may not be fully appreciated with static imaging alone (Reinero and Masseau, 2021). Despite its clinical utility, the BAR has several limitations that may have influenced measurements in this study. Slice thickness artifacts could have contributed to measurement errors. Additionally, oblique orientation of bronchi and vessels may have affected the accuracy of BAR calculations. The assumption of a normal pulmonary artery in BAR calculations may not always hold true. In diseased lung lobes, pulmonary arterial blood flow may be decreased, with compensatory increased flow to unaffected lobes due to altered regional ventilation. This redistribution of blood flow could result in a higher BAR in diseased lobes and a lower BAR in unaffected lobes, potentially skewing the interpretation of results. Moreover, four dogs in which the BAR was measured showed signs of mild to moderate pulmonary hypertension (PH). The presence

of PH may have significantly influenced BAR measurement, as increased pulmonary arterial pressure leads to pulmonary artery dilation. This arterial enlargement can artificially lower the BAR, even if the bronchial diameter remains unchanged. Consequently, dogs with true BE may present with a falsely normal or low BAR, potentially resulting in the underdiagnosis of BE when relying on BAR criteria alone. Another methodological limitation is the inability to calculate BAR at the exact locations specified by Cannon *et al.* (2009), particularly in the caudal lung lobes. In many dogs, we were unable to measure the BAR at the level of the 11th rib in cross-sectional images because there was no lung parenchyma left at this point, or the lung stopping cranially on some animals due to their small size and/or obesity. In cases where parenchyma was present, the bronchi were often too small (e.g., ~1 mm in diameter), approaching the spatial resolution limits of most clinical CT scanners, which hampered reliable detection of subtle differences between inspiratory and expiratory phases. Consequently, we initially attempted to perform BAR measurements at more cranial levels in these lobes. However, this may have affected measurement accuracy, as caudal lung regions might exhibit less dynamic airway variation due to differences in compliance, airway morphology, and gravitational influence. The cranial shift in measurement location also meant that smaller bronchi were assessed, which may inherently show less collapsibility and thus reduce the likelihood of detecting significant differences. Given these combined challenges, we ultimately chose not to include BAR measurements from the caudal lung lobes.

This study contains some limitations. First, during the time period of the retrospective study, bronchoscopies were realized by various specialists without standardized recording protocol. Parts of the lower airways may not have been recorded leading to misclassification of the patients. However, this limitation is of minor importance in our opinion. Recordings might not be standardized, and thus, the complete respiratory tract might not be visualized, but it is a general accepted rule under the different clinicians that all macroscopic abnormalities are recorded and can thus be evaluated retrospectively. Secondly, the grading system, only based on endoscopy videos, remains a subjective evaluation, even though all videos were evaluated independently by two diplomates experienced in respiratory medicine. In 16 out of 65 dogs, interobserver disagreement occurred, necessitating a re-evaluation by both observers together. This was followed by a consensual appraisal conducted blindly to the previous assessments. Next, due to the retrospective nature of this study, no standardized validated questionnaire was used to evaluate the degree and severity of the clinical signs, which remained thus subjective. Additionally, due to the retrospective nature of this study, dogs receiving anti-inflammatory drugs and antimicrobials were included. The only dog receiving antimicrobial treatment was excluded from the statistical analysis

related to microbiological and inflammation data. However, the 13 dogs that had been on anti-inflammatory drugs within the two weeks prior to presentation were not excluded, and their results regarding inflammation and clinical signs may have been influenced by the recent drug administration. Moreover, the depth of anesthesia was not standardized, and in some cases, it was more superficial, leading to occasional coughing during video recording. While we made efforts to avoid capturing videos during coughing, this remains a minor limitation that could potentially affect image quality and interpretation, as we were focusing on dynamic images. Lastly, CT allows for quantitative measurement such as the BAR. However, a limitation of a quantitative measure such as the BAR is interobserver variability. In the majority of the cases, the margins of the bronchus and the airways were well delineated, so the chance of measurement error was less likely. In order to confirm objectively bronchial collapsibility during paired CT, the bronchial collapsibility can be calculated (Yoon *et al.*, 2020). This feature was not included during re-evaluation of the CT images in this study.

Conclusion

This study describes an association between BE and BM based on endoscopy findings. However, it highlights limited clinicopathological distinctions between endoscopy-based diagnoses of BE, BM, and BEBM, as well as the absence of significant differences in BALF analysis and bacteriological findings across the three groups. The limited diagnostic agreement between imaging modalities and bronchoscopy further highlights the challenges in differentiating these conditions based solely on clinical, endoscopic, and imaging data. These findings raise questions about the clinical relevance of distinguishing between these endoscopic diagnoses, as they currently do not provide clear guidance for selecting specific treatment strategies. Treatment decisions should thus rely on a thorough assessment of all available data, including BALF cytological analysis, bacterial culture results on BALF, and qPCR results on BALF, rather than strictly relying on the endoscopic diagnosis. Importantly, these results do not support the routine use of antibiotics for all animals with these diagnoses.

Prospective studies are needed to determine whether therapeutic approaches and follow-up care should vary between these entities and to evaluate the impact of tailored treatments on clinical outcomes. While the macroscopic endoscopic distinctions between the three groups may not appear clinically relevant at this stage, future investigations may reveal a role for these differences in guiding treatment strategies.

Experimental section

Study 2 :

Proposed treatment algorithms for dogs with chronic bronchitis
associated with irreversible airway changes: bronchiectasis and/or
bronchomalacia

Preface

Chronic bronchitis (CB) is a common cause of chronic cough in dogs and may be associated with structural airway abnormalities such as bronchiectasis (BE) and bronchomalacia (BM). Although these conditions are increasingly recognized in veterinary medicine, clear and standardized treatment guidelines are currently lacking. As a result, therapeutic management often varies between clinicians and is frequently based on empirical decisions.

In human medicine, treatment algorithms integrating antimicrobial stewardship and targeted anti-inflammatory therapy have been developed for chronic airway diseases. However, comparable structured approaches are largely absent in veterinary medicine.

This study aimed to propose a structured approach for dogs with CB associated with BE and/or BM. Two treatment algorithms were developed based on the presence or absence of bacterial infection identified by cytology, culture, or qPCR. By prioritizing objective diagnostic criteria and a stepwise therapeutic strategy, this approach supports rational antimicrobial use and targeted anti-inflammatory management, providing a practical framework for clinical application and future validation.

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Experimental section

Study 2 :

Proposed treatment algorithms for dogs with chronic bronchitis
associated with irreversible airway changes: bronchiectasis and/or
bronchomalacia

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Abstract

Chronic bronchitis (CB) in dogs involves persistent inflammation of the bronchial walls and excessive mucus production within the airways, with or without bronchial infection, and may lead to degenerative airway changes such as bronchiectasis (BE) and bronchomalacia (BM). Standardized treatment protocols for CB with concurrent BE and/or BM (BEBM) are lacking. This article proposes a therapeutic approach for dogs with CB and BEBM, based on veterinary literature and relevant human medical data. Two treatment algorithms are outlined, depending on the presence or absence of cytological evidence of bacterial infection in bronchoalveolar lavage fluid (BALF) and/or bronchial brush samples. For cases with suspected infection, indicated by intracellular bacteria on cytology, first-line therapy with oral doxycycline is recommended pending BALF culture and quantitative polymerase chain reaction (qPCR) results. If warranted, antibiotic therapy should be escalated stepwise after culture/qPCR confirmation, in accordance with antimicrobial stewardship principles. In non-infectious inflammatory cases, inhaled glucocorticoids are advised as first-line therapy and may also be used in infectious cases unresponsive to antibiotics alone. Mucoactive agents and cough suppressants are not recommended in the initial protocol but may be considered as adjunctive, symptom-targeted treatments on a case-by-case basis, avoiding unnecessary or unsupported interventions. These proposed algorithms are not intended as definitive clinical guidelines, but as a starting point for discussion and future validation. They emphasize rational and prudent use of antibiotics, alone or alongside anti-inflammatory therapy, to improve patient outcomes while minimizing antimicrobial resistance risks. Further research is needed to assess the long-term efficacy of this approach.

1 Introduction

2 Chronic bronchitis (CB) in dogs is characterized by persistent inflammation of the bronchial walls
3 and excessive mucus production within the lumen of the airways, with or without concurrent
4 bronchial infection, and may progress to degenerative airway changes such as bronchiectasis (BE)
5 and bronchomalacia (BM) (Hawkins *et al.*, 2003; Boyton and Altmann, 2016; Rozanski, 2020; Reinero
6 and Masseau, 2021). BE is characterized by a progressive and irreversible dilatation of the bronchial
7 wall while BM is defined as a regional to diffuse dynamic airway collapse of segmental bronchi,
8 subsegmental bronchi, or both (Hawkins *et al.*, 2003; Boyton and Altmann, 2016; Rozanski, 2020;
9 Reinero and Masseau, 2021). BE is reported in both human and canine patients, while BM is mostly
10 observed in canine medicine and only sporadically described in human paediatric cases (Wallis *et*
11 *al.*, 2019; Reinero and Masseau, 2021). The pathogenesis of both BE and BM remains unclear, but
12 current evidence suggest they result from a complex interplay of chronic infection, inflammation,
13 degenerative changes and impaired mucociliary clearance (Marolf *et al.*, 2007; Boyton and Altmann,
14 2016; Della Maggiore, 2020).

15 Chronic bacterial infection is a hallmark of BE in humans, significantly influencing disease
16 progression (Rogers *et al.*, 2014). Currently, no standardized clinical guidelines define the
17 therapeutic approach for managing BE and BM in humans or dogs (Flume *et al.*, 2018; Wallis *et al.*,
18 2019; Reinero and Masseau, 2021). Treatment guidelines for human BE, such as those from the
19 European Respiratory Society (ERS, 2017) and the British Thoracic Society (BTS, 2019), rely primarily
20 on expert opinion and low-quality evidence from small randomized trials (Polverino *et al.*, 2017; Hill
21 *et al.*, 2019). Current medical management of BE and BM in humans and dogs includes antibiotics,
22 oral or inhaled anti-inflammatory agents, antitussives, bronchodilators, antimuscarinic agents, or
23 mucolytics, though robust evidence supporting their efficacy is lacking (Polverino *et al.*, 2017; Wallis
24 *et al.*, 2019; Reinero and Masseau, 2021). Despite limited evidence of bacterial infection in canine BE
25 and/or BM, antibiotics are often used empirically, raising antimicrobial stewardship and One Health
26 concerns (Hawkins *et al.*, 2003; Johnson *et al.*, 2019; Reinero and Masseau, 2021; Lyssens *et al.*,
27 2025a, Lyssens *et al.*, 2025b).

28 Based on the current available knowledge from veterinary and human literature, the aim of this
29 article is to propose two standardized treatment protocols for dogs affected with CB and airways
30 changes including BE and/or BM with or without concurrent airway bacterial infection.

1 Reaching a diagnosis

2 The diagnostic process for CB associated with BE and/or BM in dogs requires a systematic approach
3 to confirm these conditions while excluding other common causes of chronic cough that may
4 necessitate specific treatments, such as cardiac disease, neoplasia, parasitic infection, or tracheal
5 collapse (Rozanski, 2020). Moreover, known underlying causes of BE, such as aspiration-related lung
6 injury, inhaled foreign bodies, parasitic or fungal infections, idiopathic eosinophilic
7 bronchopneumopathy, idiopathic pulmonary fibrosis, and primary ciliary dyskinesia should be
8 considered and systematically ruled out. Such diseases require a specific diagnostic and therapeutic
9 approach, which is beyond the scope of this article. For an in-depth description of the diagnostic
10 process, readers are directed to other relevant publications (Hawkins *et al.*, 2003; Clercx and Peeters,
11 2007; Johnson *et al.*, 2013; Merveille *et al.*, 2014; Roels *et al.*, 2017; Mortier *et al.*, 2018; Nafe *et al.*,
12 2018; Della Maggiore, 2020; Yang *et al.*, 2020; Reinero and Masseau, 2021, Lyssens *et al.*, 2025a). The
13 diagnosis of BE and BM is ideally established using a combination of diagnostic imaging, such as
14 computed tomography, together with bronchoscopy (Hawkins *et al.*, 2003; Reinero and Masseau,
15 2021). Based on bronchoscopy, dogs diagnosed with BM can be subclassified into three
16 subcategories based on a previously described grading system: 25%–50% reduction in airway
17 diameter, 50%–75% reduction in airway diameter and >75% reduction in airway diameter (Reinero
18 and Masseau, 2021). Results of quantitative polymerase chain reaction (qPCR) assays on
19 bronchoalveolar lavage fluid (BALF) for lower respiratory pathogens including *Bordetella*
20 *bronchiseptica* (Bb), *Mycoplasma cynos* (Mc), *Angiostrongylus vasorum*, and *Crenosoma vulpis* may
21 aid in diagnosis. It is important to note that the causal relationships between CB, BE, and/or BM in
22 dogs remain unclear. While CB-related inflammation may contribute to BE and BM, the reverse is
23 also plausible. Many canine BE cases remain idiopathic (Hawkins *et al.*, 2003). Therefore, the
24 associations described in this study should be interpreted with caution. Dogs without cytological
25 signs of inflammation [total cell count (TCC) < 600 cells/ μ L and neutrophil percentage in BALF <12%
26 of TCC] are outside the scope of this protocol. Also, patients with BE or BM and concurrent bacterial
27 pneumonia were not included, as these conditions represent a distinct disease process requiring a
28 different diagnostic and therapeutic approach.

1 Proposal of treatment approaches

2 The present proposal was developed through consensus among 4 diplomates of the European
 3 College of Veterinary Internal Medicine (ECVIM), each with experience in respiratory clinical practice
 4 (ranging from 5 years to over 30 years). The proposal includes two distinct treatment algorithms:
 5 one for dogs with CB and evidence of BE and/or BM where bacterial infection is confirmed based on
 6 BALF and/or bronchial brushing cytology (Figure 1), and another for non-infectious inflammatory
 7 cases (Figure 2). This distinction reflects the necessity of tailoring clinical management. The
 8 numbered steps in brackets in the algorithm correspond directly to the clinical criteria and
 9 treatment considerations detailed in the main text, and should be interpreted in conjunction with
 10 it. For clarity, throughout this section, the term ‘improvement’ is used to describe a reduction in the
 11 frequency and severity of clinical signs, together with an improvement of the dog’s quality of life.

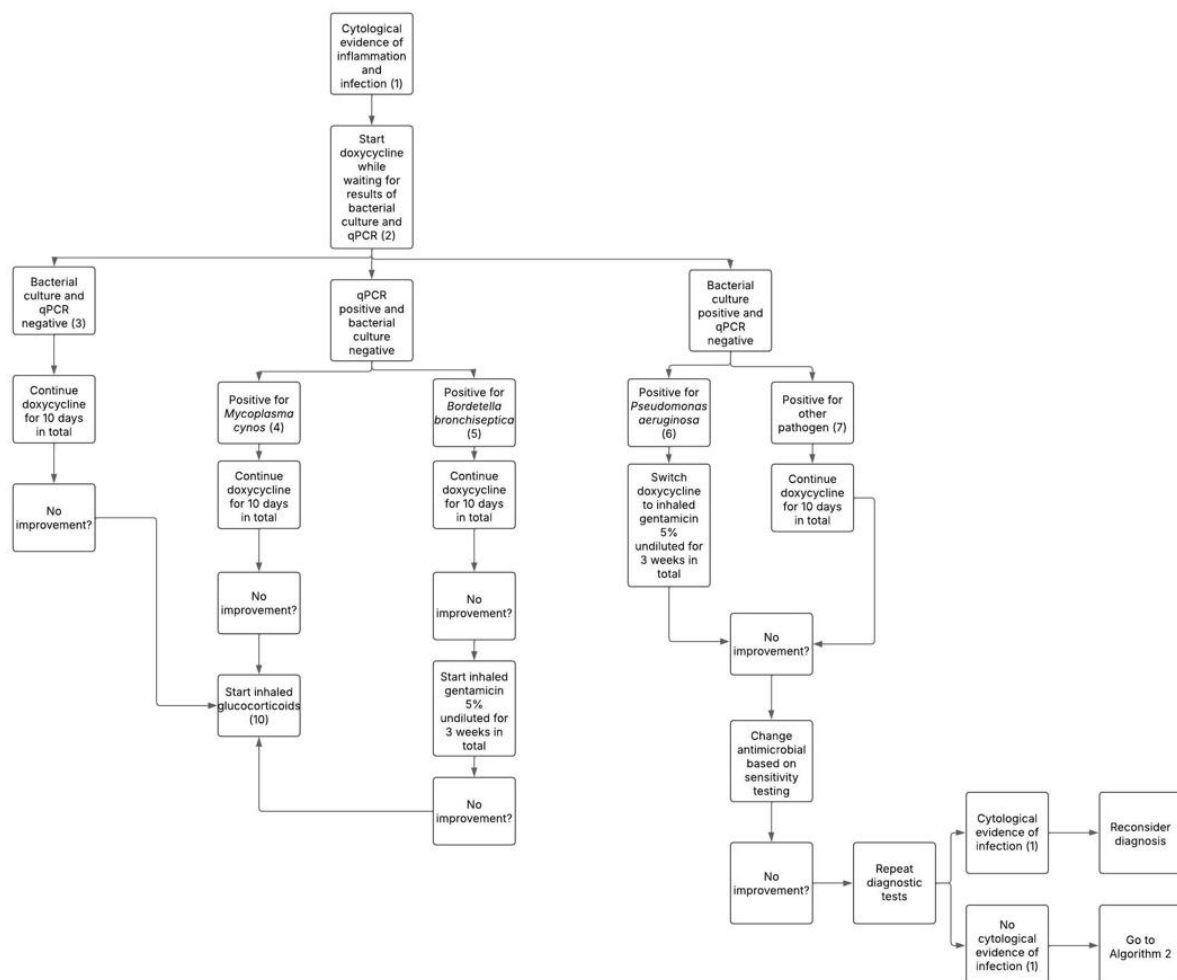


Figure 1. Proposed treatment protocol for dogs with chronic bronchitis associated with bronchiectasis and/or bronchomalacia in the presence of overt infection based on bronchoalveolar lavage fluid cytology. Numbers in parentheses refer to explanatory notes within the main text.

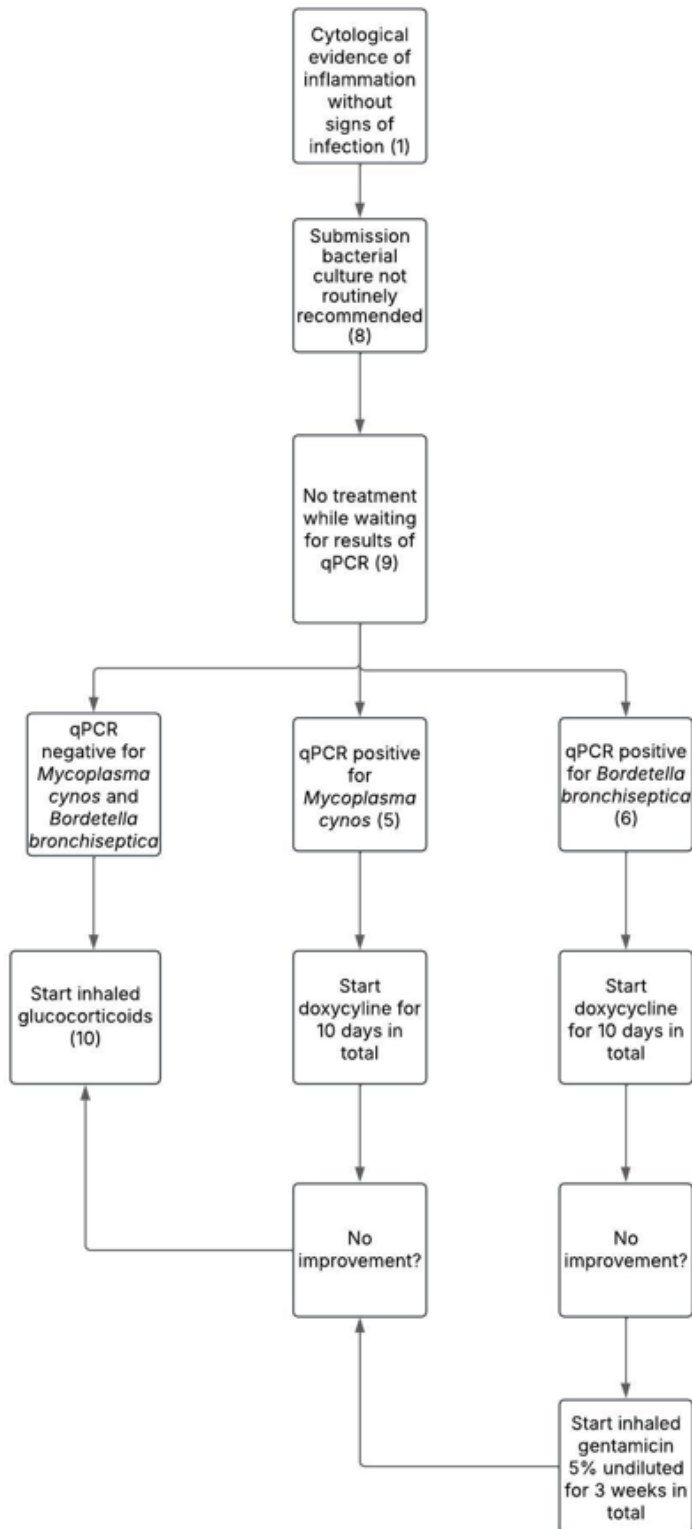


Figure 2. Proposed treatment protocol for dogs with chronic bronchitis associated with bronchiectasis and/or bronchomalacia in the absence of overt infection based on bronchoalveolar lavage fluid cytology. Numbers in parentheses refer to explanatory notes within the main text.

Cytological evidence of bacterial infection, evaluated either in-house when expertise is available on site or via an external laboratory, is primarily based on the identification of bacteria within the cytoplasm of airway neutrophils (intracellular bacteria) on cytological evaluation of BALF and/or bronchial brushing. When possible, bacteria should also be classified either using Gram-coloration or bacteria morphology, as this can provide useful preliminary information about the likely bacterial pathogens involved and interpretation of bacterial culture results. This criterion, used to distinguish infectious from non-infectious cases, forms the basis for selecting between the two proposed treatment algorithms (Note 1). It is essential to interpret cytological findings in the context of any recent or ongoing antimicrobial treatment, as prior antibiotic use may suppress visible cytological indicators of infection and lead to false-negative interpretations. Clinical judgment and further diagnostics should guide management in these cases.

If overt signs of infection are observed on BALF and/or bronchial brush cytology, initiating antimicrobial therapy promptly is recommended to prevent complications or disease progression. Doxycycline (10 mg/kg SID), the first-line empirical treatment for bacterial bronchitis recommended by the International Society for Companion Animal Infectious Diseases (ISCAID) guidelines, is effective against a wide range of canine respiratory pathogens and is associated with a low incidence of adverse effects (Lappin *et al.*, 2017) (Note 2).

In cases of negative culture and qPCR results on BALF, a 10-day course of doxycycline is recommended (Lappin *et al.*, 2021). False-negative results can occur due to prior antibiotic use, which may suppress or eliminate bacterial growth, reducing microbial load below detection thresholds (Leekha *et al.*, 2011). Sampling limitations such as insufficient BALF volume or collection from unaffected lung regions may also compromise diagnostic sensitivity (Patel *et al.*, 2024). Laboratory errors such as processing delays or suboptimal transport conditions are additional sources of false-negative results (Patel *et al.*, 2024). A positive clinical response within 7–10 days supports a diagnosis of bacterial infection; antibiotic treatment should then be continued for 1 week beyond clinical resolution (Lappin *et al.*, 2017). Conversely, lack of improvement suggests that infection is unlikely the primary cause of the clinical signs (Note 3).

A positive qPCR result for Mc despite negative culture results, is not unexpected as Mc requires specialized culture media but can be reliably detected by qPCR (Hong and Kim, 2012). A Ct value below 34 is considered a positive result (Canonne *et al.*, 2016). Doxycycline is recommended as the first-line antimicrobial treatment for Mc infection in dogs (Note 4) (Lappin *et al.*, 2017). If no improvement occurs, treatment may be extended or the diagnosis reconsidered, including repeating diagnostic tests to confirm the presence of Mc or identify other respiratory pathogens (Lappin *et al.*, 2017). Fluoroquinolones and azithromycin have shown *in vitro* efficacy against

Mycoplasma spp. and resistance to doxycycline has been documented in some *Mycoplasma* species in other animals (Kenny and Cartwright, 1991; Cooper *et al.*, 1993; Gautier-Bouchardon, 2018). However, no resistance to doxycycline in canine *Mycoplasma* species has been reported (Framst *et al.*, 2025). It is important to note that some *Mycoplasma* spp. may be found in the lower airways of healthy dogs, suggesting they can be part of the commensal microbiota (Chalker *et al.*, 2004). However, Mc is rarely identified in healthy individuals and is more consistently associated with respiratory disease, indicating its likely pathogenic role (Chalker *et al.*, 2004). Lack of clinical response to doxycycline suggests that inflammation, rather than infection, is the predominant cause of signs. This is frequently observed in chronic airway diseases such as BE and/or BM, where non-infectious inflammation may persist. In these cases, treatment should be redirected toward inhaled glucocorticoids (Note 10). Other *Mycoplasma* species, such as *Mycoplasma canis*, were not considered in this algorithm due to its currently unproven pathogenic role in canine respiratory disease (Chalker, 2005).

Doxycycline is also recommended as the first-line antimicrobial treatment for Bb infection in dogs, following the same dosage regimen as for Mc (Lappin *et al.*, 2017) (Note 5). However, studies suggest that doxycycline may not achieve therapeutic concentrations at the apical surface of the bronchial epithelium, limiting its effectiveness in treating infections within the airway lumen (Steinfeld *et al.*, 2012). Aerosolized delivery of 3-5 mL (depending on the speed of the nebulizer to aerosolize the product) of 5% undiluted gentamicin via a mask during a minimum of 10 min, twice daily for 3 weeks, has been shown to effectively induce clinical cure in dogs with Bb infection (Canonne *et al.*, 2020). For small dogs, nebulization can alternatively be performed by placing the animal in a closed container or box covered with a towel to retain the aerosol. Additionally, a 3-min inhalation protocol was investigated, confirming its efficacy and safety (Lyssens *et al.*, 2024). If there is no improvement following treatment with oral doxycycline and subsequent inhaled gentamicin, inflammation rather than active infection is likely responsible for the clinical signs and treatment with inhaled glucocorticoids is recommended (Note 10).

Haemophilus influenzae and *Pseudomonas aeruginosa* (*P. aeruginosa*) are among the most common pathogens identified in humans with BE, with *P. aeruginosa* being linked to more severe disease and frequent exacerbations (Rogers *et al.*, 2014). Given its clinical impact and intrinsic resistance profile, targeted management is required when this organism is detected (Choi *et al.*, 2024). In cases of *P. aeruginosa* infection, defined as clinically relevant growth exceeding 1.7×10^3 colony-forming units per milliliter (CFU/mL) of BALF (Peeters *et al.*, 2000), doxycycline should be stopped and switched to inhaled aminoglycosides since *P. aeruginosa* is intrinsically resistant to tetracyclines (Morita *et al.*, 2014) (Note 6). In humans with BE, current treatment guidelines for *P. aeruginosa* infections

recommend inhaled aminoglycoside antibiotics or, if not tolerated, macrolides (Polverino *et al.*, 2017; Hill *et al.*, 2019). Treatment duration typically ranges from 6 weeks to 3 months (Polverino *et al.*, 2017; Hill *et al.*, 2019). In this proposal, a 3-week treatment is recommended, followed by reassessment. If improvement is absent or partial, the antimicrobial regimen should be adjusted based on culture and sensitivity testing, prioritizing oral drugs with proven efficacy in penetrating the blood-bronchus barrier (BBB), such as macrolides, fluoroquinolones, tetracyclines and lincosamides (Rodvold *et al.*, 2011; Lappin *et al.*, 2017). If no improvement is observed, diagnostic tests, such as repeat imaging and BALF analysis, should be repeated to confirm *P. aeruginosa* persistence, detect other pathogens or reveal other pathologies. Treatment can be extended up to 6 weeks, with evaluations and repeat testing every 3 weeks as clinically indicated.

For bacterial infections not caused by *P. aeruginosa*, based on a positive culture exceeding the clinical relevance threshold of 1.7×10^3 CFU/ μ L (Peeters *et al.*, 2000), first-line treatment in humans consists of oral macrolides such as azithromycin or erythromycin, followed by inhaled gentamicin or doxycycline (Polverino *et al.*, 2017; Hill *et al.*, 2019). Based on this rationale, continuation of doxycycline is recommended while monitoring the patient's clinical response (Note 7). If no improvement is seen after 7–10 days, the antimicrobial regimen should be tailored based on culture and sensitivity testing, prioritizing oral drugs known to effectively penetrate the BBB (Lappin *et al.*, 2017).

If no intracellular bacteria are seen on BALF cytology and/or bronchial brushing (Note 1), bacterial culture is generally not recommended, particularly when cytological evaluation can be performed immediately on-site by experienced personnel, as this allows rapid triage and avoids unnecessary costs. Conversely, in the absence of on-site cytological expertise, it is advisable to submit samples for both cytology and bacterial culture immediately. This ensures that, if intracellular bacteria are later identified on external cytology review, the corresponding sample has already been processed for culture, thereby avoiding delays and potential loss of bacterial viability (Note 8). In either case, antimicrobial therapy should be withheld pending qPCR and/or bacterial culture results to support antimicrobial stewardship principles (Gerding, 2001) (Note 9). Importantly, a positive bacterial culture alone, without cytological evidence of infection, does not confirm infection, as the lungs are not sterile and bacteria may be present without causing disease (Ericsson *et al.*, 2016).

If bacterial infection is excluded and qPCRs are negative, inhaled glucocorticoids should be initiated (Note 10). In human medicine, anti-inflammatory use in BE is controversial (Polverino *et al.*, 2017; Hill *et al.*, 2019). ERS and BTS guidelines caution against long-term corticosteroids due to unclear benefits and frequent side effects (Polverino *et al.*, 2017; Hill *et al.*, 2019). Similarly, there is no evidence supporting their use in either human or canine BM (Wallis *et al.*, 2019; Reinero and Masseau,

2021). However, glucocorticoids have been shown to be effective in managing CB in dogs (Rozanski, 2020; Reiner and Masseau, 2021; Chan *et al.*, 2023). Given that CB frequently coexists with BE and BM, particularly in the canine population, the use of corticosteroids may still be appropriate to target the inflammatory component. While oral glucocorticoids provide rapid short-term relief, clinical signs often relapse after discontinuation, necessitating long-term use (Chan *et al.*, 2023). Prolonged oral therapy carries a risk of systemic side effects (Elkholly *et al.*, 2020). Although inhaled formulations may also lead to systemic side effects with long-term use, they are generally preferred in CB (Chan *et al.*, 2023). Fluticasone propionate by inhalation via a spacer, with a starting dose of 1 puff of 100 µg twice daily for 6 to 8 breaths in dogs weighing less than 20 kg, and 2 puffs of 100 µg (total dose 200 µg) twice daily for 6 to 8 breaths in dogs weighing over 20 kg should be considered (Chan *et al.*, 2023). In the long-term, the dose may be gradually tapered every 3–4 weeks based on the dog's clinical response to determine the optimal dosing regimen to control the clinical signs without causing substantial side effects (Chan *et al.*, 2023). Although most studies have focused on this dry aerosol formulation delivered via a spacer, other inhaled corticosteroids (e.g., budesonide) may also be used, depending on availability and clinical preference (Van den Bosch *et al.*, 1993; Melamies *et al.*, 2012). However, literature on this subject in veterinary medicine is lacking.

Discussion

This study proposes two structured treatment algorithms for dogs with CB associated with BE and/or BM, providing a practical approach based on the cytological presence or absence of bacterial infection. These protocols support evidence-based clinical decision-making, emphasizing the judicious use of antimicrobials and glucocorticoids, aiming to optimize treatment outcomes minimizing unnecessary medication use.

Determining true infection and the need for antibiotic therapy in dogs with BE and/or BM remains a clinical challenge, particularly due to overlap in BALF characteristics cytological findings between infectious and non-infectious inflammation. In this study, infection is defined as the concurrent presence of intracellular bacteria on BALF cytology and/or bronchial brushing, thereafter supported by a positive bacterial culture and/or qPCR results. This multimodal definition aims to increase diagnostic confidence while minimizing unnecessary antimicrobial use. However, each of these markers alone has limitations. For instance, cytology may underestimate infections: intracellular bacteria were found in only 32%–79% of culture-confirmed BALF samples in dogs with lower respiratory disease (Van den Bosch *et al.*, 1993; Peeters *et al.*, 2000; Johnson *et al.*, 2013).

Furthermore, pathogens such as Mc and Bb are poorly visualized on cytology due to their structural characteristics (Johnson *et al.*, 2013; Canonne *et al.*, 2020). In these cases, bronchial brushing or qPCR may enhance detection (Canonne *et al.*, 2016). Similarly, relying solely on semiquantitative bacterial culture thresholds can be misleading. A recent study showed that although a cut-off of 1.7×10^3 CFU/mL is commonly used to define clinically significant bacterial growth, nearly half of the dogs with clinical and cytological signs of infection had lower CFU counts (Lebastard *et al.*, 2022). Moreover, dogs with high CFU counts but lacking cytological signs of infection may be colonized rather than infected (Ericsson *et al.*, 2016). Therefore, this approach uses the 1.7×10^3 CFU/mL threshold as a reference point, but not as a strict criterion. Importantly, cultures resulting solely from enrichment procedures should be interpreted with caution, as they may reflect contamination rather than true infection (Smith *et al.*, 2014).

The use of inhaled glucocorticoids in non-infectious cases is a key component of the proposed treatment protocol. Although there is currently no evidence-based data supporting their efficacy in CB cases associated with BE and/or BM, their benefit has been demonstrated in cases of CB without dynamic airway collapse (Chan *et al.*, 2023). Given that CB commonly coexists with BE and/or BM, and shares similar inflammatory mechanisms, it is reasonable to expect a therapeutic benefit in these patients as well. This expectation is further supported by the clinical experience of the authors, who have observed improved outcomes and quality of life in affected dogs treated with inhaled glucocorticoids.

Other medications, such as mucoactive agents, cough suppressants, and bronchodilators, are not included in the proposed treatment protocols due to limited and inconclusive evidence supporting their routine use in dogs with CB and BE and/or BM (Reinero and Masseau, 2021). However, cough suppressants may be considered for non-inflammatory forms of CB (Reinero and Masseau, 2021). While mucoactive agents may theoretically help with thick mucus and cough suppressants may alleviate severe cough, both of which can significantly affect quality of life, their efficacy remains unproven, and guidelines from human medicine do not strongly support their use (Polverino *et al.*, 2017; Hill *et al.*, 2019; Wallis *et al.*, 2019; Reinero and Masseau, 2021). Therefore, these treatments are reserved as adjunct options outside the standard protocols presented here.

Despite its structured approach, this protocol has limitations. A key limitation of this work is that the proposed protocol arises from joint discussions among a small group of 4 ECVIM diplomates, all with a particular interest in respiratory medicine. While it reflects expert clinical opinion, it does not yet represent a broader consensus. Establishment of a multi-institutional panel in order to critically evaluate and validate the relevance and applicability of this protocol would be interesting.

Secondly, the proposed protocol relies mainly on limited veterinary literature and extrapolations from human medicine. It is primarily based on the authors' clinical expertise, reflecting expert opinion rather than strong, high-level evidence. Another limitation is that access to equipment and expertise for BALF analysis is generally restricted to university or specialized centers, limiting the applicability of the proposed algorithm to well-equipped referral practices.

To conclude, this protocol emphasizes judicious antibiotic use, targeted bacterial infection management, and anti-inflammatory treatment, while minimizing antibiotic resistance risks. It is ready for use in future prospective studies in dogs with CB associated with BE and or BM to assess its clinical safety and efficacy. These studies should not only evaluate clinical outcomes but also consider quality of life. The protocol may furthermore be applicable to cases of CB without degenerative airway changes.

Experimental section

Study 3 :

Increased neutrophil extracellular traps formation in the
bronchoalveolar lavage fluid of dogs with bronchiectasis

Preface

Chronic bronchial diseases, including bronchiectasis (BE) and bronchomalacia (BM), are frequently encountered in dogs presenting with chronic cough. Although these conditions are well described morphologically, their underlying pathogenesis remains incompletely understood, and increasing evidence suggests that BE and BM may represent overlapping disease processes rather than distinct clinical entities.

In human medicine, neutrophil-driven inflammation plays a central role in chronic airway diseases, with growing attention given to the contribution of neutrophil extracellular traps (NETs). These structures, composed of DNA and antimicrobial proteins, are involved in host defense but may also contribute to tissue damage when excessively produced. Despite their recognized importance in human respiratory diseases, the role of NETs in canine BE and BM has not been previously investigated.

This study aimed to investigate NETs in dogs with chronic bronchial disease associated with BE and/or BM. Using cell-free DNA, MPO-DNA ELISA and immunofluorescence, NETs were detected and quantified in BALF. Cell-free DNA concentrations were found to be higher in dogs with bacterial bronchopneumonia compared to dogs with chronic bronchial disease or healthy dogs. MPO-DNA complexes were higher in dogs with chronic bronchial diseases compared to healthy dogs, with dogs with bronchiectasis showing the greatest increase in MPO-DNA complexes. The results suggest that NET formation may contribute to chronic airway inflammation and could play a role in the pathogenesis of these conditions, in particular in BE.

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Experimental section

Study 3 :

Increased neutrophil extracellular traps formation in the
bronchoalveolar lavage fluid of dogs with bronchiectasis

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Abstract

Chronic bronchial diseases (CBD) associated with bronchiectasis (BE) and/or bronchomalacia (BM) are commonly diagnosed in dogs. Their pathogenesis remains incompletely understood, and recent studies have questioned the clinical relevance of distinguishing BE and/or BM, suggesting that their pathogenic mechanisms may overlap and that differentiating between them may have limited clinical significance. To date, the role of neutrophil extracellular traps (NETs) in BE and/or BM remains undetermined. This study aimed to validate NETs detection techniques in banked canine bronchoalveolar lavage fluid (BALF) using healthy(H) dogs, as negative controls, dogs with bacterial bronchopneumonia (BBP), as positive controls, and to compare NET detectability in dogs with CBD associated with BE and/or BM. A retrospective observational study was conducted using client-owned dogs presented with CBD and BE, BM, or BEBM based on endoscopic findings, or BBP. NETs were measured in BALF by quantification of cell-free DNA (cfDNA), detection of myeloperoxidase (MPO)-DNA complexes via enzyme-linked immunosorbent assay (ELISA) and by confocal laser scanning microscopy of immunofluorescent labelled samples. cfDNA concentrations were significantly higher in BBP dogs than in CBD or H-dogs, with no significant differences among the CBD groups. MPO–DNA complex levels were significantly higher in dogs with CBD and BBP than in H-dogs, with BE dogs showing the greatest increase compared with H and BM dogs, and BEBM dogs also exceeding H-dogs. Across all dogs, MPO–DNA complexes correlated positively with total and absolute neutrophil counts, neutrophil percentage, cfDNA concentrations, and duration of clinical signs. These associations varied between groups, with the strongest and most consistent correlations observed in BE-dogs. This study indicates that NETs are detected and quantifiable in the BALF of dogs with CBD and BE and/or BM and dogs with BBP. Their presence suggests that NET formation may play a role in the pathogenesis of CBD, especially in cases involving BE. Further research is needed to explore the role of NETs as well as the therapeutic potential of NET modulation in respiratory diseases.

1 Introduction

2 Chronic bronchitis (CB), frequently diagnosed in both human and veterinary medicine, is
3 characterized by chronic inflammation and excessive mucus production (Rozanski, 2020). In dogs,
4 such persistent inflammation can lead to degenerative bronchial changes, including bronchiectasis
5 (BE) and bronchomalacia (BM), or both (BEBM) (Hawkins *et al.*, 2003; Boyton and Altmann, 2016;
6 Reinero and Masseau, 2021; Lyssens *et al.*, 2025). The exact pathogenesis of BE and/or BM is not fully
7 understood, although morphological differences have been identified between BE and BM using
8 various imaging modalities, such as thoracic radiography, computed tomography, and
9 bronchoscopy (Hawkins *et al.*, 2003; Reinero and Masseau, 2021), only limited clinical and
10 pathological differences between dogs with BE and/or BM have been found (Lyssens *et al.*, 2025),
11 raising questions about the clinical relevance of distinguishing these entities. This suggests that the
12 underlying pathogenic mechanisms may potentially overlap, while factors leading to one or another
13 of these specific conditions remain yet to be discovered. Since neutrophilic inflammation is
14 commonly observed in CB, the role of neutrophil extracellular traps (NETs) is gaining more and more
15 attention in human medicine (Rozanski, 2020; Nery Porto and Tetelbom Stein, 2016; Keir and
16 Chalmers, 2022). NETs are web-like structures, composed of DNA fibers coupled to citrullinated
17 histone 3 (cit-H3), and antimicrobial proteins such as myeloperoxidase (MPO), neutrophilic elastase
18 (NE), cathepsin G, LL37 and defensins, that entrap bacteria, viruses, protozoa and fungi (Brinkmann
19 *et al.*, 2004; Kano *et al.*, 2016; Masucci *et al.*, 2020; Janssen *et al.*, 2022). Hereby, pathogens are
20 immobilized and exposed to a locally high and lethal concentration of antimicrobial proteins (Fuchs
21 *et al.*, 2010; Kano *et al.*, 2016; Masucci *et al.*, 2020; Keir *et al.*, 2022; Janssen *et al.*, 2022). However,
22 excessive NETs production or inadequate NETs clearance may be harmful and induce deleterious
23 effects on the host tissue (Wright *et al.*, 2016; Papayannopoulos, 2018; Janssen *et al.*, 2022). In human
24 medicine, it has been shown that NETs formation plays an important role in the pathogenesis of
25 many chronic neutrophilic lung diseases such as asthma, cystic fibrosis and, more recently, BE (Nery
26 Porto and Tetelbom Stein, 2016; Keir and Chalmers, 2022). The role of NETs in BM and in bacterial
27 bronchopneumonia (BBP) remains unclear. Studies in murine models of BBP have demonstrated
28 NETs formation, particularly in infections that cause significant lung injury and necrosis, such as
29 *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, and *Escherichia coli* (Sanders *et al.*, 2022).
30 Additionally, research on humans with ventilator-associated pneumonia has shown increased NETs
31 in the alveolar spaces (Mikacenic *et al.*, 2018). These data indicate that acute bacterial infection of
32 the lower airways is a potent natural trigger of NETs release. Limited literature is available on NETs
33 formation in BM, likely because this condition rarely occurs in humans. In veterinary medicine, NETs

have been described in blood, tracheal wash fluid, and effusion samples, but exclusively in the context of systemic, infectious, or non-respiratory diseases, including sepsis, immune-mediated hemolytic anemia, canine leishmaniosis, *Trypanosoma cruzi* infection, trauma, and heat stroke, as well as in cats with FeLV infection and in horses with asthma (Wardini *et al.*, 2010; Kano *et al.*, 2016; Jeffery *et al.*, 2017; Pereira *et al.*, 2017; de Buhr *et al.*, 2018; Letendre and Goggs, 2018a; Letendre and Goggs, 2018b; Radermecker *et al.*, 2021; Janssen *et al.*, 2022). These studies consistently reported increased NETs markers, supporting the involvement of NETs. However, various methods were used across these studies to detect NETs. Each of the commonly used methods for NETs detection have important limitations: quantification of cell-free DNA is sensitive but nonspecific; MPO–DNA ELISAs provide a more specific yet indirect measurement; and immunofluorescent co-localization of markers such as cit-H3 and MPO enables direct visualization but remains low-throughput and labour-intensive (Kano *et al.*, 2016; Radermecker *et al.*, 2021; Janssen *et al.*, 2022). Despite these advances, NETs formation has never been investigated in canine respiratory disease, and no study has assessed their presence in the bronchoalveolar lavage fluid (BALF). This study aimed to validate a technique previously applied in human and equine medicine for the detection and assessment of NETs in BALF samples from dogs. Healthy (H) dogs were included as a negative control group, and dogs with BBP served as a positive control group. The inclusion of BBP dogs as a positive control was based on evidence from human and experimental animal studies showing that acute bacterial infection of the lower airways elicits a NET response, making BBP a biologically justified model for NET-positive pulmonary inflammation (Mikacenic *et al.*, 20158; Sanders *et al.*, 2022). After validation, the technique was applied to dogs with CBD associated with BE and/or BM to compare NETs detectability among groups. We hypothesized that NETs can be detected in the BALF of H-dogs, that their levels are increased in BBP-dogs, and that significant differences may exist between BE- and/or BM- and H-dogs, as well as between H- and BBP-dogs.

Material and methods

Study population

H-dogs included clinically healthy client-owned dogs and healthy experimental Beagle dogs. All individuals had no clinical signs of disease, showed normal findings on physical examination, haematology, and serum biochemistry, and displayed a normal gross bronchoscopic appearance with no abnormalities detected on BALF analysis. Samples from healthy client-owned dogs and experimental Beagles were obtained from previously collected and stored material, originally

gathered under approved experimental protocols (Ethical Committee of the University of Liège; protocol #1435 and #2299, respectively) and with informed owner consent where applicable. BALF samples of client-owned dogs diagnosed with acute BBP at the Small Animal Teaching Hospital, Liège, Belgium, but without con-current BE and/or BM, were also retrospectively included. The diagnosis of BBP was based on compatible clinical and physical examination findings, characteristic abnormalities on thoracic radiographs (focal, multifocal, or diffuse alveolar patterns), bronchoscopy findings, and BALF analysis showing septic inflammation, combined with a positive bacterial culture and/or quantitative polymerase chain reactions (qPCR) result (Dear, 2020). Lastly, client-owned dogs, presented at the same institution for chronic cough between February 2015 and October 2022 and diagnosed with CBD with BE, BM or BEBM based on endoscopy findings according to previously described criteria, were retrospectively included in the study (Lyssens *et al.*, 2025). Briefly, BE was defined as an obvious macroscopic lack of tapering or increased diameter of the bronchial lumen and BM was defined as dynamic airway collapse of the bronchi whereby changes in luminal diameter of at least 25% were observed during respiration. Data collected from the medical records included age, sex, breed, body weight, duration of clinical signs, previous or ongoing treatment, results of total cell count (TCC), differentiated cell count (DCC) and absolute neutrophil count (ANC) on BALF, results of traditional semi-quantitative bacterial culture and qPCR results for *Bordetella bronchiseptica* and *Mycoplasma cynos* on BALF. Normal TCC was considered less than 600 cells per microliter, and neutrophilic inflammation was concluded if >12% of neutrophils were observed (Canonne *et al.*, 2020). The ANC in BALF was calculated by multiplying the TCC by the percentage of neutrophils obtained from the DCC. Aerobic culture testing was performed by a commercial veterinary diagnostic laboratory (Antech, Liège, Belgium) and qPCR testing for *Bordetella bronchiseptica* and *Mycoplasma cynos* was performed at the clinical pathology laboratory (Department of Veterinary Pathology, Liège, Belgium). Infection was defined as the combined presence of an increased TCC, presence of neutrophilic inflammation, presence of intra- or extracellular bacteria and positive culture [$>1.7 \times 10^3$ colony-forming units (CFU) per millilitre of BALF] and/or positive qPCR results [cycle threshold (Ct) < 34] (Peeters *et al.*, 2000; Canonne *et al.*, 2016). Dogs that tested positive for *Bordetella bronchiseptica* were excluded for further NET analysis since it has been shown that *Bordetella pertussis* inhibits NET formation by suppressing the oxidative burst necessary for NETosis (Storisteanu *et al.*, 2017). For all dogs with CBD and BE and/or BM based on endoscopy, a final diagnosis was also attributed based on all available data. Dogs were diagnosed with eosinophilic bronchopneumopathy (EBP) if they exhibited a moderate to severe broncho-interstitial pattern, abundant yellow-green mucopurulent material on bronchoscopy, and more than 50% eosinophils in the BALF (Johnson *et al.*, 2019). A diagnosis of parasitic bronchitis was made

when parasites were observed either during bronchoscopy, identified in BALF analysis by qPCR, or detected through a Baermann test on faeces. Dogs were suspected to have idiopathic pulmonary fibrosis (CIPF) based on the signalment, clinical signs at presentation, compatible CT findings, exclusion of other cardiopulmonary diseases, and histopathology of the lungs, if available (Clercx *et al.*, 2018). Lastly, dogs were diagnosed with CB based on history of coughing for more than 2 months, an increased TCC and non-septic neutrophilic inflammation on BALF analysis, and no other specific underlying cause (Rozanski, 2020).

BALF sample collection and processing

BALFs were obtained in each dog under anaesthesia as previously described (Fastrès *et al.*, 2019). Dogs were anesthetized without intubation. The paediatric bronchoscope was cleaned and disinfected using an endoscopy washing machine (Serie 4-PA, Soluscope®, France) before each use. The bronchoscope was then inserted into the trachea, taking care to avoid contact with the oral and tracheal mucosa. The BAL was performed by injecting 3–4 mL/kg of sterile saline solution divided into 3 aliquots, including 2 aliquots with the endoscope inserted into the right diaphragmatic lobe, followed by a 3rd aliquot placed into the left diaphragmatic lobe. Each aliquot was directly aspirated by gentle suction and the fluids recovered from the 3 aliquots were pooled. A small amount of naïve BALF was directly used for calculation of the TCC as well as for the cytospin preparation. For the latter, 150 µL of BALF was added to a cytospin funnel, which was subsequently centrifuged at 1000 rpm ($112 \times g$) for 4 min using a rotor JC 301 Cellspin I 1206-14 (Tharmac). Cytospin preparations were stained by Diff Quick and were used for differential cell count (DCC) determination by counting a minimum of 100 cells. After centrifugation of BALF, supernatant of BALF were transferred into cryotubes and stored at -80°C until analysis.

Low specificity quantification of cfDNA in BALF supernatant

This method was applied in this study to verify the presence of extracellular DNA in the BALF supernatant (Hui *et al.*, 2015; Kowarsky *et al.*, 2017). It is a highly sensitive method, allowing rapid and quantitative detection of low concentrations of double-stranded DNA, and is relatively cost-effective (Hui *et al.*, 2015; Volik *et al.*, 2016; Kowarsky *et al.*, 2017; Grilz *et al.*, 2017; Grabuschnig *et al.*, 2020). However, it does not provide information on the origin of the detected DNA and therefore cannot discriminate between DNA released through NETs formation, apoptosis, or other cellular processes (Masuda *et al.* 2016; van Breda *et al.*, 2019). To quantify the concentration of cfDNA in the supernatant, the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, P7589) was used following the manufacturer's protocol. A standard curve ranging from 0.4 µg/mL to 3.125×10^{-3}

µg/mL was prepared to estimate the double-stranded (ds)DNA concentration in the BALF samples. Samples from dogs in the H, BE, BM, and BEBM groups were diluted 1:5, while samples from dogs with BBP were diluted 1:10 for the quantification of cfDNA. Quant-iT PicoGreen reagent was added directly to the wells. Fluorescence signals were measured using an Infinite 200 PRO multimode plate reader (Tecan Group Ltd., Switzerland), with filter settings of 485 nm and 535 nm.

ELISA detecting MPO-DNA complexes in BALF supernatant

To confirm the presence of NETs and provide a more specific assessment than cfDNA quantification alone, an adapted sandwich ELISA was performed in duplicate to detect MPO–DNA complexes, as previously described (Kano *et al.*, 2016; Janssen *et al.*, 2022). MPO–DNA complexes are considered more specific indicators of NET formation than either DNA or MPO alone, as extra-cellular DNA may originate from any dying cell, and MPO is present in neutrophils regardless of NET release (Kano *et al.*, 2016; Janssen *et al.*, 2022; Islam *et al.*, 2023). The simultaneous detection of both MPO and DNA within a complex therefore strongly suggests active NET formation (Kano *et al.*, 2016; Janssen *et al.*, 2022). For the assay, 96-well flat-bottom plates were coated overnight at 4 °C with goat anti-human/mouse MPO antibody (3.125 µg/mL, R&D Systems, AF3667) diluted in PBS. The following day, the plates were blocked for 1 h with PBS containing 1% bovine serum albumin (BSA, Sigma, A7906) to prevent non-specific binding. After blocking, the BALF samples were added to the wells. To enhance the accessibility of the MPO-DNA complexes for antibody binding and detection, the samples were treated with small amounts of DNase I (RNase-free, 125 U, Sigma, 11284932001) for 15 min, as previously described (Kano *et al.*, 2016; Janssen *et al.*, 2022). A DNase concentration of 125 U was chosen to maximize the detection of MPO-DNA complexes while preventing excessive degradation of the NETs, as previously reported for this ELISA method (Kano *et al.*, 2016; Janssen *et al.*, 2022). After the DNase treatment, the enzymatic reaction was stopped by adding 1 µL of PBS-EDTA (0.05 M), and the plates were incubated for 90 min at room temperature. Mouse anti-DNA detection antibodies (1.10–2 µg/mL, clone BV16-13, Sigma-Aldrich, MAB030) were then added and incubated for 1 h. Subsequently, biotinylated poly-clonal rat anti-mouse IgG2a (1.10–2 µg/mL, BD Biosciences, 553388) was added and incubated for 90 min. Following these incubations, the plates were washed and streptavidin-conjugated horseradish peroxidase (HRP) (1:500 dilution, ThermoFisher, 18-4100-94) was added for a 30-min incubation. After washing the plates again, the substrate 3,3',5,5'-Tetramethylbenzidine (TMB, Lifetechnologies, SB02) was added and incubated in the dark, allowing the enzymatic reaction to proceed. The reaction was then stopped by adding 1 M H₂SO₄. The absorbance was measured at 450 nm using a Multiskan FC plate reader (ThermoScientific, 51119000), and no background or reference wave-length correction was applied.

Throughout the procedure, 3 to 5 washes were performed between each step using PBS-Tween-20 0.05% (ThermoFisher, 233360010) to ensure optimal signal clarity.

Immunofluorescence staining and analysis of BALF

Following quantitative assessment of MPO–DNA complexes, immunofluorescence (IF) staining was performed to obtain morphological visualization of NETs within BALF samples (Zambrano *et al.* 2025). Immunofluorescence staining was performed on BALF cytopspins from 1 dog with BE and 1 dog with BBP, as previously described (Janssen *et al.* 2022). The cytopspin slides were permeabilized with 2% Triton X-100 in PBS. To block non-specific binding, the samples were incubated with a blocking buffer consisting of PBS with 2% bovine serum albumin (BSA) and 2% fetal donkey serum (FBS; Sigma-Aldrich) for 1 h at room temperature. The samples were then stained with rabbit anti-human citrullinated histone H3 (Cit-H3, Abcam, Ab5103) at a 1:100 dilution and goat anti-human myeloperoxidase (MPO, R&D Systems, AF3667) at a 1:40 dilution in blocking buffer for 1 h at room temperature. Following incubation, the samples were washed with PBS, and secondary antibodies were applied: donkey anti-rabbit Alexa Fluor 568 (ThermoFisher, A10042, 1:200 dilution) and donkey anti-goat Alexa Fluor 488 (ThermoFisher, 11055, 1:200 dilution). These secondary antibodies were prepared in blocking buffer containing 4',6-diamidino-2-phenylindole (DAPI, ThermoFisher, D3571, 1:1000 dilution) to stain the nuclei. The samples were incubated for 2 h in the dark at room temperature. After incubation, the samples were mounted with 10 μ L of ProLong Antifade reagent (Thermo Fisher, P36961) on glass slides and stored in the dark at room temperature overnight. All samples were analysed using fluorescence microscopy with standard filter sets. For image acquisition, a Zeiss LSM 880 Airyscan Elyra S.1 confocal microscope (Zeiss) was used. Images were processed using ImageJ software. Negative and isotype control staining were not performed for the immunofluorescence analyses. However, the primary antibodies used have been previously validated and applied in our laboratory in studies involving murine, human, and equine samples, where appropriate specificity controls were included (Radermecker *et al.*, 2019; Radermecker *et al.*, 2021; Janssen *et al.*, 2022).

Statistical analysis

Categorical variables were described using frequency tables (numbers and percentages) and continuous variables were summarized using mean $\pm$ standard deviation (SD) when normally distributed, median and quartiles (Q1, Q3) and minimum and maximum values. Characteristics of groups were compared using chi-square test (or Fisher exact test) for categorical variables and ANOVA (or Kruskal–Wallis for asymmetric variables) for continuous variables. Symmetric variables

were described by mean $\pm$ SD and asymmetric variables were described by median (Q1; Q3). Pairwise comparisons were performed when significant differences are detected. To explore the relationships between MPO–DNA complex levels and clinical or cytological variables, including age, body weight, duration of clinical signs, TCC, ANC, and the percentages of macrophages, neutrophils, eosinophils, and lymphocytes in BALF, as well as cfDNA concentrations in BALF, Spearman correlation coefficients were calculated across all dogs and within diagnostic groups. Significant level was set at 5% ($p < 0.05$). Calculations were conducted using SAS software (version 9.4) and graphics were produced using R software (version 4.4.1). No power calculation was performed, as this was a retrospective exploratory study based on the availability of archived bronchoalveolar lavage fluid samples.

Results

Study population and BALF cell analysis

A total of 53 dogs was included in the study and categorized into 5 groups according to the diagnosis: BE, BM, BEBM, BBP and H. Groups characteristics are reported in Table 1. No statistically significant differences in weight and sex were observed among the 5 groups. Dogs diagnosed with BEBM were significantly older than those with BBP ($p = 0.025$). The duration of clinical signs also differed among diagnostic groups, with a longer clinical course observed in dogs with BE and/or BM compared to those with BBP ($p < 0.001$). Group characteristics regarding BALF cell analysis are reported in Table 2. TCC was markedly higher in BBP-dogs compared to dogs with BE, BM, and BEBM, and all disease groups showed higher TCCs than H-dogs ($p < 0.0001$). The DCC also differed significantly among groups. Neutrophil percentage in BALF was significantly higher in BBP compared with BM and H-dogs ($p = 0.0023$) and macrophage percentage in BALF was significantly lower in BBP than in BM and H-dogs ($p = 0.0011$). Lymphocyte percentage in BALF was significantly lower in BBP than in H-dogs ($p = 0.023$). No significant differences were observed in eosinophil percentage among the 5 groups. ANC was significantly higher in BBP compared to BE, BM, BEBM, and H-dogs ($p < 0.0001$), while ANC in H-dogs was lower than in BEBM ($p = 0.001$). Of the six dogs with BBP, semi-quantitative bacterial culture identified *Pasteurella canis* in two dogs, *Staphylococcus pseudintermedius* in two dogs, *Pseudomonas aeruginosa* in one dog and *Streptococcus equi* subsp. *zooepidemicus* in one dog. Bacterial culture and qPCR analysis was negative in all BE and/or BM and H-dogs.

Table 1. Characteristics of the dogs according to the endoscopic diagnosis and the results of bacterial culture and quantitative polymerase chain reaction results.

Variable	BE	BM	BEBM	BBP	H
N	9	11	12	6	15
Sex (M/F)	4/5	5/6	7/5	3/3	7/8
Age (year)	9.22 ± 2.73	9.09 ± 2.81	10.17 ± 2.55	5.67 ± 3.33	8.07 ± 2.55
Weight (kg)	13.97 ± 9.42	10.94 ± 9.19	15.01 ± 5.96	24.28 ± 10.73	17.61 ± 9.05
Duration of clinical signs (months)	9.00 (3.00;12.00)	8.00 (4.00;24.00)	8.00 (6.00;24.00)	1.00 (0.50;1.00)	0.00 (0.00;0.00)
Years of BALF sample collection	2016-2021	2015-2021	2015-2021	2015-2022	2012-2021
Breeds	3 crossbreed, 2 Dachshund, 1 Beagle, 1 Fox Terrier, 1 Malinois, 1 Maltese	3 crossbreeds, 2 Sheltie, 1 Poodle, 1 Labrador retriever, 1 Yorkshire Terrier, 1 Jack Russel Terrier, 1 WHWT, 1 Pomerian	4 WHWT, 3 crossbreeds, 1 Whippet, 1 Munsterlander, 1 Samoyed, 1 Basset Fauve, 1 Beagle	1 American Staffordshire terrier, 1 Bouvier de Flandres, 1 American Pitbull, 1 Griffon Bruxellois, 1 Australian shepherd, 1 Malinois	4 Beagle, 2 Border collie, 1 Jack Russel Terrier, 1 American Staffordshire Terrier, 1 German Shepherd, 1 Dobermann Pinscher, 1 Malinois, 1 Yorkshire Terrier, 1 CKCS, 1 Labrador retriever, 1 crossbreed
Final diagnosis	8 chronic bronchitis, 1 EBP	10 chronic bronchitis, 1 CIPF	8 chronic bronchitis, 3 CIPF, 1 EBP	5 not specified, 1 suspicion of foreign body in lung lobe	/

Data are expressed as mean ± standard deviation regarding 'age' and 'weight' and expressed as median and interquartile range regarding duration of clinical signs.

BE = bronchiectasis; BM = bronchomalacia; BEBM = combination of bronchiectasis and bronchomalacia; BBP = bacterial bronchopneumonia; H = healthy; M = male; F = female; BALF = bronchoalveolar lavage fluid; WHWT = West Highland White Terrier; CKCS = Cavalier King Charles Spaniel; EBP = eosinophilic bronchopneumopathy; CIPF = canine idiopathic pulmonary fibrosis

Table 2. Characteristics of the total and differential cell count in bronchoalveolar lavage fluid of dogs in each diagnostic group.

Group	TCC (cells/ μ l)	Macrophages (%)	Neutrophils (%)	Lymphocytes (%)	Eosinophils (%)	ANC (cells/ μ l)
BE	1024 (720-1320)	50.22 $\pm$ 31.66	16.00 (9.00-65.00)	2.00 (0.00-5.00)	2.00 (0.00-4.00)	152 (64.8-665.6)
BM	800 (340-2000)	63.64 $\pm$ 27.19	17.00 (9.00-40.00)	4.00 (1.00-5.00)	0.00 (0.00-2.00)	417.6 (19.8-720.8)
BEBM	1620 (900-2315)	52.00 $\pm$ 29.87	21.50 (8.00-47.50)	1.50 (0.00-11.00)	3.00 (0.00-12.00)	246.5 (73.9-987)
BBP	9750 (2700-24475)	20.67 $\pm$ 20.96	85.00 (58.00-95.00)	1.00 (0.00-2.00)	0.00 (0.00-0.00)	9267.5 (1215-22950)
H	320 (250-390)	74.30 $\pm$ 14.10	9.00 (6.00-15.00)	11.00 (5.00-23.00)	2.00 (0.50-3.00)	25.2 (18.9 -48)

Data are expressed as median and interquartile range for TCC, ANC, % neutrophils, % lymphocytes and % eosinophils. Data are expressed as mean $\pm$ standard deviation for % macrophages.

TCC = Total cell count per μ l; ANC = absolute neutrophil count; BE = bronchiectasis, BM = bronchomalacia, BEBM = combination of bronchiectasis and bronchomalacia; BBP = bacterial bronchopneumonia; H = healthy

Low specificity quantification of cell-free DNA in BALF

Low-specificity quantification of cfDNA in BALF was performed on 51 dogs (8 dogs with BE, 11 dogs with BM, 11 dogs with BEBM, 6 BBP-dogs and 15 H-dogs). cfDNA concentrations in BALF were significantly higher in dogs with BBP compared to H-controls ($p = 0.0004$). cfDNA concentrations of BBP-dogs were also significantly greater than those observed in dogs with CBD ($p < 0.05$ for all pairwise comparisons). No significant differences were observed among the CBD groups (BE, BM, BEBM) or between these and H-controls (Figure 1).

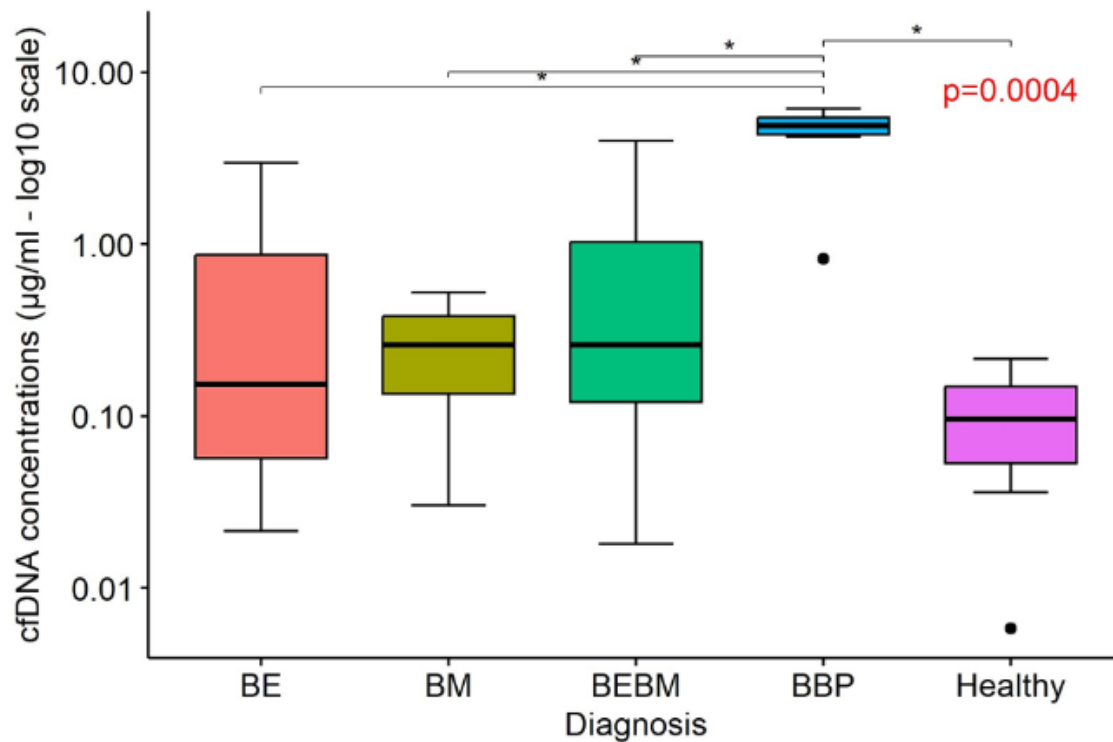


Figure 1. Boxplots illustrating the concentration of cell-free DNA (cfDNA; µg/ml) in bronchoalveolar lavage fluid supernatants from dogs with bronchiectasis (BE), bronchomalacia (BM), a combination of both conditions (BEBM), bacterial bronchopneumonia (BBP), and healthy control dogs. Each box represents the interquartile range (IQR: 25th–75th percentile), with the horizontal line indicating the median cfDNA concentration. Outliers are shown as individual dots. Logarithmic scaling (log₁₀) was applied to the y-axis. CFDNA concentrations were significantly higher in dogs with BBP compared to healthy dogs and to those with chronic airway disease (BE and/or BM).

ELISA detecting MPO-DNA complexes

An ELISA was performed on a cohort of 47 dogs (9 dogs with BE, 11 dogs with BM, 11 dogs with BEBM, 6 BBP-dogs and 10 H-dogs). The intra-assay coefficient of variation, calculated across all samples combined, was 5.75%. All samples were analysed in duplicate on a single 96-well plate during one assay run; therefore, inter-assay variability was not applicable and only intra-assay coefficient of variation was calculated. MPO–DNA complex levels, expressed as optical density, were overall significantly higher in dogs with CBD and BBP compared with H-dogs ($p < 0.0001$). Pairwise analysis showed higher levels of MPO–DNA concentrations in BE-dogs compared with H-dogs ($p < 0.05$) and BM-dogs ($p < 0.05$), while BEBM-dogs also had higher levels of MPO–DNA complexes in their BALF

compared to H-dogs ($p < 0.05$) (Figure 2). No statistically significant difference was found between BBP-dogs and dogs with CBD.

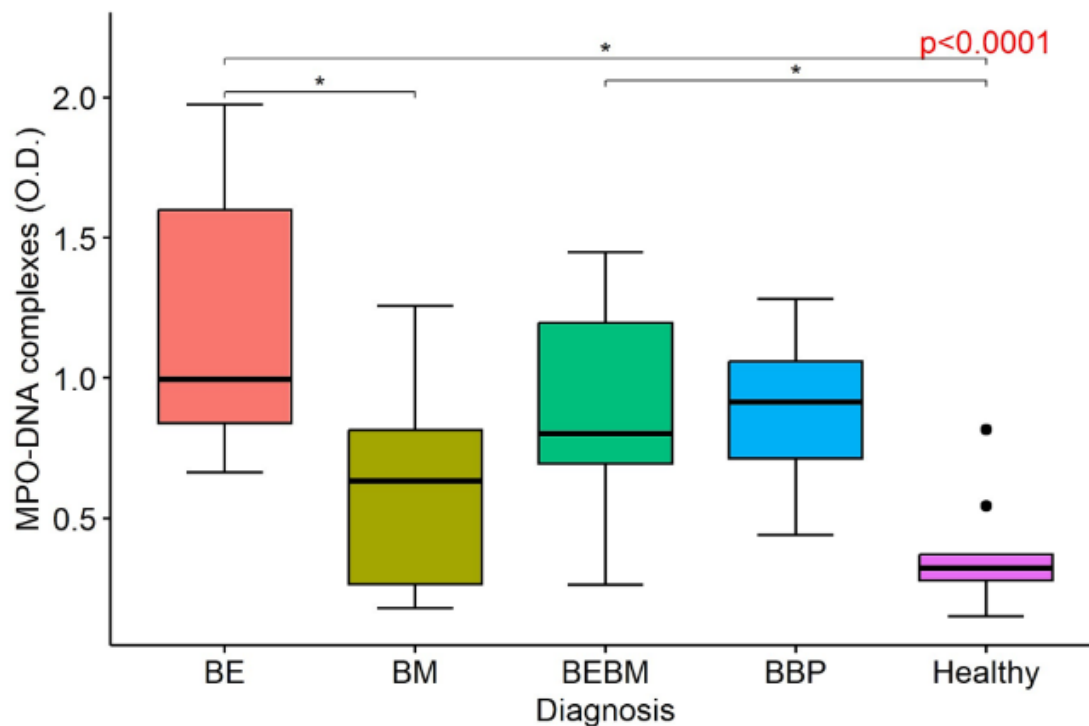
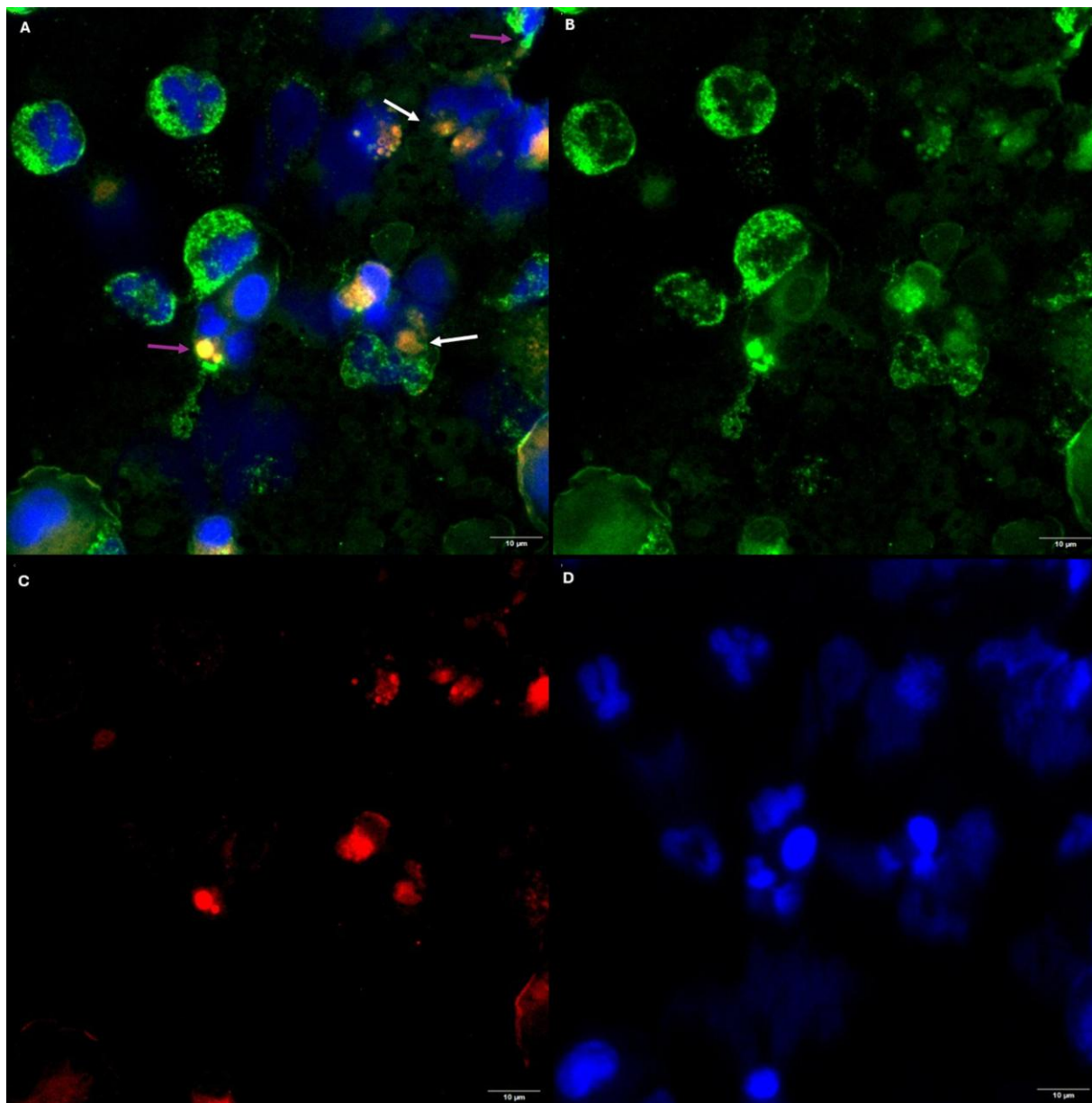


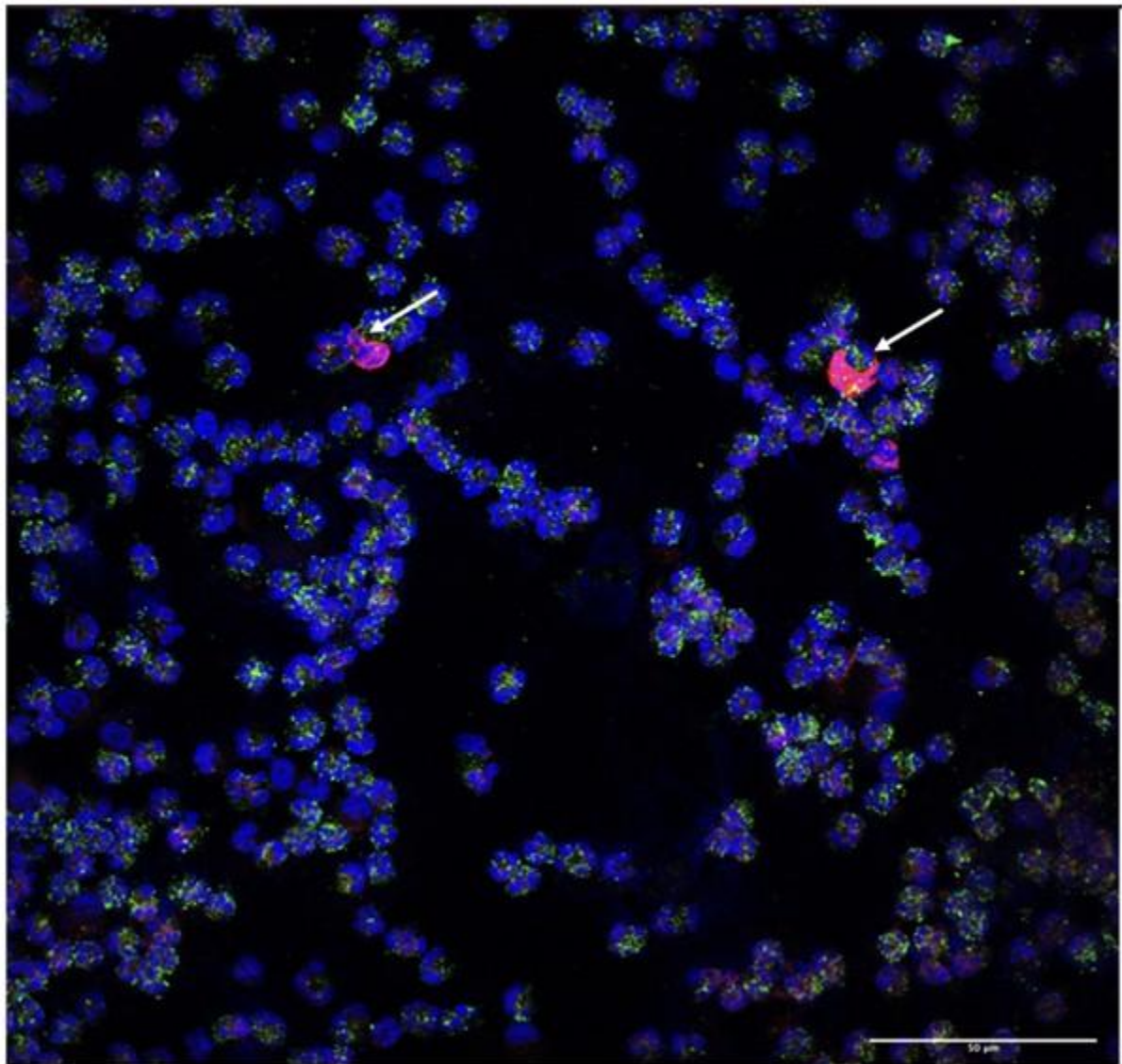
Figure 2. Boxplots illustrating the concentration of myeloperoxidase (MPO)–DNA complexes, expressed as optical density (O.D.), in bronchoalveolar lavage fluid from dogs with bronchiectasis (BE), bronchomalacia (BM), a combination of both conditions (BEBM), bacterial bronchopneumonia (BBP), and healthy control dogs. Each box represents the interquartile range (IQR: 25th–75th percentile), and the horizontal line within the box indicates the median value. Outliers are displayed as individual dots.

Immunofluorescence staining

IF staining was processed in one dog with BE (Supplementary Figure S1) and in one dog with BBP (Supplementary Figure S2). In both slides, extracellular web-like structures were observed that stained positive for MPO and cit-H3, consistent with the presence of NETs. The extracellular DNA backbone of these structures was counterstained with 4', 6-diamidino-2-phenylindole (DAPI). These structures were observed in close proximity to neutrophils and appeared as diffuse, filamentous material extending into the extracellular space, suggestive of neutrophil decondensation and chromatin release.



Supplementary Figure S1. Illustrative confocal microscopy pictures (objective 40x) of BALF neutrophils in bronchoalveolar lavage fluid of a dog with bronchiectasis (A). Activated neutrophils showing extracellular myeloperoxidase (MPO; green; B)- and citrullinated histones 3 (Cit-H3; red; C)-positive structures, consistent with ongoing NETs formation, are indicated by purple arrows on A. The white arrows indicate presence of intracellular MPO and Cit-H3-positive structures on A. DAPI (4',6-diamidino-2-phenylindole) is a fluorescent dye used to label DNA (D). The scale bar indicates a length of 10 µm



Supplemental Figure S2. Illustrative merged confocal microscopy pictures (objective 20x) of BALF neutrophils in bronchoalveolar lavage fluid of a dog with bacterial bronchopneumonia. NETs are defined as extracellular structures double positive for myeloperoxidase (MPO; green) and citrullinated histones (Cit-H3; red) and are indicated by white arrows. DAPI (4',6-diamidino-2-phenylindole) is a fluorescent dye used to label DNA. The scale bar indicates a length of 50 μm.

Association between MPO-DNA complexes and other measures

Across all dogs, MPO–DNA complexes were positively correlated with TCC ($r_s = 0.38$; $p = 0.0078$), ANC ($r_s = 0.37$; $p = 0.0098$), neutrophil percentage ($r_s = 0.33$; $p = 0.024$), cfDNA concentrations ($r_s = 0.36$; $p = 0.017$), and duration of clinical signs ($r_s = 0.36$; $p = 0.014$), and negatively correlated with macrophage percentage ($r_s = -0.40$; $p = 0.0052$) (Supplementary Table 1; Figures 3A–E). However, within diagnostic subgroups, several correlations differed in direction or lost significance. In

1 particular, the positive global association between MPO–DNA complexes and TCC or ANC was
2 reversed in dogs with BBP (TCC: $r_s = -0.94$; $p = 0.0048$; ANC: $r_s = -0.89$; $p = 0.019$), whereas cfDNA
3 concentrations remained positively and significantly associated with MPO–DNA complexes only in
4 dogs with BEBM ($r_s = 0.68$; $p = 0.029$). In contrast, in dogs with BE, MPO–DNA complexes showed
5 weak to moderate positive correlations with selected variables, with all Spearmann correlation
6 coefficients remaining below 0.5, although they did not consistently reach statistical significance
7 (Table 3). Additionally, in dogs with BE, MPO–DNA complexes were positively correlated with
8 duration of clinical signs ($r_s = 0.72$; $p = 0.028$), a relationship not observed in the other diagnostic
9 groups (Table 3; Figure 3D).

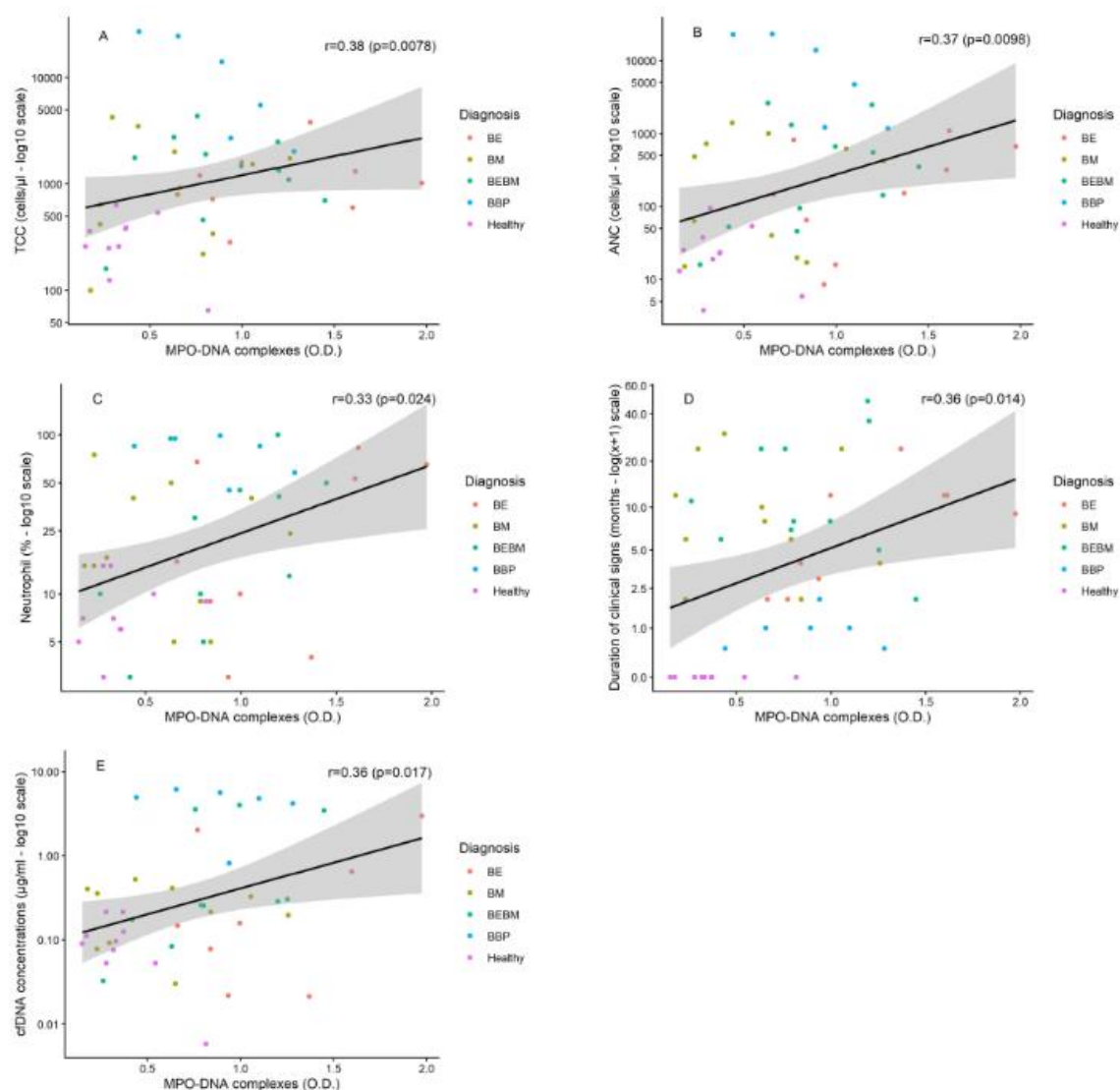


Figure 3. Scatterplots illustrating the associations between MPO–DNA complexes (expressed as optical density, O.D.) and selected clinical or cytological variables, including total cell count (TCC), absolute neutrophil count (ANC), percentage of neutrophils in BALF, duration of clinical signs, and cfDNA concentrations (A–E, respectively). Each point represents one dog and is color-coded by diagnosis. The solid black line represents the global linear association across all dogs, with the shaded area indicating the 95% confidence interval. Logarithmic scaling (log₁₀) was applied to y-axes for TCC, ANC, neutrophil percentage and cfDNA concentrations. BE, bronchiectasis; BM, bronchomalacia; BEBM, combined bronchiectasis and bronchomalacia; BBP, bacterial bronchopneumonia.

Table 3: Spearman correlation coefficients between MPO-DNA complexes and other variables in dogs stratified by endoscopic diagnosis.

Variable	BE		BM		BEBM		BBP		H	
	Correlation	p-value	Correlation	p-value	Correlation	p-value	Correlation	p-value	Correlation	p-value
Age (years)	-0.05	0.90	0.30	0.37	-0.33	0.33	0.09	0.87	-0.02	0.96
Weight (kg)	-0.13	0.73	0.44	0.17	0.33	0.33	-0.43	0.40	0.33	0.35
Duration of clinical signs (months)	0.72	0.028*	-0.16	0.65	-0.21	0.54	0.06	0.91	/	/
TCC (cells/ μ l)	0.22	0.58	0.17	0.61	-0.15	0.65	-0.94	0.0048*	0.15	0.69
Macrophage (%)	-0.35	0.36	-0.13	0.70	-0.39	0.23	0.37	0.47	-0.05	0.88
Neutrophil (%)	0.30	0.43	-0.12	0.73	0.45	0.17	-0.49	0.32	0.22	0.54
Lymphocyte (%)	0.48	0.18	0.51	0.11	-0.32	0.33	0.83	0.039*	-0.01	0.99
Eosinophil (%)	0.03	0.93	-0.10	0.77	0.56	0.076	0.39	0.44	0.09	0.81
ANC (cells/ μ l)	0.42	0.26	0.03	0.94	0.26	0.43	-0.89	0.019*	0.01	0.99
cfDNA (μ g/ml)	0.24	0.57	-0.16	0.63	0.68	0.029*	-0.66	0.16	-0.37	0.29

* Indicates a statistically significant correlation

TCC = Total cell count per μ l; ANC = absolute neutrophil count; cfDNA = cell-free DNA; BE = bronchiectasis, BM = bronchomalacia, BEBM = combination of bronchiectasis and bronchomalacia; BBP = bacterial bronchopneumonia; H = healthy

Supplementary Table 1: Spearman correlation coefficients between MPO-DNA complexes and other variables for all dogs

Variables	Correlation	p-value
Age (years)	0.10	0.49
Weight (kg)	0.15	0.30
Duration of clinical signs (months)	0.36	0.014*
TCC (cells/ μ l)	0.38	0.0078*
Macrophage (%)	-0.40	0.0052*
Neutrophil (%)	0.33	0.024*
Lymphocyte (%)	-0.11	0.45
Eosinophil (%)	0.15	0.32
ANC (cells/ μ l)	0.37	0.0098*
cfDNA (μ g/ml)	0.36	0.017*

* Indicates a statistically significant correlation

TCC = Total cell count per μ l; ANC = absolute neutrophil count; cfDNA = cell-free DNA

Discussion

To the author's knowledge, this study is the first to demonstrate the presence and quantifiable detection of NETs in the BALF of healthy dogs, dogs with BBP and dogs with CBD (BE and/or BM). By combining low-specificity cfDNA quantification, ELISA for detection of MPO-DNA complexes, and in a limited way, IF staining, we provide evidence that NETs are present in the airways of canine inflammatory bronchopulmonary diseases.

Although NETs have previously been described in veterinary medicine across various diseases contexts and biological fluids, as out-lined in the introduction, direct comparison with those studies

1 is limited. This is largely due to the heterogeneity of NET detection methods employed, including
2 cfDNA quantification, ELISA-based assays, and IF, which differ in specificity and interpretability and
3 complicates meaningful quantitative comparison across studies and sample types.

4 Dogs diagnosed with CBD (BE, BM, BEBM) and BBP exhibited significantly higher concentrations of
5 MPO-DNA complexes in BALF compared with healthy controls, confirming NETs presence in diseased
6 airways. MPO-DNA detection is considered a specific indicator of NETs formation, as it reflects the
7 biochemical coupling of MPO and DNA during chromatin decondensation and extrusion, unlike
8 cfDNA quantification alone, which lacks cellular specificity (Brinkmann *et al.*, 2004; Kano *et al.*, 2016;
9 Janssen *et al.*, 2022).

10 Across all dogs, MPO-DNA complexes correlated positively with TCC, ANC and neutrophil
11 percentage. These associations indicate that NETs formation parallels the degree and presence of
12 neutrophilic inflammation. However, subgroup analyses revealed disease-specific differences.
13 Notably, dogs with BE in particular showed the highest amount of MPO-DNA complexes compared
14 to BM and H-dogs, suggesting a potential greater contribution of NETs to the pathophysiology of BE.
15 These findings are consistent with observations in human medicine, where elevated NETs markers
16 have been detected in the sputum and BALF of patients with BE and other chronic neutrophilic lung
17 diseases (Roghani *et al.*, 2006; Keir *et al.*, 2021). In the BE-group, the high amount of MPO-DNA
18 complexes and the lack of a significant positive correlation between MPO-DNA complexes and either
19 neutrophil percentages or ANC indicates that the elevation of MPO-DNA in BE is not simply a
20 consequence of increased neutrophil numbers, but may rather reflect heightened neutrophil
21 activation and NETosis. In other words, NETs formation in BE appears to be driven by qualitative
22 changes in neutrophil behaviour rather than by the quantitative presence of these cells. This
23 observation may support the hypothesis that chronic airway injury in dogs with BE might involve
24 dysregulated neutrophil function, as described for human BE and chronic neutrophilic airway dis-
25 ease, leading to increased NETs release even in the absence of proportionally greater neutrophil
26 infiltration (Wright *et al.*, 2016; Keir *et al.*, 2021; Keir *et al.*, 2022). Interestingly, dogs classified within
27 the BEBM group also showed elevated MPO-DNA complex levels compared to healthy controls. This
28 likely reflects the contribution of BE changes within this subgroup, as BE was associated with the
29 highest degree of neutrophil activation and NETs formation among the chronic respiratory
30 conditions evaluated.

31 The positive correlation observed between MPO-DNA complexes and the duration of clinical signs,
32 specifically in dogs with BE, may further supports the role of persistent neutrophil activation and

NETs formation in the pathophysiology of chronic airway inflammation in this subgroup. Prolonged clinical disease likely reflects chronic epithelial injury and ongoing inflammatory stimulation, which may result in persistent or cumulative NETs presence within the airways (Keir *et al.*, 2022). Such chronic NETs accumulation may perpetuate inflammation by releasing cytotoxic and proinflammatory components, thereby promoting tissue remodelling and airway damage (Farrera and Fadeel, 2013; Zou *et al.*, 2018; Keir *et al.*, 2022).

Although MPO-DNA complexes differed significantly between CBD and healthy dogs, cfDNA concentrations did not differ significantly between groups. This discrepancy suggests that cfDNA quantification alone may not specifically reflect NET-associated DNA, as it can also originate from other forms of cell death such as apoptosis, necrosis or tissue injury (Grabuschnig *et al.*, 2020; Janssen *et al.*, 2022). In this context, dogs with BBP exhibited the highest cfDNA concentrations among all groups but their MPO-DNA complex levels were low. Spearman correlation coefficient analysis revealed that, in contrast to the global cohort, BBP dogs showed negative correlations between MPO-DNA complexes and TCC and ANC, and no significant association with neutrophil percentage. Together, these findings suggest that the elevated cfDNA in BBP primarily reflects extensive tissue damage and necrotic cell death due to the active inflammation, rather than ongoing NETs formation. The low MPO-DNA complex levels observed in BBP may reflect a predominance of neutrophil phagocytosis rather than NETs formation during bacterial infection (Yousefi *et al.*, 2009; Kobayashi *et al.*, 2023). In certain bacterial infections, neutrophils initially rely on phagocytosis, and NETs formation is not immediate (Yousefi *et al.*, 2009; Kobayashi *et al.*, 2023). For example, in the case of *S. aureus* (Yousefi *et al.*, 2009), demonstrated that during the early minutes to hours of interaction, phagocytosis predominates. *S. aureus* triggered NETs formation only after an extended period in vitro, and within the first 60 min, neutrophils mainly killed *S. aureus* through phagocytosis rather than NETs release (Yousefi *et al.*, 2009; Kobayashi *et al.*, 2023). Low MPO-DNA levels in BBP may also result from neutrophil exhaustion or previous NETs release during the acute phase of infection, as well as subsequent degradation of NETs by proteolytic enzymes and nucleases in the inflamed lung (Wang *et al.*, 2024). In addition, active bacterial evasion strategies may further contribute to the relatively low MPO-DNA levels observed in BBP, as degradation or suppression of NET formation would directly reduce measurable MPO-DNA complexes despite ongoing neutrophilic inflammation. Although NETs effectively ensnare pathogens, many microorganisms have developed mechanisms to evade this defence. Among the species identified in this study, *Staphylococcus pseudintermedius* has been shown to degrade NETs through the release of a thermostable nuclease (NucB), while *Pseudomonas aeruginosa* exhibits multiple strategies to resist

NET mediated killing, including suppression of NET formation and development of resistant phenotypes in chronic airway disease (Wang *et al.*, 2024; Haller *et al.*, 2025). In addition, *Streptococcus equi subsp. zooepidemicus* produces extracellular nucleases capable of degrading the DNA backbone of NETs (Ma *et al.*, 2016). In contrast, NET-evasion mechanisms have not been described for *Pasteurella canis* to date.

Several limitations should be considered when interpreting our findings. First, due to its retrospective nature, the study relied on previously collected clinical data and BALF samples. This may have introduced variability in sample handling, and sample storage. Although BALF supernatants were stored at -80 °C to minimize DNase activity, bacterial growth, and degradation, the time between collection and freezing was not standardized, which could affect cfDNA concentrations through evaporation or oxidative or hydrolytic changes. Moreover, the retrospective design of this study did not allow us to perform simultaneous measurement of cfDNA and MPO-DNA complexes in all samples. Secondly, BALF was collected at a single time point per dog, limiting assessment of the dynamics of NETs formation during disease progression or in response to therapy. This is especially relevant in BBP, where neutrophil exhaustion or previous NETs release could affect measurable MPO-DNA complexes. Another limitation is the small sample size per group. The BBP group in particular was limited in size, which may reduce generalizability of subgroup analyses leading to potential increased risk of type I errors. Additionally, the low number of cytospin slides available for IF restricted direct visualization of NETs to only two samples (one BE and one BBP dog). Also, no quantification of the NETs based on IF could be performed. Moreover, IF analyses were performed without negative or isotype controls, which limits the ability to fully exclude non-specific staining. Although the antibodies used have been previously validated in our laboratory in other species with appropriate specificity controls, the absence of such controls in the present study limits definitive interpretation of the IF findings (Radermecker *et al.*, 2019; Radermecker *et al.*, 2021; Janssen *et al.*, 2022). Therefore, these findings should be interpreted as illustrative and supportive rather than confirmatory. Future prospective studies should expand IF analysis to a larger cohort, include longitudinal sampling, and combine NETs quantification with IF imaging to better characterize NETs dynamics and their role in CBD.

Overall, the detection of NETs in BALF of canine BE and/or BM highlights a potential link between chronic neutrophilic inflammation and airway remodelling. These findings may provide new insights into the pathogenesis of CBD, suggesting that persistent NETs formation may contribute to airway damage. In conclusion, this study provides the first evidence of NETs in the lower airways of dogs with both chronic (BE and/or BM) and acute (BBP) bronchopulmonary diseases, reinforcing the

- 1 translational parallels with human bronchial disorders and highlighting potential therapeutic
- 2 strategies focused on NETs modulation (Wright *et al.*, 2016; Keir *et al.*, 2021; Keir *et al.*, 2022).

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Experimental section

Study 4 :

Assessment of lung microbiota in bronchoalveolar lavage fluid in dogs
with bronchiectasis, bronchomalacia, and combined
bronchiectasis/bronchomalacia

Preface

Chronic bronchial diseases in dogs are increasingly recognized as complex and multifactorial conditions, often associated with structural airway abnormalities such as bronchiectasis (BE) and bronchomalacia (BM). Although these conditions are commonly identified in dogs with chronic cough, their underlying pathophysiology remains incompletely understood, particularly regarding the role of the lung microbiota (LM).

In human medicine, alterations in the LM have been shown to contribute to the development and progression of chronic airway diseases, with dysbiosis influencing inflammation, disease severity, and clinical outcomes. In veterinary medicine, however, the characterization of LM in dogs with chronic bronchial disease is still limited, and the relationship between airway structural abnormalities and microbial communities remains unclear. In particular, BE and BM are inconsistently associated with bacterial infection, raising questions about whether microbial alterations occur independently of overt infection.

This study aimed to characterize the LM in dogs with chronic cough associated with BE, BM, or their combination (BEBM), and to compare these findings with healthy dogs. Using 16S rRNA gene sequencing of bronchoalveolar lavage fluid, the study further sought to evaluate differences in microbial composition and diversity between healthy and diseased dogs, as well as among specific disease phenotypes. Alpha diversity was significantly lower in diseased dogs than in healthy dogs, reflecting reduced community evenness and increased dominance of specific bacterial taxa, while richness did not differ significantly between groups. Beta diversity differed significantly between diseased and healthy dogs, and between BM and healthy dogs. Diseased dogs showed dominance of *Pseudomonadota*, *Bacillota* and *Bacteroidota*, while healthy dogs had a more balanced LM and higher *Actinomycetota* abundance. These findings suggest that chronic respiratory diseases in dogs is associated with reduced microbial diversity and altered LM composition, suggesting its potential role in disease pathogenesis.

By improving the understanding of LM alterations in canine chronic bronchial disease, this work aims to provide new insights into disease mechanisms and support the development of more targeted diagnostic and therapeutic approaches.

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Experimental section

Study 4 :

Assessment of lung microbiota in bronchoalveolar lavage fluid in dogs
with bronchiectasis, bronchomalacia, and combined
bronchiectasis/bronchomalacia

Submitted in Heliyon

Aurélie Lyssens, Bernard Taminiau, Aline Fastrès, Frédéric Billen, Georges Daube, Cécile Clerx

Abstract

Lung microbiota (LM) alterations are increasingly recognized in chronic respiratory diseases (CRD) in dogs and humans. In dogs, bronchiectasis (BE), bronchomalacia (BM), or their combination (BEBM) are inconsistently associated with bacterial infection, whose role in disease pathogenesis and progression remains unknown. The primary objective of this study was to assess LM composition in bronchoalveolar lavage fluid (BALF) from dogs with chronic cough and airway abnormalities (BE, BM, or BEBM) compared to healthy dogs (H). The secondary objective was to compare LM differences among disease groups. Client-owned H-dogs and dogs with CRD (BE and/or BM, based on bronchoscopy) were retrospectively included. Dogs with concomitant bacterial infection, based on bacterial culture and quantitative polymerase chain testing, were excluded from LM analysis. LM was analyzed via 16S rRNA gene amplicon sequencing of DNA from naïve BALF. Statistical analyses included alpha and beta diversity analyses, non-metric multidimensional scaling, permutational multivariate analysis of variance, linear discriminant analysis, and DESeq2 differential abundance analysis. Bacterial composition and diversity were compared using MOTHUR v1.48.1, STAMP v2.1.3, XLStat v2.2, and the R vegan package v0.4.2. Eighty-one dogs were included: 28 H and 53 diseased (BE = 14, BM = 24, BEBM = 15). Alpha diversity was significantly lower in diseased dogs than in H-dogs, reflecting reduced community evenness and increased dominance of specific bacterial taxa, while richness did not differ significantly between groups. Beta diversity differed significantly between diseased and H-dogs, and between BM and H-dogs. Diseased dogs showed dominance of *Pseudomonadota*, *Bacillota* and *Bacteroidota*, while H-dogs had a more balanced LM and higher *Actinomycetota* abundance. These findings suggest that CRD in dogs is associated with reduced microbial diversity and altered LM composition, suggesting its potential role in disease pathogenesis.

1 Introduction

2 The term “microbiota” refers to all the bacteria present in a specific region or habitat (Segal *et al.*,
3 2014). In healthy dogs, the lung microbiota (LM) consists of microbial population similar to that
4 found in healthy humans, predominantly including the major phyla *Pseudomonadota*, *Bacillota*,
5 *Actinomycetota* and *Bacteroidota* (Ericsson *et al.*, 2016; Richardson *et al.*, 2019; Fastrès *et al.*, 2020;
6 Vientos-Plotts *et al.*, 2023).

7 In human, alterations in the LM community structure have been described in various chronic lung
8 diseases such as chronic bronchitis, chronic obstructive pulmonary disease (COPD), asthma, lung
9 cancer and idiopathic pulmonary fibrosis (IPF) although it remains unclear whether these changes
10 in the LM are a cause or a consequence of the disease (Han *et al.*, 2014; Arrieta *et al.*, 2018; Jin *et al.*,
11 2019; Haldar *et al.*, 2020; Yagi *et al.*, 2021). In humans with bronchiectasis (BE), LM studies
12 documented reductions in microbial diversity and increased relative abundance of pathogenic or
13 opportunistic bacteria (Rogers *et al.*, 2013; Yagi *et al.*, 2021). Furthermore, a positive linear
14 correlation has been found between lung function and measures of microbiota richness and
15 evenness (Rogers *et al.*, 2013). Additionally, several studies have reported that the frequency of
16 exacerbations in BE, defined as the sudden appearance or worsening of symptoms, prompting an
17 adjustment in respiratory therapy or the start of a specific treatment (e.g. antibiotics), is higher in
18 patients with a microbiota predominantly consisting of *Pseudomonadota*, such as *Pseudomonas* and
19 *Haemophilus* genera and *Bacillota*, such as the anaerobic *Veillonella* genus (Rogers *et al.*, 2013; Chen
20 *et al.*, 2024; De Angelis *et al.*, 2024). Since bronchomalacia (BM) is a rare disease in humans, no
21 microbiota analysis data is currently available.

22 In dogs, dysbiosis has been described in several chronic respiratory diseases, including chronic
23 bronchitis, bacterial pneumonia, and IPF (Vientos-Plotts *et al.*, 2019; Ericsson *et al.*, 2020; Fastrès *et al.*,
24 2020; Vientos-Plotts *et al.*, 2023). However, LM alterations associated specifically with the airway
25 abnormalities BE and BM have not yet been investigated. Dogs can be affected with BE, BM or a
26 combination (BEBM), mostly as sequela of CRD such as IPF, eosinophilic bronchopneumopathy,
27 chronic bronchitis and chronic recurrent bacterial infections (Hawkins *et al.*, 2003; Roels *et al.*, 2017;
28 Lyssens *et al.*, 2025). The exact underlying mechanism leading to development of BE or BM or both
29 remain unclear, although chronic inflammation, recurrent infection, and degenerative airway
30 changes are suspected to contribute to disease development (Marolf *et al.*, 2007; Boyton and
31 Altmann, 2016; Chandrasekaran *et al.*, 2018; Della Maggiore, 2020; Reinero and Masseau, 2021).

Traditional culture-based diagnostic microbiology is commonly employed in canine medicine to detect lower respiratory tract pathogens (Ericsson *et al.*, 2016; Vientos-Plotts *et al.*, 2019; Vientos-Plotts *et al.*, 2023). Classical bacterial culture of bronchoalveolar lavage fluid (BALF) in dogs with BE, BM and BEBM has been reported in a limited number of cases, identifying a diverse range of bacterial species, which makes the role of bacteria in the development of BE, BM and BEBM unclear (Hawkins *et al.*, 2003; Johnson and Pollard, 2010; Adamama-Moraitou *et al.*, 2012; Johnson *et al.*, 2016; Lyssens *et al.*, 2025). However, bacterial species, especially those that are difficult to grow under standard diagnostic conditions, may remain undetected (Fastrès *et al.*, 2020; Vientos-Plotts *et al.*, 2023). Considering the potential role of bacterial infections in sustaining CRD such as BE, BM and BEBM, it is important to gain deeper understanding of the relationship between airway bacterial populations and these conditions. Therefore, investigating the role of the LM using culture-independent molecular sequence approaches such as 16S ribosomal ribonucleic acid (rRNA) gene amplicon sequencing, in BE, BM and BEBM might identify previously undetected bacterial targets and reveal possible different management guidelines for these conditions.

The primary objective of this study was to analyse the LM composition in BALF of dogs diagnosed with chronic cough and airway alterations such as in BE, BM or BEBM, compared to healthy dogs, in order to determine how LM profiles are impaired in these chronic conditions. A secondary aim was to differentiate the LM profiles among the three conditions to determine whether tailored management approaches might be warranted.

Material and methods

Study population

Client-owned dogs, presented to the Small Animal Teaching Hospital (XXX) for chronic cough (mean duration: 10.5 months) between February 2015 and October 2021 and diagnosed with BE, BM or BEBM based on bronchoscopy findings according to previously described criteria, were retrospectively included in the study (Lyssens *et al.*, 2025). Briefly, BE was defined as an obvious macroscopic lack of tapering or increased diameter of the bronchial lumen. BM was defined as dynamic airway collapse of the bronchi whereby changes in luminal diameter of at least 25% were observed during respiratory cycle. Samples from healthy (H) client-owned dogs were obtained from previously collected and stored material, originally gathered under approved experimental protocols (Ethical Committee of the XXXX; protocol #1435) and with informed owner consent where

1 applicable. H-dogs had no clinical signs, normal findings on physical examination, normal
2 haematology and serum biochemistry results, normal gross appearance during bronchoscopy, and
3 no abnormalities detected in the BALF analysis.

4 Data collected from the medical records included age, sex, breed, body weight, previous ongoing
5 treatment, and results of the total cell count (TCC), differentiated cell count (DCC), traditional
6 bacterial culture and quantitative polymerase chain reactions (qPCR) for *Bordetella bronchiseptica*
7 and *Mycoplasma cynos* (formerly *Mycoplasma cynos* since 2018) on BALF. For clarity and
8 consistency, the more commonly used name *Mycoplasma cynos* will be used throughout this article.
9 Normal TCC was considered less than 600 cells per microliter (Canonne *et al.*, 2020). Neutrophilic
10 inflammation was concluded if >12% of neutrophils were observed (Canonne *et al.*, 2020; Lebastard
11 *et al.*, 2022). Semi-quantitative aerobic culture testing on BALF was performed by a commercial
12 veterinary diagnostic laboratory (XXXX) and qPCR testing for *Bordetella bronchiseptica* and
13 *Mycoplasma cynos*, was performed at the clinical pathology laboratory (XXXX). Infection was defined
14 by the simultaneous presence of an increased TCC together with neutrophilic inflammation,
15 presence of intra- or extracellular bacteria and positive culture ($>1.7 \times 10^3$ colony-forming units (CFU)
16 per millilitre of BALF) and/or positive qPCR results (cycle threshold (Ct) < 34) in BALF (Lyssens *et al.*,
17 2025). Dogs in which bacterial infection was revealed, were excluded for further LM analysis. None
18 of the included dogs had received antimicrobial treatment within 4 weeks prior to sampling. The 2-
19 week washout period was selected based on previous work demonstrating substantial recovery of
20 LM composition within 14 days after antimicrobial discontinuation in dogs (Fastrès *et al.*, 2019).

21 For all dogs with BE and/or BM based on bronchoscopy, a final diagnosis was also attributed based
22 on all available data. Dogs were diagnosed with eosinophilic bronchopneumopathy if they exhibited
23 a moderate to severe broncho-interstitial pattern, abundant yellow-green mucopurulent material
24 on bronchoscopy, and more than 50% eosinophils in the BALF (Johnson *et al.*, 2019). A diagnosis of
25 parasitic bronchitis was established when parasites were identified during bronchoscopy, detected
26 by qPCR on BALF, or identified using a Baermann faecal examination. Dogs were suspected to have
27 IPF based on compatible signalment, clinical presentation, thoracic computed tomography (CT)
28 findings, exclusion of other cardiopulmonary diseases, and histopathology when available (Clercx
29 *et al.*, 2018). Lastly, dogs were diagnosed with CB based on history of coughing for more than 2
30 months, an increased TCC and non-septic neutrophilic inflammation on BALF analysis, and no other
31 specific underlying cause (Rozanski, 2020).

32

1 *Sample collection*

2 BALF was obtained in each dog under anaesthesia as previously described (Fastrès *et al.*, 2019). Dogs
3 were anesthetized without intubation in an attempt to minimize potential contamination of BALF
4 samples from the upper airways and endotracheal tube during LM assessment. The bronchoscope
5 was cleaned and disinfected using an endoscopy washing machine (Serie 4-PA, Soluscope®, France)
6 before each use. A procedural control specimen was obtained prior to each BAL procedure by
7 injection of 10 mL of sterile saline solution through the bronchoscope channel followed by
8 aspiration through the same channel into a sterile container using a low-power suction pump. The
9 bronchoscope was then inserted into the trachea, taking care to avoid contact with the oral and
10 tracheal mucosa.

11 The BAL was performed by injecting 3-4 mL/kg of sterile saline solution divided into 3 aliquots. BAL
12 was preferentially performed in bronchial regions showing gross macroscopic abnormalities during
13 bronchoscopy. In dogs with focal lesions, the bronchoscope was wedged into the most visibly
14 affected bronchial segment. In cases without clear focal abnormalities or in the presence of diffuse
15 disease, BAL was performed in the diaphragmatic lobes (two aliquots in the right diaphragmatic lobe
16 and one in the left diaphragmatic lobe), following routine institutional practice. Each aliquot was
17 directly aspirated by gentle suction and the fluids recovered from the 3 aliquots were pooled. After
18 sampling, BALF was transferred into cryotubes and stored at -80°C until analysis. A small amount of
19 BALF was used for calculation of the TCC as well as for the cytospin preparation (centrifugation at
20 221g, for 4 minutes at 20°C, Thermo Shandon Cytospin©4). Cytospin preparations were stained by
21 Diff Quick and were used for DCC determination by counting a minimum of 100 cells.

23 *16S rDNA extraction and high throughput sequencing*

24 DNA extraction

25 Analysis of the LM was performed as previously described (Fastrès *et al.*, 2019). Total DNA was
26 extracted from BALFs according to the manufacturer's instructions with the DNeasy Blood and
27 Tissue kit (QIAGEN Benelux BV, Antwerp, Belgium), using the pretreatment for Gram-positive
28 bacteria protocol. This protocol was preceded by a beat beating step with glass beads > 106 µm and
29 glass beads soda-lime (Sigma-Aldrich, Overijse, Belgium, Cat. G4649 and Z265926). DNA was eluted
30 into DNase/RNase-free water for a final volume of 30 µl and the concentration and purity were
31 evaluated using a ND-1000 spectrophotometer (NanoDrop ND-1000, Isogen, De Meern, The
32 Netherlands). Three negative controls were included in the procedure up to the sequencing, the first

was sterile saline wash of the channel, a second was a blank extraction control and finally a blank 16S PCR control.

16S rDNA amplicon sequencing

For bacterial identification, PCRs targeting the V1-V3 hypervariable regions of the 16S rDNA were performed for all samples with the following primers: forward (5'-GAGAGTTTGATYMTGGCTCAG-3') and reverse (5'-ACCGCGGCTGCTGGCAC-3') and Illumina overhand adapters as earlier described (Fastrès *et al.*, 2019). Amplicons obtained were purified, quantified and submitted to a 2nd PCR round for indexing. A final quantification of all samples was performed by qPCR before normalization and pooling of the amplicons to form libraries. Libraries obtained were then sequenced on a MiSeq Illumina sequencer (Illumina, San Diego, CA, USA) using V3 reagents at the GIGA sequencing platform (XXX). Sequence reads processing was performed with MOTHUR v1.48 using standard operating procedure, coupled with VSEARCH for chimera detection and removal (Schloss *et al.*, 2009; Rognes *et al.*, 2016). SILVA SSU ribosomal database v1.38.1 was used for Operational Taxonomic Unit (OTU) clustering (0.03 dissimilarity cut-off) and for taxonomic assignation. Sequence treatment was performed on 8,877,884 raw sequences keeping 7,064,557 sequences after cleaning and chimera removal. A subsampled set of 10,000 read per sample was generated and further used for OTU clustering and taxonomic assignation. A total of 49,250 OTU was obtained spanning 1,066 different genera. An *in silico* analysis of possible laboratory contaminants using Decontam v1.28 package with ds DNA V1V3 16S PCR quantification and abundance methodology was not applied to the data because, negative controls PCR quantification was nearly 0 and negative samples reads count were low (below 500 reads). Possible laboratory contaminant were considered as unlikely.

Data analysis

Statistical analyses were conducted using XLStat version 2.2 (Addinsoft, Paris, France) to assess significant differences in the mean values of age, weight, TCC, and DCC between the four diagnostic groups (BE, BM, BEBM, and H). Normality of data distributions was assessed using the Shapiro-Wilk test. For normally distributed data, differences among groups were analysed using ANOVA followed by Tukey's post hoc test with Bonferroni correction. Results were reported as mean $\pm$ standard deviation (SD). For non-normally distributed data, group comparisons were performed using the

Kruskal-Wallis test, followed by Dunn's post hoc test with Bonferroni correction, with results expressed as median and interquartile range (IQR).

For LM analysis, Good's coverage index and ecological indicators, including the bacterial richness (Chao1 index), evenness (Simpson index-based measure) and alpha diversity (inverse Simpson's index) were calculated. Comparisons of these parameters were first conducted between H-dogs and dogs with CRD (BE, BM, and BEBM, collectively referred to as the "diseased" group) using the non-parametric Mann-Whitney U test in XLStat v2.2. Statistical differences in the relative abundance of taxa (genus and phylum levels) between groups were assessed using ANOVA followed by Tukey-Kramer post hoc tests with Bonferroni correction via STAMP software version 2.1.3 (Parks *et al.*, 2014). Non-metric multidimensional scaling (NMDS) plots, based on a Bray-Curtis dissimilarity matrix, were generated to visualize global bacterial composition (beta diversity) between groups using the R *vegan* package v0.4.2. Significant differences regarding beta diversity were evaluated using permutational multivariate analysis of variance (PERMANOVA), followed by pairwise tests with Bonferroni correction in the R *vegan* package. Beta-dispersion analysis, using *betadisper* in *vegan* package was used to assess heterogeneity in microbiota profile. Distance-based redundancy analysis (dbRDA) was also performed using R *vegan* package v0.4.2 to assess the influence of host variables (age, weight, and their interaction) on microbiota composition. Statistical significance of the explanatory variables was tested using permutation tests ($n = 999$).

To further investigate LM profiles across the three conditions relative to healthy controls, comparisons of ecological indicators and Good's coverage index among groups were conducted using Kruskal-Wallis tests and Dunn's post hoc tests with Bonferroni correction in XLStat v2.2. These tests were similarly applied to assess statistical differences in the relative abundance of taxa (genus and phylum levels). Beta diversity differences were assessed separately using PERMANOVA followed by pairwise comparisons with Bonferroni correction, as described above. Linear discriminant analysis (LDA) was employed to identify discriminant bacterial genera between groups using MOTHUR v1.48.1. A p -value < 0.05 and a LDA score > 2.0 were considered indicative of significant differences. DESeq2 analysis was performed to assess differentially abundant taxa between groups. Across all analyses, statistical significance was set at $p < 0.05$.

Results

Study population

A total of 81 dogs were included in the study and categorized into 4 groups based on bronchoscopy findings: H, BE, BM and BEBM. A total of 9 dogs were considered infected, including 4 dogs with BE, 3 with BEBM, and 2 with BM. Semi-quantitative aerobic culture identified *Pseudomonas aeruginosa* (n=2; one BE and one BEBM), *Escherichia coli* (n=2; both BE), *Streptococcus canis* (n=1; BEBM), and *Serratia marcescens* (n=2; BM). In two additional dogs, infection was supported by positive qPCR results for *Mycoplasma cynos* (BE) or *Bordetella bronchiseptica* (BEBM) despite negative culture.

Consequently, the final analysis was conducted on 72 dogs. Groups characteristics are reported in Table 1. H-dogs were younger than BE-dogs ($p=0.002$), BM-dogs ($p<0.000$) and BEBM-dogs ($p<0.000$). Additionally, BM-dogs had a lower weight compared to the BE-dogs, BEBM-dogs and the H-dogs ($p=0.000$). Results of the BALF cell analysis are reported in Table 1. Significant increased TCC was found in dogs with BE ($p=0.005$), BM ($p=0.001$), and BEBM ($p<0.000$) compared with H-dogs. Macrophage percentages were significantly lower in dogs with BE ($p<0.000$) and BEBM ($p=0.001$) compared with H-dogs and in dogs with BE compared with BM-dogs ($p=0.012$). Neutrophil percentages were higher in BE ($p=0.016$), BM ($p=0.035$) and BEBM ($p<0.000$) dogs, compared with H and in dogs with BEBM compared with BM-dogs ($p=0.04$). Lymphocyte percentages were also significantly higher in healthy dogs compared to BEBM ($p=0.006$). Lastly, although only 1 dog with BEBM was diagnosed with eosinophilic bronchopneumopathy, eosinophil percentages were significantly higher in dogs with BEBM ($p=0.017$) compared to H-dogs.

1 **Table 1:** Characteristics of groups according to the endoscopic diagnosis, after excluding dogs with
 2 concurrent bacterial infection

	BE	BM	BEBM	H
N	10	22	12	28
Sex (M/F)	5/5	13/9	6/6	12/16
Age (year)	9.4 ± 3.17 ^a	9.95 ± 3.2 ^a	10.5 ± 2.6 ^a	5.89 ± 2.4 ^b
Weight (kg)	9.8 (7.7 – 15.5) ^b	5.2 (7.2 – 9.1) ^a	13.9 (8.0 – 18.0) ^b	23.5 (9.2 – 30.8) ^b
Breeds	3 crossbreed, 1 dachshund, 1 Whippet, 1 WHWT, 1 Jack Russel Terrier, 1 Maltese, 1 Beagle, 1 Malinois	3 Jack Russel Terrier, 3 Yorkshire Terrier, 5 crossbreed, 2 Shih Tzu, 2 Poodle, 2 CKCS, 2 Maltese, 1 Pomerian, 1 Whippet, 1 Labrador	2 crossbreed, 1 Shih Tzu, 1 WHWT, 1 Tibetan Terrier, 1 Whippet, 1 Basset hound, 1 Chihuahua, 1 Beagle, 1 Epagneul, 1 Samoyède, 1 Sheltie	10 terrier breeds other than WHWT, 10 shepherd, 5 retriever, 1 Beauceron, 1 Dalmatian
Total cell count (cells/μl)	860 (643 – 1290) ^a	860 (555 – 1805) ^a	1220 (700 – 2045) ^a	360 (260 – 660) ^b
% Macrophages	38.0 (18.7 – 54.7) ^{a,b,c}	80 (51.7 – 86.2) ^b	57.5 (20 – 74.7) ^a	82.5 (76 – 89.7) ^c
% Neutrophils	20 (9.5 – 48.7) ^b	12.5 (6.5 – 25.5) ^b	35.5 (14.5 – 56.2) ^a	8 (5 – 11.2) ^c
% Lymphocytes	2.5 (0.5 – 11) ^a	2 (0 – 6.2) ^a	1 (0 – 2.5) ^b	5 (2 – 11.5) ^a
% Eosinophils	2.5 (0 – 26.2) ^b	0.5 (0 – 3.5) ^b	3.5 (0.7 – 11) ^a	1 (0 – 1.2) ^b
Final diagnosis	6 CB, 4 EBP	21 CB, 1 EBP	10 CB, 1 IPF, 1 EBP	/

Data are presented as mean $\pm$ standard deviation for 'age' and as median with interquartile range for weight, total cell count, and the percentages of macrophages, neutrophils, lymphocytes, and eosinophils. Superscript letters indicate statistically significant differences ($p < 0.05$), determined using ANOVA with Tukey's post hoc test for 'age' and the Kruskal-Wallis test with Dunn's post hoc test for the other variables.

BE = bronchiectasis, BM = bronchomalacia, BEBM = combination of bronchiectasis and bronchomalacia, H = healthy, M = male, F = female, WHWT = West Highland White Terrier, CKCS = Cavalier King Charles Spaniel, CB = chronic bronchitis, EBP = eosinophilic bronchopneumopathy, IPF = idiopathic pulmonary fibrosis

BALF microbiota analysis

Good's coverage was $> 99.5\%$ in all samples for all analyses. In diseased dogs, alpha diversity was lower than in H-dogs ($p=0.035$) while no differences were observed among the groups (H, BE, BM, BEBM). No significant differences in bacterial richness were observed between H-dogs and diseased dogs (BE, BM and BEBM). Similarly, no significant differences were detected among the groups (H, BE, BM, BEBM). Bacterial evenness was lower in diseased compared to H-dogs ($p = 0.029$). Similarly, it was lower in BM-dogs compared to H-dogs ($p = 0.030$).

Differences in beta diversity between diseased dogs and H-dogs were visualised using an NMDS plot (Figure 1A) and confirmed statistically by PERMANOVA ($p=0.001$). Beta-dispersion analysis revealed significantly greater dispersion of microbiota profiles in diseased dogs compared with H-dogs ($p=0.04$), suggesting increased heterogeneity of the lung microbiota among diseased dogs. In contrast, H-dogs exhibited a more conserved microbiota composition. No significant differences in dispersion were detected among the BE, BM and BEBM groups ($p>0.05$). When comparing H-dogs and each disease group separately, the only difference was found between H and BM ($p=0.012$) (Figure 1B). Based on a dbRDA, no influence of neither age ($p=0.125$), weight ($p=0.227$) nor the interaction between age and weight ($p=0.055$) was detected on this microbiota model.

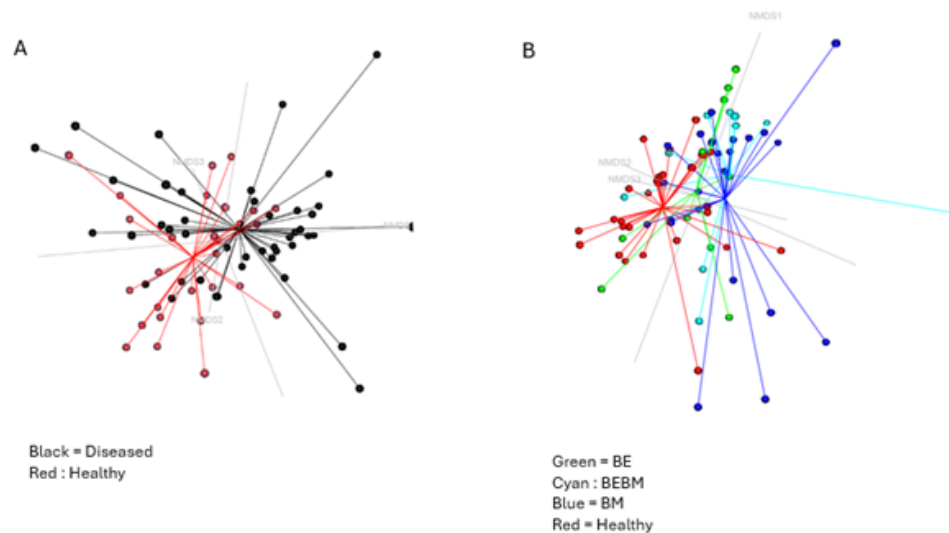


Figure 1. Three-dimensional non-parametric representation of the beta diversity at the genus level between each group of dogs based on a Bray-Curtis matrix of dissimilarity. Panel A shows comparisons in beta diversity between healthy (Healthy) and diseased dogs (Diseased). The NMDS was performed in 3 dimensions with a final stress value of 0.109. Panel B illustrates bacterial beta diversity for each group: bronchiectasis (BE), bronchiectasis/bronchomalacia (BEBM), bronchomalacia (BM), and healthy dogs (Healthy). The NMDS was performed in 3 dimensions with a final stress value of 0.109. A significant difference in beta diversity was present between healthy dogs and diseased dogs ($p=0.001$) and between healthy dogs and BM-dogs ($p=0.012$). NMDS = non-metric multidimensional scaling.

1 Table 2 represents the results of a DESeq2 analysis, based on beta diversity results, comparing
 2 bacterial abundances at genus level between BALF samples from H-dogs, diseased dogs and dogs
 3 with BM.

4 **Table 2:** Different abundance analysis at genus level of bacterial taxa in bronchoalveolar samples

Bacterium	H vs D Log2FC (p-value)	H vs BM Log2FC (p-value)
Akkermansia [*]	-	9.151043 (0.001176)
Serratia [§]	8.652494 (1.82 E-10)	9.128631 (3.39 E-11)
Weeksellaceae_ge [*]	5.038539 (3.24 E-06)	5.953477 (6.53 E-07)
Azomonas [*]	4.18994 (9.8 E-06)	3.668062 (0.007944)
Chryseobacterium [§]	4.551436 (0.0025)	5.406177 (0.000839)
Parcubacteria_ge [*]	-4.38953 (0.000173)	- 4.01525 (0.018151)
Citrobacter [§]	5.520063 (0.000947)	-
Filifactor [§]	3.87671 (0.020627)	-
Propionibacterium [*]	7.313242 (0.001393)	-
Sphingobacterium [*]	6.148505 (0.003421)	5.84866 (0.027965)
Escherichia_Shigella [§]	2.190921 (0.030971)	-
Pasteurellaceae_ge [§]	2.91491 (0.000785)	-
Diaphorobacter [*]	5.642444 (0.020627)	-
Pseudomonadales_ge [§]	4.328862 (0.001161)	5.699366 (4.74 E-05)
Proteocatella [*]	4.815741 (0.007335)	5.322555 (0.038846)
TM7x [*]	4.35484 (0.013609)	-
Microbacteriaceae_ge [*]	-3.06808 (0.011438)	-

SN8 [*]	-5.60132 (0.008745)	-
Flavobacterium [§]	-2.00759 (0.013609)	-2.505 (0.015347)
Enhydrobacter [*]	-3.13949 (0.023158)	-
Sphingomonadaceae_ge [*]	-	3.210656 (0.04784)
Rothia [§]	-3.26518 (0.048513)	-
Pasteurella [§]	2.434537 (0.030971)	2.887036 (0.041121)
Enterobacteriaceae_ge [§]	-	2.379527 (0.044257)
Bacteria_ge [*]	2.067945 (0.042076)	3.249083 (0.000974)
Sphingobium [*]	3.704746 (0.02305)	-
Pseudomonas [§]	-4.06834 (2.45 E-07)	-5.04064 (1.61 E-08)
Cutibacterium [§]	-1.63682 (0.027511)	-2.30712 (0.004276)
Corynebacteriaceae_ge [§]	-1.99296 (0.030971)	-
Citreitalea [*]	-2.61536 (0.040674)	-
Comamonas [*]	-3.0033 (0.04541)	-4.35632 (0.010052)
Lachnospiraceae_ge [*]	-2.46647 (0.048513)	-
Lactobacillales_ge [*]	1.771132 (0.048513)	-
Enterobacterales_ge [§]	-	5.543355 (1.57 E-05)
Gammaproteobacteria_ge [*]	-	4.463827 (0.000214)
Thermus [*]	-	8.93955 (0.013427)
Proteobacteria_ge [§]	-	6.067428 (0.015938)
Massilia [*]	-	-4.62201 (0.041311)
Hydrogenophilus [*]	-	7.165088 (0.030116)

This table presents the results of a DESeq2 analysis comparing bacterial abundances at genus level between bronchoalveolar lavage fluid (BALF) samples from healthy dogs (H), diseased dogs (D) and dogs with bronchomalacia (BM). Log2FoldChange (log2FC) indicates the direction and magnitude of differential abundance, with positive values, mentioned in bold, indicating higher abundance in diseased dogs and negative values indicating higher abundance in healthy dogs. The p-value assess the significance of differences, accounting for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) correction. A p-value < 0.005 was considered statistically significant. \$ indicates that the bacterium is considered as an opportunistic pathogen. * indicates that the bacterium is considered as non-pathogenic pathogen and non-opportunistic pathogen based on literature in human and veterinary medicine.

When evaluating specific groups (H, BE, BM and BEBM), the LDA identified 17 taxa that were discriminant between groups. Specifically, 8 taxa were found to be associated with H-dogs. For the diseased groups, 3 taxa were associated with BM dogs (*SN8*, *Pasteurella* and *Propionibacterium*), 1 taxus with BEBM dogs (*Azomonas*) and 5 taxa with BE dogs (*Frederiksenia*, *Pseudomonadaceae_ge*, *Diaphorobacter*, *Sphingobacterium* and *Citrobacter*) (Figure 2).

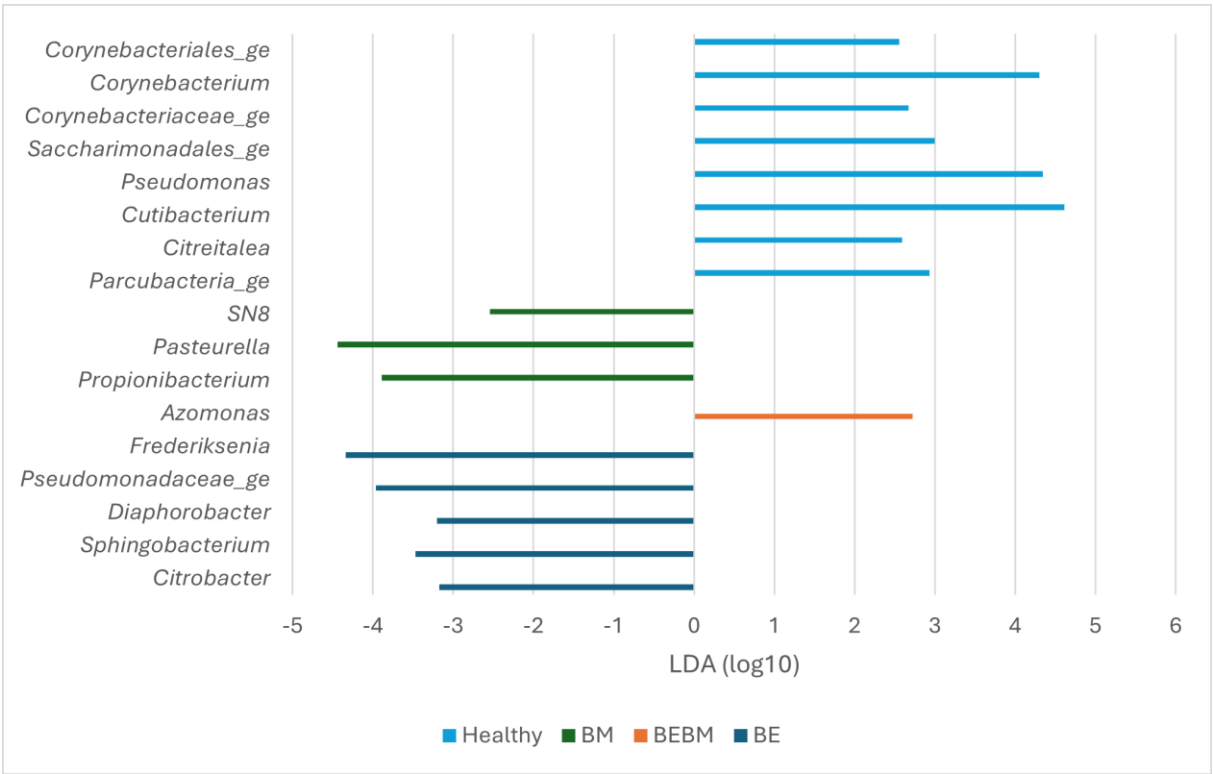


Figure 2. Linear Discriminant Analyse (LDA) to identify taxa that distinguish between the different groups of dogs based on their microbiota composition. The groups analyzed include healthy dogs, dogs with bronchomalacia (BM), dogs with a combination of bronchiectasis and bronchomalacia (BEBM), and dogs with bronchiectasis (BE).

Figure 3 illustrates the distribution of bacterial relative abundance at the phyla level in the LM of H versus diseased dogs. Diseased dogs exhibited a pronounced dominance of the phylum *Pseudomonadota*, which accounted for a significant proportion of the microbiota, alongside high representation of *Bacillota* and *Bacteroidota*. In contrast, H-dogs displayed a more balanced microbial composition with an increased relative abundance of *Actinomycetota*.

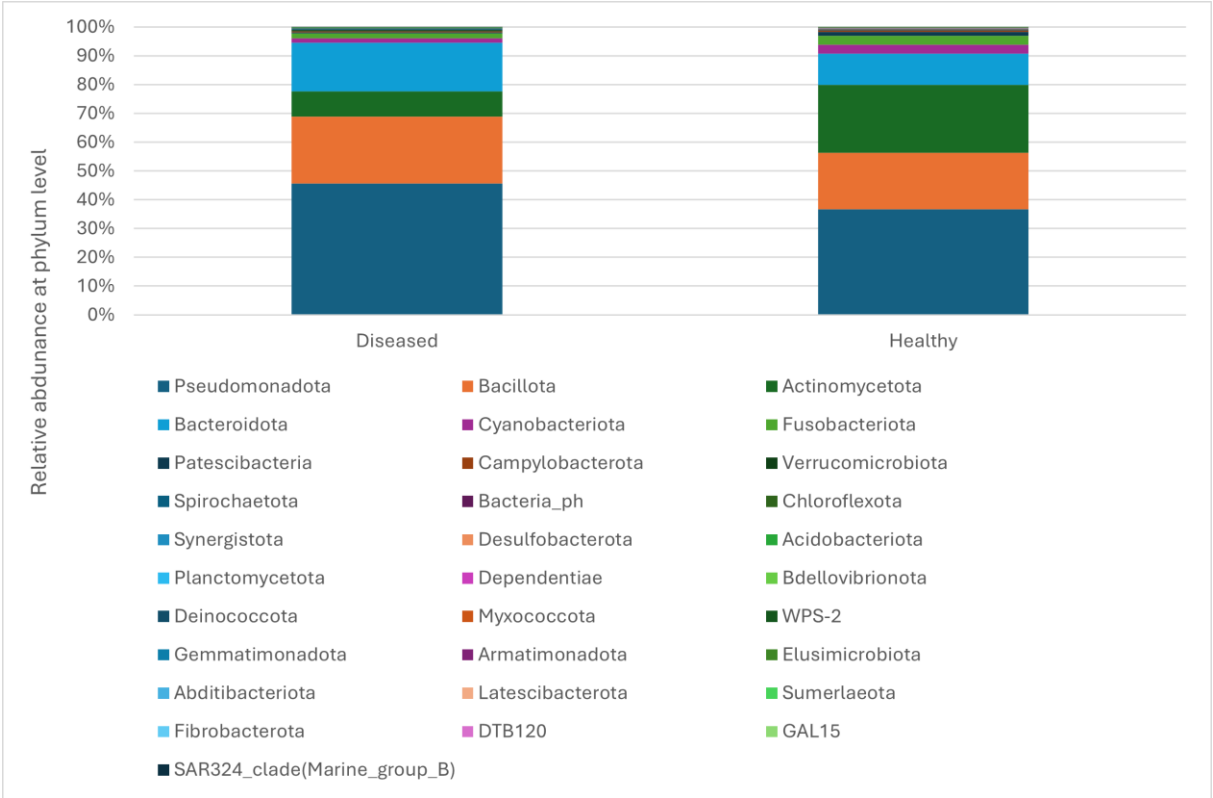


Figure 3. Phylum-level composition of bronchoalveolar lavage fluid microbiota for diseased dogs and healthy dogs.

Figure 4 expands on these findings by examining specific groups. Across dogs with BE, BM and BEBM, the same four phyla dominated. In dogs with BE, BM and BEBM, the dominating phylum was *Pseudomonadota*, followed by *Bacillota*, *Bacteroidota* and *Actinomycetota*. In dogs with BE, there was an increased dominance of *Pseudomonadota*. In contrast, H-dogs exhibited a different composition, with *Pseudomonadota* and *Actinomycetota* as the dominating phyla.

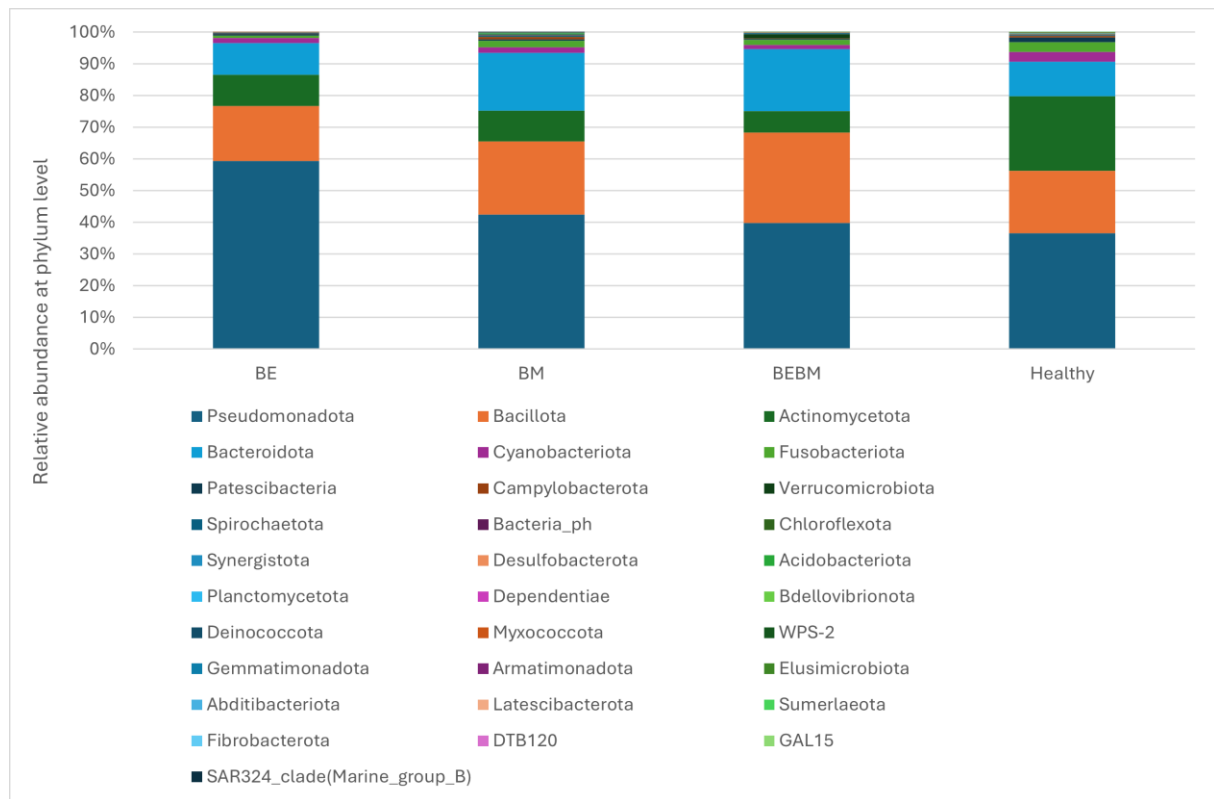


Figure 4. Phylum-level composition of bronchoalveolar lavage fluid microbiota for dogs with bronchiectasis (BE), bronchomalacia (BM), a combination of bronchiectasis and bronchomalacia (BEBM) and for healthy dogs (Healthy).

Figure 5 illustrates the distribution of the relative abundance of bacterial genera in the LM of H versus diseased dogs. Overall, the bacterial composition appears variable between both groups. For both diseased and H-dogs, genera contributing less than 2% of the overall microbiota were dominant, followed by *Streptococcus*, *Conchiformibius* and *Pasteurellaceae_ge* in descending order in diseased dogs.

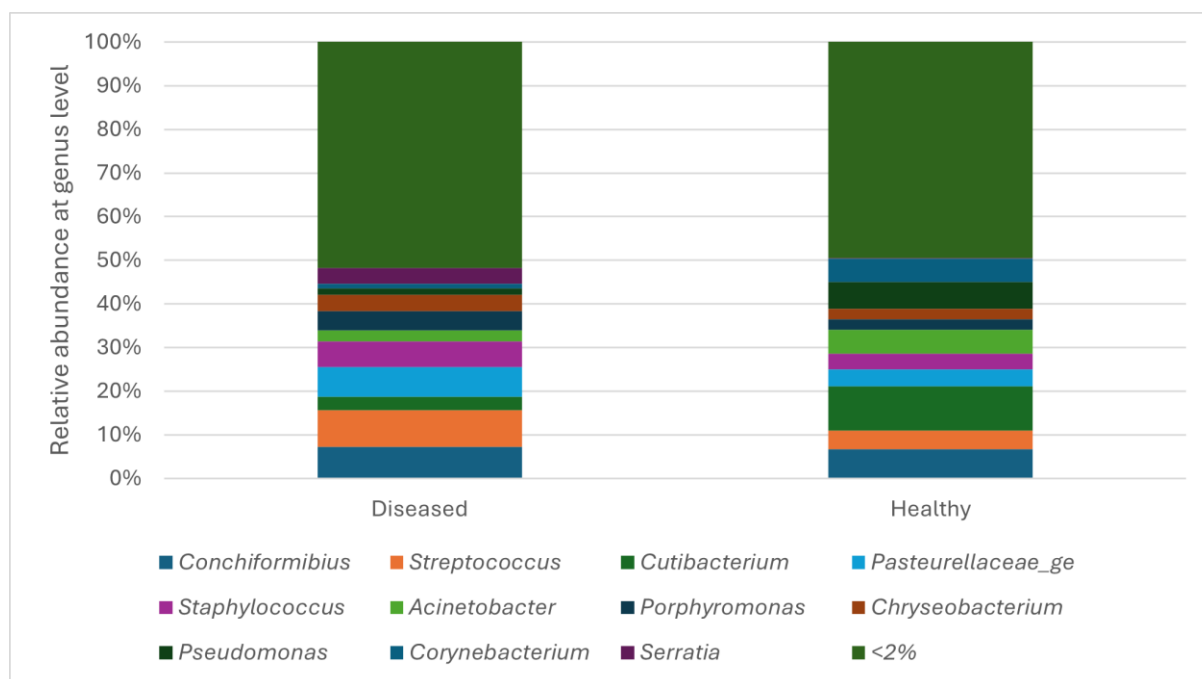


Figure 5. Genus-level composition of bronchoalveolar lavage fluid microbiota for dogs presenting with bronchiectasis and/or bronchomalacia, and healthy dogs, concerning only phyla of more than 2%.

Figure 6 expands on these findings by examining specific groups. Across all groups, genera contributing less than 2% of the overall microbiota were dominant. In BE dogs, this was followed by *Pasteurellaceae_ge* and *Conchiformibius*. In BM-dogs, it was followed by *Streptococcus* and *Porphyromonas*. In BEBM-dogs, *Streptococcus* was the next most abundant genus. In H-dogs, *Cutibacterium*, *Pseudomonas*, and *Corynebacterium* followed in descending order.

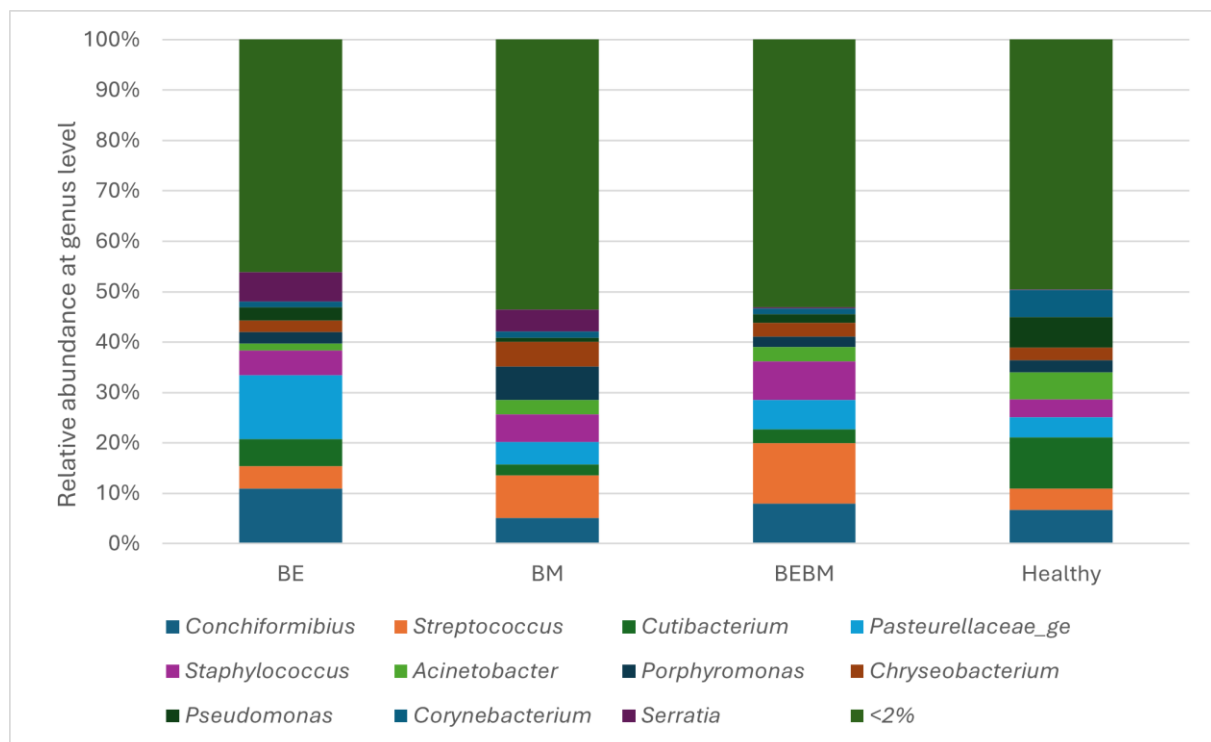


Figure 6. Genus-level composition of bronchoalveolar lavage fluid microbiota for dogs with bronchiectasis (BE), bronchomalacia (BM), a combination of bronchiectasis and bronchomalacia (BEBM) and for healthy dogs (Healthy), concerning only phyla of more than 2%.

Discussion

The present study provides new insights into the LM of dogs with chronic cough and airway alterations such as BE, BM and BEBM, without concomitant bacterial infection, compared to healthy control dogs. Our findings reveal a significant reduction in alpha diversity in diseased dogs compared to healthy controls. This reduction was primarily driven by decreased evenness rather than reduced richness, indicating a shift toward dominance of specific bacterial taxa rather than a loss of taxa. No significant differences in alpha diversity were detected among the different disease

groups. Additionally, beta diversity analysis indicates a shift in overall microbial composition between healthy and diseased dogs, as well as among disease groups.

In the present study, 4 major phyla were detected across all dogs, including Pseudomonadota, Actinomycetota, Bacillota and Bacteroidota, as shown in Figure 3. These phyla are consistent with those found in the core lung microbiome of healthy dogs, although the relative abundance of these phyla varied (Ericsson *et al.*, 2016; Richardson *et al.*, 2019; Fastrès *et al.*, 2020; Vientos-Plotts *et al.*, 2023). Pseudomonadota has been reported as the predominant phylum in the lower airways of healthy dogs (Vientos-Plotts *et al.*, 2023). This finding was confirmed in this study, where it was the most abundant phylum in healthy and also in diseased dogs. However, notable shifts in the relative abundance of certain genera and phyla were observed, as shown in Figure 3 and Figure 5. Specifically, Actinomycetota was decreased in dogs with CRD compared to healthy dogs. The potential beneficial effects of the Actinomycetota phylum have been explored in human studies, particularly in patients with asthma. One study found that an increase in Actinomycetota was associated with a positive clinical response to steroid treatment (Huang *et al.*, 2015). Furthermore, Actinomycetota is metabolically diverse and produces a range of secondary metabolites, including antimicrobial and anti-inflammatory compounds (Huang *et al.*, 2015). The reduced abundance of Actinomycetota in dogs with BE, BM, and BEBM might indicate a disrupted microbial balance in the lungs, potentially contributing to the severity and progression of these respiratory conditions.

The LM primarily consisted of a diverse array of low-abundance genera, a pattern observed across healthy dogs, the combined diseased group, and the disease subgroup classifications (BE, BM, and BEBM). In diseased dogs, *Streptococcus*, *Conchiformibius*, and *Pasteurellaceae_ge* were the most prevalent genera, in descending order, as illustrated in Figure 5. These genera are commonly found in both healthy and diseased lungs of dogs but their relative abundance may differ (Ericsson *et al.*, 2016; Fastrès *et al.*, 2019; Fastrès *et al.*, 2020). In humans with BE, pathogens such as *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* are associated with disease severity and exacerbations (Rogers *et al.*, 2013; Dickson *et al.*, 2016; Flume *et al.*, 2018). In contrast, none of these taxa showed consistent overrepresentation in our cohort, likely reflecting the exclusion of dogs with concurrent bacterial infection.

LDA analysis identified 17 discriminant genera (Figure 2), suggesting a link between certain bacterial taxa and disease states. No significant increase in *Pseudomonas*, *Haemophilus*, or *Streptococcus* was observed. Instead, BM dogs were associated with *SN8*, *Pasteurella*, and *Propionibacterium*, while BEBM dogs were characterized by *Azomonas*. In BE dogs, *Frederiksenia*, *Pseudomonadaceae_ge*,

Diaphorobacter, *Sphingobacterium*, and *Citrobacter* were more prominent. The clinical significance of such findings remains uncertain, as many identified genera are environmental contaminants or commensals, potentially reflecting incidental detection rather than disease involvement. However, *Pasteurella*, *Pseudomonadaceae*_ge, and *Citrobacter* may act as opportunistic pathogens, though *Pasteurella* is also part of the normal respiratory flora (Doran, 1998; Michael *et al.*, 2018; Lalucat *et al.*, 2022). In light of the increased microbiota heterogeneity observed in diseased dogs, these findings suggest that the identified taxa may not be equally representative of all diseased individuals and should therefore be interpreted with caution.

The lower alpha diversity in diseased dogs compared to healthy ones suggests that disease may negatively affect the microbial ecosystem by promoting dysbiosis, reducing the variety of microbes that can survive in the environment created by the disease. However, the lack of significant differences in alpha diversity among the different disease groups suggests that while disease status impacts lung microbiota composition, the specific type of CRD (BE, BM, or BEBM) does not substantially alter overall microbial diversity. Nevertheless, we cannot exclude the possibility that a larger sample size might be needed to detect potential differences between disease groups. These findings align with previous research on both human and canine respiratory microbiomes, which indicates that alpha diversity changes are not always straightforwardly associated with disease and disease severity (Cox *et al.*, 2017; Ericsson *et al.*, 2020; Avalos-Fernandez *et al.*, 2022; Mac Aogain *et al.*, 2024). Avalos-Fernandez *et al.* reviewed multiple studies on chronic lung diseases in humans, including asthma, COPD, cystic fibrosis (CF) and non-CF BE, and found no consistent trend in respiratory microbiota alpha diversity when comparing healthy individuals to those with stable or exacerbated disease states, underscoring the complexity of microbiota changes in chronic lung diseases. However, Ericsson *et al.* reported that dogs with chronic bronchitis and other respiratory diseases had lower richness compared to healthy research dogs. In contrast, we were unable to detect difference in richness between groups in our study. Instead, we observed changes in evenness, which may indicate alterations in bacterial dominance rather than species loss. This discrepancy could be due to differences in study populations, sampling methods, or disease classifications.

We found a difference in beta diversity, or overall microbial community composition, in diseased dogs compared to the healthy ones (Figure 1A). In addition, diseased dogs exhibited significantly greater beta-dispersion than healthy dogs, indicating increased inter-individual variability in lung microbiota composition. Accordingly, a study by Ericsson *et al.* also showed that dogs with chronic bronchitis exhibited a greater range of community profiles compared to both healthy dogs and

those with non-inflammatory airway disease, suggesting increased microbial variability in dogs affected with chronic bronchitis. In humans, beta diversity has been explored in patients with BE: significant differences were shown between patients with BE and those with COPD by Chen *et al.*, while Lee *et al.* (2018) found no significant differences between patients with mild to moderate and severe BE. Further analysis comparing H-dogs to each disease group separately showed that the only significant difference in beta diversity was between H-dogs and BM-dogs (Figure 1B). This finding highlights that while the microbial communities of diseased dogs as a whole differ from H-dogs, only BM dogs exhibit a distinct shift in microbial composition when compared to healthy dogs. The lack of significant differences between healthy dogs and the other disease groups (BE and BEBM) may point to more subtle or less pronounced changes in their LM. Alternatively, this finding could be due to the limited sample size in each subgroup and may require confirmation in studies with larger populations. Overall, these results suggest that disease impacts the LM in a way that is observable at the community level, but the extent of this shift may vary depending on the specific type of disease. Future studies should control for these variables to better isolate the impact of disease on the LM.

Results of the different abundance analysis (Table 2) highlights significant shifts in the LM of dogs with CRD, and underscores the complexity of the respiratory microbiome in chronic canine respiratory disease. Differential abundance analysis was only performed between H-dogs and diseased dogs as a whole, and specifically between the H-dogs and BM-dogs, because these were the only comparisons that showed statistically significant differences in beta diversity analysis. Other pairwise comparisons (H vs BEBM, H vs BE, BE vs BEBM, BE vs BM, and BM vs BEBM) did not show significant differences in beta diversity and were therefore not pursued in the differential abundance analysis. Across different comparisons, the most consistently enriched bacterial taxa in diseased dogs included *Serratia*, *Weeksellaceae_ge*, and *Azomonas*, with additional contributions from *Pseudomonadales_ge* and *Enterobacterales_ge* in the BM group. *Serratia*, an opportunistic pathogen associated with nosocomial infections, has been reported to colonize the respiratory tract in compromised hosts (Golemi-Kotra, 2008). Its increased detection in diseased dogs may reflect secondary colonization of an altered airway environment rather than a primary pathogenic role. In contrast, *Weeksellaceae_ge* and *Azomonas* were also significantly more abundant in diseased dogs but are considered as environmental contaminants (Miransari, ElNaker *et al.*, 2018). Their presence is likely due to environmental exposure and possibly impaired lung defenses, and microbiome dysbiosis rather than direct pathogenicity. The inclusion of *Pseudomonadales_ge* and *Enterobacterales_ge* in the BM group is notable, as both taxa belong to bacterial orders that include

many opportunistic pathogens. However, because these groups were identified at a general taxonomic level (genus not yet classified), it is not appropriate to directly associate them with specific genera such as *Pseudomonas* or *Enterobacter*. Instead, their enrichment in diseased dogs may point to the presence of previously uncharacterized microbial agents potentially involved in the pathophysiology of CRD. These taxa warrant further investigation, including genomic or culture-based characterization, to determine their identity, ecological role, and potential contribution to disease processes. Their consistent presence in the BM group also underscores the importance of exploring novel microbial signatures in canine respiratory dysbiosis

In this study, we excluded dogs with bacterial infections from further LM analysis to obtain a more accurate characterization of the microbial communities in dogs with CRD, without being influenced by concurrent bacterial pathogens, which are known to significantly alter microbial diversity and structure (Vientos-Plotts *et al.*, 2023). A previous study by the same authors found that the prevalence of bacterial co-infections in dogs diagnosed with BE, BM and BEBM was relatively low (Lyssens *et al.*, 2025). However, even low levels of bacterial infection can impact the LM profile, as infections often lead to local inflammation and changes in mucus production, all of which may disrupt the native microbial community (Vientos-Plotts *et al.*, 2023). Given that bacterial infections could introduce variability unrelated to the underlying CRD, we opted to exclude these cases. This approach allowed us to focus specifically on the intrinsic LM characteristics of diseased dogs, ensuring that our findings more accurately reflect the microbial environment associated with chronic airway pathology rather than secondary infections.

This study has limitations. As it relies on retrospective data, there is a potential for selection bias that may have influenced the results. Secondly, the study included a limited number of dogs, particularly within the BE and BEBM subgroups, which may restrict both the generalizability of the findings and the statistical power to detect subtle disease-specific differences in LM composition. Furthermore, the unequal distribution of dogs among disease groups, with the BM group containing substantially more dogs than the BE and BEBM groups, may have influenced the ability to detect significant differences between groups. This may partly explain why significant beta-diversity differences were identified between healthy and BM dogs, but not between healthy and BE or BEBM dogs. Therefore, the absence of statistically significant differences between disease groups and between healthy dogs and the smaller BE and BEBM subgroups should be interpreted cautiously and does not exclude the possibility of a Type II error. In addition, diseased dogs exhibited significantly greater beta-dispersion than healthy dogs, indicating increased heterogeneity of LM composition within the disease population. This increased inter-individual variability may limit the

generalizability of individual taxa identified through LDA and DESeq2 analyses, as some disease-associated taxa may characterize subsets of affected dogs rather than being consistently observed across all diseased individuals. Thirdly, because of the low bacterial biomass of the respiratory tract, strict contamination control is essential in LM studies. Exogenous DNA may be preferentially amplified and thereby bias microbial profiles. Potential contamination can arise at multiple stages, including sampling, endoscopic equipment, and laboratory processing. To limit reagent-derived contamination, strict laboratory quality control procedures were applied. Procedural control specimens were collected prior to each BAL to monitor potential contamination originating from the bronchoscope, saline solution, or lavage system. During bronchoscope insertion, contact with the oropharyngeal, laryngeal, and tracheal mucosae was carefully avoided. Although limited upper airway carryover cannot be entirely excluded, previous studies indicate that its impact on bronchoscopically obtained lung microbiota profiles is minimal (Bassis *et al.*, 2015; Dickson *et al.*, 2015). Because of the retrospective nature of the study, cytological indicators of oropharyngeal contamination, such as squamous epithelial cells or *Simonsiella spp.*, were not systematically recorded and could therefore not be retrospectively assessed in all cases. Consequently, the presence of clinically relevant upper airway contamination cannot be completely excluded. Another limitation relates to the diagnostic classification of BE, BM, and BEBM, which was based on bronchoscopic findings. Although bronchoscopy is routinely used in veterinary medicine for the diagnosis of airway abnormalities, it does not allow complete assessment of peripheral bronchi or certain lung regions (Johnson *et al.*, 2016). Therefore, mild or localized BE may have been underdiagnosed, particularly in dogs classified as having BM alone. The absence of systematic thoracic CT imaging may have led to potential misclassification in some cases. Future prospective studies incorporating high-resolution CT could improve diagnostic accuracy and subgroup stratification. The BAL sampling site was primarily determined by the presence of gross macroscopic bronchoscopic abnormalities, with diaphragmatic lobes sampled when no focal lesions were identified. Although this reflects routine clinical practice, regional heterogeneity of lung microbiota cannot be excluded and may have influenced microbial profiles (Dickson *et al.*, 2015). While similar data in dogs are limited, this may reduce the likelihood that sampling site substantially biased the overall comparisons. However, regional heterogeneity in focal disease cannot be completely excluded and warrants further investigation in future studies using standardized multi-lobar sampling strategies.

Healthy control group differed significantly in age and weight from the diseased groups. Although dbRDA analysis did not identify age, weight, or their interaction as significant drivers of microbiota

composition, residual confounding effects cannot be entirely excluded (Lloyd *et al.*, 2017). Future studies using age- and weight-matched control groups would further strengthen causal interpretation. Also, dogs with bacterial infections were excluded, potentially underestimating the impact of secondary infections on LM. Additionally, the absence of longitudinal data and limited clinical correlation restrict the interpretation of microbiota dynamics and their association with disease progression. Further studies are needed to confirm these findings and explore the role of specific bacterial taxa in CRD. The definition of bacterial infection used for exclusion purposes may also represent a limitation. Although based on a combination of BALF cytology, culture, and qPCR findings and consistent with previous work, no universally accepted gold standard exists for the diagnosis of lower respiratory tract infection in dogs. In particular, the clinical significance of positive qPCR results for organisms such as *Mycoplasma cynos* or *Bordetella bronchiseptica* remains uncertain, as these microorganisms may also be detected in dogs without active infection. Consequently, some dogs classified as infected and excluded from the study may not have represented true bacterial infections. However, given the primary objective of characterizing lung microbiota in dogs without concurrent bacterial infection, a conservative exclusion approach was considered preferable to the inclusion of potentially infected dogs.

In conclusion, this study provides new insights into the LM of dogs with CRD such as BE, BM and BEBM. While alpha diversity was lower in dogs with CRD compared to H-dogs, no statistically significant differences were observed among the BE, BM and BEBM groups in this cohort; however, the relatively small subgroup sizes may have limited the ability to detect disease-specific microbiota differences. In contrast, significant differences in beta diversity were observed, indicating a possible shift in microbial composition rather than a loss of bacterial richness and evenness. While this study provides a strong foundation for understanding LM shifts in canine respiratory disease, further research is needed to explore the functional roles of identified bacterial taxa and their potential impact on disease progression and treatment outcomes.

1

2

Discussion - Perspectives

Chronic cough in dogs is a frequent reason for referral in small animal internal medicine and represents the clinical expression of a broad spectrum of airway and pulmonary disorders. Among these, chronic bronchitis with or without BE and/or BM, which constitutes irreversible outcomes of chronic airway injury, is one of the most frequent causes of persistent cough in dogs. BE and BM represent distinct morphological airway abnormalities resulting from chronic airway injury, yet their biological and clinical relationship remains poorly understood. This thesis provides an integrated investigation of dogs with BE, BM, and the concurrent presence of both abnormalities. The overarching hypothesis was that these airway abnormalities would exhibit distinct clinicopathological and microbiological characteristics, supporting the existence of biologically different phenotypes. However, across the complementary studies presented in this thesis, no consistent differences could be demonstrated between dogs with BE, BM, or both with respect to their clinical presentation, BAL cytology, NET formation, or LM composition. These findings indicate that, using the approaches employed in the present work, the three groups exhibited quite similar biological profiles. Based on these observations, an evidence-based treatment algorithm was proposed that focuses on the underlying inflammatory or infectious phenotype rather than on the specific morphological airway abnormality. LM analysis identified disease-associated dysbiosis characterized by reduced microbial diversity and a relative overrepresentation of Proteobacteria, whereas NET analyses demonstrated increased neutrophil activation within the lower airways of dogs with chronic bronchial disease, particularly in dogs with BE. Together, these findings improve our understanding of the inflammatory and microbiological environment associated with chronic bronchial disease in dogs. Importantly, the absence of detectable differences between BE and BM should not be interpreted as evidence that these airway abnormalities share an identical pathophysiology. Rather, the present work demonstrates that no biological differences could be identified using the clinicopathological, microbiological, and immunological approaches employed. Additional mechanisms, including differences in airway biomechanics, histopathology, extracellular matrix remodelling, or other molecular pathways, may contribute to the development of these distinct morphological abnormalities but were beyond the scope of this thesis. Consequently, BE and BM should be regarded as distinct structural manifestations that may arise in the context of chronic bronchial disease, while the extent to which they share common pathogenic mechanisms remains to be determined by future studies.

The first study of this thesis sought to determine whether dogs diagnosed with BE and/or BM represent distinct clinicopathological phenotypes, based on endoscopy and imaging findings. Despite their distinct morphological definitions, no meaningful differences were identified among the BE, BM, and BEBM groups with regard to clinical presentation, BALF cytology, or other clinicopathological variables. These findings indicate that, using the clinicopathological variables investigated in the present study, the three groups exhibit quite similar biological profiles. When considered together with the NET and lung microbiota studies, this observation suggests that the inflammatory and microbiological mechanisms evaluated in this thesis are largely shared across these airway abnormalities. However, these findings should not be interpreted as evidence that BE and BM share an identical pathophysiology, as additional disease-specific mechanisms may exist but were beyond the scope of the present work. Collectively, these findings suggest that the structural phenotype, namely bronchial dilation versus bronchial collapse, is less closely associated with the inflammatory and microbiological variables investigated in this thesis than initially hypothesized. Rather than demonstrating biologically distinct phenotypes, the present studies identified largely overlapping inflammatory and microbial profiles among the three groups. Whether additional molecular, biomechanical, or structural mechanisms distinguish BE from BM remains to be determined. In this context, both bronchial dilation and bronchial collapse may reflect different structural consequences of chronic inflammatory injury affecting the bronchial wall. This concept aligns with the current understanding of BE pathogenesis in human medicine, where chronic airway diseases are often conceptualized within the framework of the “vicious cycle” model (Marolf and Blaik, 2006; Chandrasekaran *et al.*, 2018). According to this model, persistent airway inflammation, impaired mucociliary clearance, and microbial colonization interact to perpetuate progressive airway damage. Structural changes in the bronchial wall may arise as a consequence of chronic inflammatory injury and enzymatic degradation of airway components (Marolf and Blaik, 2006; Chandrasekaran *et al.*, 2018). Within this framework, both bronchial dilation and dynamic airway collapse may represent structural outcomes of prolonged inflammatory processes affecting the bronchial wall and surrounding tissues. However, the present studies do not establish that both abnormalities develop through identical pathogenic mechanisms. Rather, they indicate that the inflammatory pathways investigated here are common to dogs presenting with either morphological abnormality.

The frequent coexistence of BE and BM observed in the present study suggests that these airway abnormalities may develop concurrently in some dogs with chronic bronchial disease. This parallels human medicine, where a combination of BE and BM is described (Chang *et al.*, 2002; Nishino *et al.*,

2006). Whether this reflects shared pathogenic mechanisms, common predisposing factors, or independent consequences of chronic airway injury remains to be clarified.

A potential explanation for the apparently higher frequency of BM in dogs compared to humans may relate to species-related differences. In particular, anatomical and biomechanical characteristics of the canine airways may hypothetically predispose dogs to develop airway collapse more readily under chronic inflammatory conditions, although this remains speculative.

Another important finding of the first study was the limited agreement between imaging modalities and bronchoscopic findings for the diagnosis of airway abnormalities. Thoracic radiography and CT are commonly used in the evaluation of chronic respiratory disease in dogs. However, the results of this thesis highlight the limitations of these modalities in reliably identifying bronchial abnormalities when compared with direct bronchoscopic visualization. This finding has important clinical implications, as it suggests that imaging alone may underestimate the presence of structural airway disease. Bronchoscopy therefore remains a critical diagnostic tool for the evaluation of dogs with chronic cough and suspected bronchial disease. However, it should also be considered that bronchoscopy may overestimate dynamic BM, as airway manipulation, anaesthesia, or changes in intrathoracic pressure during the procedure could induce or accentuate bronchial collapse that may not be apparent under physiological conditions. This may partly explain the discrepancies observed between imaging and bronchoscopic findings. When combined with the microbiota and NETs studies, this diagnostic mismatch further highlights how complex chronic bronchial disease in dogs is. The absence of clear differences in clinicopathological, microbiological, and inflammatory variables further illustrates the complexity of chronic bronchial disease in dogs. These findings indicate that BE, BM, and BEBM share many biological characteristics measurable using current approaches. Nevertheless, because only selected aspects of disease biology were investigated, these results should not be interpreted as demonstrating that these airway abnormalities represent a single disease process.

While the first study focused primarily on clinical and diagnostic aspects of BE and BM, the subsequent investigations aimed to explore potential mechanisms underlying chronic airway inflammation in dogs. The second component of this thesis focused on neutrophil-driven inflammatory mechanisms, particularly the role of NETs.

The detection and quantification of NETs in BALF from dogs with chronic bronchial disease provide novel evidence that neutrophil activation is a key feature of these conditions. NETs were identified across all disease groups, indicating that neutrophil-mediated inflammation is a prominent feature of chronic bronchial disease irrespective of the morphological airway abnormality present. The

1 observed associations between NET markers, neutrophilic inflammation, and clinical variables such
2 as disease duration further support a role for NETs in sustaining airway inflammation. In particular,
3 the positive correlations between MPO–DNA complexes and neutrophil counts and duration of
4 clinical signs indicate that NET formation is closely linked to the intensity and chronicity of the
5 inflammatory process.

6 Importantly, one of the major findings of this study is that NETs appear to be more prominent in
7 dogs with BE. Indeed, MPO–DNA complex levels were significantly higher in BE dogs compared with
8 BM and healthy controls. This suggests that NET formation may play a more central role in the
9 pathophysiology of BE, potentially reflecting enhanced neutrophil activation. These findings
10 suggest that NET formation may be particularly pronounced in dogs with BE and could contribute
11 to airway injury in this subgroup. Whether increased NET formation represents a cause or a
12 consequence of bronchiectatic remodelling remains unknown.

13 When considered alongside the microbiota findings, the presence of NETs provides additional
14 evidence supporting the concept of a self-perpetuating inflammatory cycle in canine chronic airway
15 disease. Microbial dysbiosis may stimulate neutrophil recruitment and activation, leading to NET
16 release. In turn, the inflammatory and proteolytic environment generated by NET formation may
17 further damage airway structures and impair mucociliary clearance, thereby promoting the
18 persistence of microbial communities within the airways. This interaction between microbial
19 communities and host inflammatory responses may contribute to the progressive structural
20 alterations observed in diseases such as BE and BM. Importantly, both microbiota alterations and
21 NET formation were observed in dogs with BE, BM, and BEBM. Together, these findings indicate that
22 chronic airway inflammation, neutrophil activation, and microbial dysbiosis are common biological
23 features associated with these airway abnormalities. However, the present work does not
24 demonstrate that these mechanisms fully explain the development of the distinct structural
25 phenotypes.

26
27 A third objective of this thesis was to investigate the role of the LM in chronic airway disease and to
28 determine whether microbial composition differed between dogs with BE, BM, and BEBM. Based on
29 the traditional view that structural airway abnormalities may influence local microbial
30 environments, it was initially hypothesized that distinct microbiota profiles could be identified
31 across these groups. However, when considered in light of the findings of the first study, which
32 demonstrated minimal clinicopathological differences between BE, BM, and BEBM, the likelihood of
33 identifying clearly distinct microbial signatures between these phenotypes appeared less probable.

1 The LM has emerged in recent years as an important component of respiratory health. Contrary to
2 earlier assumptions that the lower airways were sterile in healthy individuals, it is now well
3 established that the respiratory tract hosts complex microbial communities that interact with the
4 host immune system. These microbial communities contribute to immune homeostasis and may
5 influence susceptibility to respiratory disease. In chronic airway disorders, structural airway
6 alterations, mucus accumulation, and impaired mucociliary clearance may disrupt the normal
7 microbial balance, leading to microbial dysbiosis.

8 The microbiota analysis performed in this thesis confirmed that dogs with chronic bronchial disease
9 exhibit significant alterations in microbial composition compared with healthy controls,
10 characterized by reduced diversity and shifts in bacterial community structure. However,
11 importantly, these alterations were consistent across BE, BM, and BEBM, with no clear separation
12 between the different structural phenotypes. These findings indicate that dysbiosis is primarily
13 associated with the chronic inflammatory airway environment rather than with a specific structural
14 abnormality.

15 When integrated with the clinicopathological findings of the first study, these findings indicate that
16 microbial dysbiosis is associated with chronic bronchial disease regardless of the morphological
17 airway abnormality identified. No disease-specific microbial signature could be demonstrated for
18 BE, BM, or BEBM. Moreover, the relatively low frequency of confirmed bacterial infection observed
19 across the studies suggests that microbial alterations in these dogs most often reflect colonization
20 or ecological imbalance rather than true infection. This distinction is critical, as it challenges the
21 traditional infection-driven paradigm of chronic airway disease. Instead, the findings support a
22 model in which dysbiosis arises as a consequence of impaired airway defence mechanisms,
23 including altered mucociliary clearance and structural airway changes, while simultaneously
24 contributing to the maintenance of chronic inflammation through persistent immune stimulation.
25 The microbiota findings complement the clinicopathological and NET analyses by demonstrating
26 that the chronic inflammatory airway environment is associated with similar microbial alterations
27 across the three groups. Whether additional mechanisms distinguish the development of BE and BM
28 requires further investigation.

29
30 Overall, the findings of this thesis demonstrate that chronic bronchial disease in dogs is
31 characterized by interconnected inflammatory, microbiological, and structural alterations. Within
32 the scope of the investigated variables, dogs with BE, BM, and BEBM exhibited quite similar
33 biological profiles. Nevertheless, the present work does not establish that these airway
34 abnormalities share identical pathogenic mechanisms, and future studies incorporating molecular,

1 biomechanical, and longitudinal approaches will be required to determine how these distinct
2 structural abnormalities develop.

3
4 All these findings also have important implications for the optimal therapeutic management of dogs
5 with chronic airway disease. Despite the high prevalence of chronic bronchial disorders and the
6 availability of advanced diagnostic tools, evidence-based treatment guidelines remain poorly
7 defined in veterinary medicine. As a result, therapeutic decisions are often empirical and vary
8 substantially between clinicians. The therapeutic algorithm proposed in this thesis therefore aims
9 to provide a structured framework for the management of dogs with chronic bronchial disease while
10 promoting rational antimicrobial use. However, this algorithm should be regarded as a hypothesis-
11 generating clinical tool rather than a validated treatment guideline. Its clinical utility and impact on
12 long-term outcomes remain to be confirmed in prospective controlled studies. Moreover, the
13 practical implementation of this algorithm may vary depending on the availability, accessibility, and
14 cost of diagnostic procedures such as bronchoscopy, BALF cytology, bacterial culture, or molecular
15 analyses. Consequently, the proposed approach should be adapted to the local clinical setting and
16 available resources.

17 Central to this approach is the distinction between infectious and non-infectious inflammatory
18 phenotypes, determined primarily by cytological evaluation of BALF, particularly the detection of
19 intracellular bacteria. Importantly, the proposed algorithm is intended for dogs presenting with
20 chronic bronchial disease rather than chronic bronchitis alone. It distinguishes between sterile
21 chronic bronchitis and chronic bronchial disease complicated by secondary bacterial infection.
22 Thus, the algorithm does not redefine chronic bronchitis as an infectious disorder but rather
23 acknowledges that dogs with chronic inflammatory airway disease may develop concurrent
24 bacterial infection requiring antimicrobial therapy. Accordingly, the decision to institute
25 antimicrobial treatment is based on objective evidence of infection, particularly the identification of
26 intracellular bacteria on BALF cytology, rather than on the diagnosis of chronic bronchitis itself.

27 In many chronic bronchial diseases, inflammation rather than infection appears to be the primary
28 driver of airway pathology. This is consistent with the findings of the first study of this thesis, in which
29 only a minority of dogs showed cytological or microbiological evidence of bacterial infection in
30 BALF. This contrasts with human BE, where chronic bacterial infection is frequently reported and
31 plays a central role in disease progression. In human patients, persistent colonization with
32 pathogens such as *Pseudomonas aeruginosa* is associated with more severe disease, increased
33 exacerbation frequency, and poorer long-term outcomes. Consequently, long-term antibiotic

1 strategies, including prolonged or rotating antimicrobial therapy, are often recommended in human
2 medicine to reduce exacerbations and improve survival.

3 In dogs, however, the relatively low frequency of confirmed bacterial infection observed in this
4 thesis suggests that chronic airway inflammation may more often occur in the absence of persistent
5 infection. In this context, empirical antibiotic use may provide only transient symptomatic
6 improvement through anti-inflammatory effects rather than addressing the underlying disease
7 mechanisms. Unnecessary antimicrobial therapy also carries the risk of promoting antimicrobial
8 resistance and disrupting the airway microbiota. The proposed treatment algorithms therefore aim
9 to focus therapeutic strategies on the underlying disease processes, reserving antimicrobial therapy
10 for cases where infection is clearly demonstrated.

11 Within the broader context of this thesis, these therapeutic considerations are closely linked to the
12 clinicopathological and mechanistic findings obtained in the preceding studies. The frequent
13 coexistence of BE and BM together with the observed alterations in airway microbiota and
14 neutrophil-driven inflammation indicate that these biological processes are relevant across dogs
15 with different chronic airway remodelling patterns. Consequently, therapeutic strategies should
16 primarily target the underlying inflammatory and infectious processes identified in each patient
17 rather than the specific structural phenotype alone.

18 Although the proposed algorithm primarily focuses on controlling the underlying inflammatory or
19 infectious process, symptomatic management of cough should also be considered in selected
20 patients. In particular, carefully selected dogs with severe BM and persistent non-productive cough
21 may benefit from antitussive therapy when excessive coughing contributes to airway irritation and
22 dynamic airway collapse. It is also possible that, in some dogs with advanced BM, cough may be
23 driven predominantly by mechanical airway collapse rather than active inflammation. Although this
24 hypothesis could not be evaluated in the present studies, as all included dogs exhibited
25 bronchoalveolar inflammation, it may explain why carefully selected patients with severe BM and
26 non-productive cough could benefit from antitussive therapy. However, antitussives should be used
27 cautiously and generally avoided in dogs with productive cough or active lower respiratory infection,
28 where cough remains an important airway clearance mechanism.

29 Finally, management should not be limited to the structural airway abnormalities themselves.
30 Whenever possible, treatment should also address the underlying disease responsible for the
31 development of BE and/or BM, such as chronic bronchitis, EBP, infectious disease, or other
32 respiratory disorders, as this remains the cornerstone of long-term management.

33

34

1 **Limitations**

2 While the studies presented in this thesis provide novel insights into the clinicopathological
3 characteristics, inflammatory mechanisms, LM, and therapeutic management of chronic bronchial
4 disease in dogs, several limitations should be considered when interpreting the findings and their
5 broader applicability. These limitations largely reflect the exploratory, retrospective, and cross-
6 sectional nature of the work.

7
8 A first major limitation of this thesis concerns its retrospective design. Both the descriptive clinical
9 study and the investigations on LM and NETs relied on retrospectively collected data from medical
10 records and archived samples of client-owned dogs, which inherently introduces variability. This
11 approach limits the ability to standardize sampling procedures, follow-up intervals, and treatment
12 protocols prior to inclusion. As a result, potential confounding factors, such as previous antibiotic or
13 corticosteroid use, environmental exposure, or concurrent disease, could not always be controlled
14 for. Moreover, retrospective analyses rely on the accuracy of record keeping and may be affected by
15 incomplete datasets or missing parameters. Another methodological consideration concerns the
16 BALF procedure. Although BALF was performed according to a standardized protocol at our
17 institution, BALF techniques vary considerably between centres, particularly regarding the volume
18 instilled (fixed volume versus body weight-adjusted volume), the number of aliquots, and the
19 handling or correction for BALF dilution. These methodological differences may influence
20 cytological and microbiological, potentially limiting direct comparison with studies using different
21 BALF protocols.

22
23 The absence of prospective control groups is another important constraint. Although healthy dogs
24 were included in the LM study and in the NET study, these controls were derived from a different
25 cohort, and not all studies shared identical sampling conditions or timelines. For instance, some of
26 the healthy control dogs were purpose-bred Beagles. Consequently, the influence of external factors
27 such as housing environment, diet, or technical variability cannot be fully excluded. Future
28 prospective cohorts using uniform inclusion criteria and synchronized control sampling will be
29 essential to validate and expand these findings.

30
31 Another important limitation concerns the composition of the study population. Although dogs
32 were classified according to the presence of BE and/or BM, the vast majority of included cases had
33 chronic bronchitis as the underlying respiratory disease. Consequently, the findings primarily reflect
34 the biological characteristics of BE and BM occurring in the context of chronic bronchitis and should

not be generalized to all dogs presenting with these morphological airway abnormalities. In particular, dogs with IPF (including traction BE), EBP, congenital airway disorders, or breeds predisposed to tracheobronchomalacic abnormalities were poorly represented or absent. These conditions may involve distinct inflammatory, microbiological, or other mechanisms that were not captured in the present work. Future studies including a broader spectrum of underlying respiratory diseases will therefore be required to determine the applicability of these findings across different clinical contexts. A related limitation concerns the inclusion of a small number of dogs with positive bacterial cultures. Although chronic bronchitis is classically defined as a sterile inflammatory disease, chronic bronchial disease complicated by BE and/or BM may predispose to secondary bacterial colonization or infection as a consequence of impaired mucociliary clearance and structural airway changes. Because of the retrospective nature of the study, it was not always possible to distinguish colonization from clinically relevant infection based on culture results alone. Consequently, some degree of biological heterogeneity may have been introduced into the study population.

While most dogs included in this thesis were part of overlapping cohorts and contributed to several complementary analyses, not all data types were available for every individual. For instance, some dogs with well-characterized clinical and imaging findings did not have corresponding LM sequencing or NET measurements, and vice versa. This partial overlap reflects the practical constraints of retrospective sample availability and the gradual development of the project over time. As a result, although the same population underpinned the majority of studies, complete multimodal datasets were not uniformly obtained for each case, limiting the possibility to establish direct, within-subject correlations between microbiota composition, NET formation, and structural or functional changes.

Another important limitation relates to the referral nature of the study population. All cases were recruited from a tertiary referral hospital, meaning that the included dogs likely represented a more severe and diagnostically complex subset of patients than those typically encountered in primary care. Many had undergone prior diagnostic investigations or empirical treatments before referral, which may have influenced bronchoalveolar cytology, microbiota composition, or inflammatory markers at the time of sampling. This referral bias introduces a potential sample selection effect and may limit the generalizability of the findings to the broader population of dogs with milder or early-stage disease managed in first-opinion practice.

1 The sample sizes within some subgroups were relatively small, especially for dogs with combined
2 BE and BM. While statistical analyses were adjusted accordingly, the modest number of cases limited
3 the power to detect subtle differences between disease categories and may have contributed to type
4 I errors. Similarly, in the LM study, exclusion of dogs with active infection was necessary to focus on
5 commensal alterations but further reduced the effective sample size, narrowing the generalizability
6 of results.

7
8 From a diagnostic and imaging perspective, the lack of standardized CT and bronchoscopy protocols
9 represents another limitation of this study. Although imaging and endoscopic evaluations were
10 double-blinded and independently reviewed, the retrospective nature of case inclusion resulted in
11 variability in imaging sequences, respiratory phases, and endoscopic recording quality across
12 patients and operators. Such inconsistencies may have influenced disease classification and
13 contributed to the limited concordance observed between imaging and endoscopic findings. In
14 addition, only a subset of dogs underwent thoracic CT examination. Because CT is considered a
15 highly sensitive imaging modality for detecting BE, the relatively limited number of CT examinations
16 reduced the ability to comprehensively compare CT findings with bronchoscopy and may have
17 decreased the detection of more subtle structural airway abnormalities. Importantly, both
18 modalities have inherent diagnostic limitations. Computed tomography is generally considered the
19 most reliable technique for identifying structural airway abnormalities such as BE, but it may
20 underestimate dynamic airway collapse when expiratory imaging is not consistently performed.
21 Conversely, bronchoscopy allows direct visualization of dynamic bronchial collapse and remains the
22 reference method for diagnosing BM, yet it may be less sensitive for detecting more subtle or
23 peripheral bronchial dilation associated with BE. Ideally, a combined diagnostic approach including
24 both bronchoscopy and inspiratory–expiratory CT would have been performed in each patient to
25 provide complementary structural and functional information on the airways. Future prospective
26 studies employing standardized imaging protocols, including paired inspiratory–expiratory CT
27 acquisitions combined with systematic bronchoscopic evaluation, would allow a more reliable
28 assessment of diagnostic agreement and improve characterization of structural and dynamic airway
29 abnormalities in dogs with chronic bronchial disease.

30
31 Regarding the therapeutic study, the proposed clinical algorithms are based primarily on
32 retrospective evaluation and literature synthesis rather than prospective data. As a result, the
33 efficacy and long-term outcomes of the recommended treatment pathways remain to be validated

in controlled, forward-looking clinical trials. Variations in owner compliance, inhalation technique, and follow-up intervals can influence treatment success.

Furthermore, breed and body weight were not specifically evaluated as independent variables. Because airway anatomy, bronchial biomechanics, and the prevalence of airway collapse vary considerably among breeds and body sizes, these factors may influence the occurrence and severity of BM. However, the available sample size and heterogeneous distribution of breeds did not allow meaningful stratified analyses. Future multicentre studies including larger numbers of dogs should investigate the influence of breed, body conformation, and body weight on the development of BM and BE.

In the LM analysis, several technical and interpretative challenges must be considered. The inherently low bacterial biomass of bronchoalveolar lavage samples increases susceptibility to contamination from reagents or the upper airways, despite strict aseptic techniques and inclusion of negative controls. Additionally, sequencing focused on the 16S rRNA gene provides compositional but not functional information, precluding conclusions about microbial metabolic activity or host interactions. The use of archived frozen BALF might also have influenced DNA quality and relative abundance of certain taxa.

In the NETs study, quantification relied on indirect surrogate markers, cfDNA and MPO–DNA complexes, rather than direct visualization. While cfDNA is a sensitive indicator of extracellular nucleic acids, it is not specific for NET formation and may originate from other processes such as cell necrosis or apoptosis. In contrast, MPO–DNA complexes are considered a more specific marker of NETs, as they reflect the association of neutrophil-derived granular proteins with extracellular DNA. Nevertheless, these assays remain indirect measures and do not provide information on the spatial distribution or morphological characteristics of NET structures within the airway lumen.

The absence of IF staining in this study represents an additional limitation. Incorporating IF-based visualization and quantitative image analysis in future research would allow morphological confirmation of NET formation, localization relative to epithelial structures, and potential colocalization with microbial aggregates within BALF samples. However, biochemical assays and BALF analyses remain indirect approaches and do not provide spatial information regarding NET formation within the airway tissue itself (van Breda *et al.*, 2019). As highlighted by de Buhr and Köckritz-Blickwede (2016), detection of NETs directly within tissue sections is particularly important because NETosis may remain undetected when relying solely on circulating or fluid-based

measurements. Immunolabelling for NET-specific markers in tissue sections has been widely described, and NET structures have been demonstrated in various organs including the placenta, intestine, kidney, lung, skin, and intracoronary material (Brinkmann *et al.*, 2004; Gupta *et al.*, 2005; , Kessenbrock *et al.*, 2009; Filio-Rodriguez *et al.*, 2017; Ginley *et al.*, 2017; Santos *et al.*, 2018; Radermecker *et al.*, 2020). Performing IF analyses on airway tissue sections could therefore provide valuable complementary information by enabling in situ visualization of NETs within the bronchial wall and surrounding tissues. Such an approach would allow assessment of the spatial relationship between NET formation and structural airway lesions, including epithelial damage or bronchial wall remodelling, and could provide insights into whether NET-mediated inflammation contributes to airway weakening or collapse

Finally, the absence of a control group of dogs with chronic bronchial disease without BE or BM, together with the cross-sectional nature of the studies, precludes definitive conclusions regarding the relationship between chronic inflammation and structural airway remodelling. Consequently, it cannot be determined whether the observed clinicopathological, microbiological, and immunological findings are specifically associated with BE and/or BM or rather reflect chronic bronchial disease itself. Furthermore, because dogs were evaluated after BE and/or BM had already developed, it cannot be determined whether BE and BM arise independently, sequentially, or concurrently during disease progression. Likewise, it remains unknown whether the observed microbiota alterations and NET formation are causes or consequences of structural airway abnormalities. Longitudinal studies including dogs with chronic bronchial disease without BE or BM and following patients from disease onset will therefore be essential to clarify these relationships.

Perspectives

The work presented in this thesis has clarified several aspects of chronic bronchial disease in dogs: its clinical presentation, pathophysiology, microbiota composition, inflammatory mechanisms, and therapeutic management. Yet, each study also revealed the limits of our current understanding and pointed toward new ideas for research.

A first key priority will be to establish prospective studies that follow dogs from the earliest presentation of chronic cough through to the development of irreversible airway changes. Such studies would allow determination of the relationship between inflammation, dysbiosis, and remodelling. These are questions that retrospective designs cannot definitively answer. Regular bronchoscopy, imaging, and BALF sampling, combined with standardized clinical scoring, would

1 make it possible to identify early biomarkers of progression and to define “at-risk” phenotypes
2 before permanent damage occurs.

3 Another important goal is the standardization of diagnostic and imaging criteria. Prospective
4 validation of objective CT and endoscopic scoring systems would improve reproducibility and
5 comparability. Integrating imaging data with cytological, molecular, and clinical markers within the
6 same cohort may facilitate the development of integrated measures that reflect ongoing disease
7 activity rather than fixed structural changes.

8
9 Improving our understanding of the structural airway changes associated with BE and/or BM will
10 also require more detailed histopathological investigation. Future studies should explore how
11 chronic inflammation contribute to weakening of the bronchial wall and cartilage structures.
12 Integrating histological analysis with NET detection, microbial localization, and inflammatory
13 markers could help determine whether specific inflammatory microenvironments are associated
14 with airway dilation or collapse. Such approaches may also clarify whether NET-associated
15 proteases and oxidative stress contribute directly to airway remodelling.

16
17 Prospective studies are critical for validating the proposed therapeutic algorithms. The treatment
18 schemes proposed in this thesis, emphasizing cytology-guided antibiotic use and inhaled
19 corticosteroids for non-infectious inflammation, now require evaluation in prospective clinical
20 trials. Such studies should compare outcomes including cough frequency, quality of life, airway
21 cytology, and imaging progression under standardized conditions. Incorporating prospective
22 control groups and predefined withdrawal phases would provide strong evidence for long-term
23 efficacy and safety, while also improving understanding of treatment response, relapse dynamics,
24 and the long-term effects of inhaled versus systemic anti-inflammatory therapy.

25
26 At the same time, deeper investigation of the interaction between the host immune response and
27 the airway microbiota will be essential. While this thesis focused primarily on characterizing
28 microbial composition, future studies should also explore microbial function and host-microbe
29 interactions. Approaches such as metagenomics, metatranscriptomics, and metabolomics could
30 help determine how microbial communities influence airway inflammation and whether specific
31 microbial signatures promote or protect against disease progression. Linking these microbial
32 patterns with inflammatory pathways and NET formation may provide a more integrated view of the
33 airway ecosystem in chronic bronchial disease.

Microbiota-based therapeutic approaches represent another promising but largely unexplored field. Strategies aimed at restoring microbial balance, including probiotics, prebiotics, or microbiota-targeted therapies, could potentially reduce airway inflammation and improve mucosal immune homeostasis. Long-term monitoring of microbial communities will be necessary to determine whether such interventions produce stable ecological changes within the airway environment.

In parallel, further characterization of neutrophil-mediated inflammation will be important for understanding the role of NETs in airway pathology. Future studies could expand the analysis of inflammatory mediators involved in NET formation, including cytokines and chemokines such as IL-8, IL-1 β , TNF- α , and IL-17, which are known to promote neutrophil recruitment and activation. Measuring these mediators in BALF alongside NET markers may help determine whether NET formation reflects a downstream consequence of neutrophilic inflammation or whether it represents an independent driver of tissue injury. Longitudinal studies quantifying NET markers during periods of disease exacerbation and remission may further clarify whether NET activity correlates with disease severity or predicts clinical deterioration.

In parallel, future work should expand the use IF-based detection techniques, which enable direct visualization and quantification of NET structures in BALF or tissue samples. Including a larger number of well-characterized IF cases, together with quantitative image analysis, would provide a more objective assessment of NET burden and spatial distribution within the airways. These approaches could complement biochemical assays such as MPO-DNA or cfDNA, offering both qualitative and quantitative confirmation of NET formation. Ultimately, integrating IF quantification with longitudinal sampling could help establish standardized NET scoring systems and strengthen their use as biomarkers of inflammation and treatment response.

Moreover, exploring NET-modulating therapies, for example PAD4 inhibitors that limit chromatin decondensation, antioxidants that reduce oxidative triggers, or mucolytic enzymes such as recombinant DNase that degrade extracellular DNA, represents an exciting translational direction. Carefully designed prospective clinical trials evaluating these interventions in dogs with naturally occurring BE and BM could clarify their clinical benefit, safety, and potential to modify the course of chronic airway disease.

Additional mechanisms that were not specifically investigated in this thesis may also play a role in the development of chronic airway disease. For example, repeated microaspiration has been proposed as a contributing factor in several chronic respiratory conditions in both human and veterinary medicine. Investigating the presence of gastric or oropharyngeal markers in BALF,

1 together with microbiota profiling, could help determine whether microaspiration contributes to
2 chronic airway inflammation or microbial dysbiosis in some patients.

4 **Conclusion**

5 Taken together, the four studies presented in this thesis provide an integrated characterization of
6 BE and/or BM in dogs. Despite their distinct morphological definitions, dogs in these groups
7 exhibited quite similar clinicopathological characteristics, bronchoalveolar lavage cytology, LM
8 composition, and evidence of NET formation. Using the approaches employed in this thesis, no
9 consistent biological differences could be demonstrated between these airway abnormalities.

10 These findings indicate that chronic airway inflammation, microbial dysbiosis, and neutrophil
11 activation are common features of chronic bronchial disease in dogs with BE and/or BM associated
12 predominantly with chronic bronchitis. Because other clinical contexts, including IPF with traction
13 bronchiectasis, EBP, congenital airway disorders, and breed-related tracheobronchomalacic
14 abnormalities, were poorly represented or absent, the present conclusions should not be
15 generalized to all dogs with BE or BM. Furthermore, the present work does not demonstrate that BE
16 and BM share an identical pathophysiology, and additional mechanisms are likely to contribute to
17 the development of these distinct structural airway abnormalities. Future prospective longitudinal
18 studies integrating advanced imaging, metagenomics, immunological profiling, and molecular
19 characterization will be essential to clarify the biological relationship between BE and BM,
20 determine the mechanisms leading to these structural airway abnormalities, and further optimize
21 the management of chronic bronchial disease in dogs.

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