Mucosal immunity in COPD
From mucociliary dysfunction to lymphoid follicles

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2016

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Manuscript in preparation

CHAPTER 9
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<th>Full Form</th>
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<tbody>
<tr>
<td>ASL</td>
<td>airway surface liquid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell-activating factor</td>
</tr>
<tr>
<td>BAFFR</td>
<td>B cell-activating factor receptor</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BCMA</td>
<td>B cell maturation antigen</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>CB</td>
<td>chronic bronchitis</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CF</td>
<td>cystic fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CS</td>
<td>cigarette smoke</td>
</tr>
<tr>
<td>DAMP</td>
<td>damage associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DI</td>
<td>destructive index</td>
</tr>
<tr>
<td>ENaC</td>
<td>epithelial natrium channel</td>
</tr>
<tr>
<td>FDC</td>
<td>follicular dendritic cell</td>
</tr>
<tr>
<td>FEV₁</td>
<td>forced expiratory capacity in one second</td>
</tr>
<tr>
<td>FVC</td>
<td>forced vital capacity</td>
</tr>
<tr>
<td>GOLD</td>
<td>global initiative for obstructive lung diseases</td>
</tr>
<tr>
<td>FRC</td>
<td>fibroblastic reticular cell</td>
</tr>
<tr>
<td>GWAS</td>
<td>genome wide association study</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>ILC</td>
<td>innate lymphoid cells</td>
</tr>
<tr>
<td>Lm</td>
<td>mean linear intercept</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MLN</td>
<td>mediastinal lymph nodes</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MUC</td>
<td>mucin</td>
</tr>
<tr>
<td>PCL</td>
<td>periciliary layer</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>ROI</td>
<td>reactive oxygen intermediate</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>SLO</td>
<td>secondary lymphoid organ</td>
</tr>
<tr>
<td>SPLUNC</td>
<td>short palate and lung and nasal epithelial clone 1</td>
</tr>
<tr>
<td>TACI</td>
<td>transmembrane activator and cyclophilin ligand interactor</td>
</tr>
<tr>
<td>Tfh</td>
<td>T follicular helper cell</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
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ABSTRACT

Chronic Obstructive Pulmonary Disease (COPD) is a highly prevalent disease which is characterized by an abnormal inflammation and destruction of lung tissue in response to noxious particles and gases, mainly cigarette smoke (CS). The pathogenesis of COPD is still not fully unravelled, which hampers the search for therapies that stop the progression of the disease.

In this thesis, we aimed to investigate 2 aspects of the mucosal immune response to CS: airway surface liquid dehydration and the formation of B cell-rich lymphoid follicles.

First, we studied the effect of airway surface liquid dehydration on CS-induced pathology in mice. Dehydration of the airway surface causes mucociliary clearance dysfunction and leads clinically to chronic bronchitis. Airway surface liquid dehydration was mimicked in mice which overexpress the β-subunit of the Epithelial Natrium Channel (ENaC). We subjected βENaC-Tg mice to CS for 4 and 8 weeks and observed that airway surface dehydration aggravates CS-induced inflammation and alveolar destruction. Furthermore, following 8 weeks of CS exposure, βENaC-Tg mice already started to develop lymphoid aggregations whereas wild-type mice did not. This suggests that dysfunction of mucociliary clearance can induce or accelerate the adaptive immune response to CS.

Next, we investigated the effect of chronic bronchitis on clinically important outcomes in patients with COPD in a large population-based cohort. We observed that the presence of chronic bronchitis in patients with COPD leads to an increased risk for being a frequent exacerbator and increases the risk of mortality. This demonstrates the importance of the symptom chronic bronchitis and consequently, the importance of a well-functioning mucociliary clearance system.

Inadequate mucociliary clearance may contribute to the chronic inflammation and lymphoid follicle formation seen in COPD. It has been demonstrated that the number of airways with lymphoid follicles is associated with disease severity. In addition, the expression of B cell-Activating Factor (BAFF) in these lymphoid follicles in patients with COPD is also correlated with disease severity. Therefore, we aimed to investigate the role of BAFF and B cell-rich lymphoid follicles in COPD. We localized BAFF expression in lymphoid follicles of patients with COPD and antagonized BAFF in mice which were chronically exposed to CS. We demonstrated that BAFF is significantly increased in lungs of patients with COPD and is present around both immune and stromal cells within lymphoid follicles. Furthermore, antagonizing BAFF in CS-exposed mice attenuates pulmonary inflammation and alveolar destruction.

The results shown in this thesis underline the importance of the mucosal immune response in the pathogenesis of COPD.
PART I: INTRODUCTION

CHAPTER 1: CHRONIC OBSTRUCTIVE PULMONARY DISEASE
1.1 Definition

Although Chronic Obstructive Pulmonary Disease (COPD) is the third leading cause of death worldwide and a major cause of morbidity, the term ‘COPD’ is not well known by the lay public\(^1,2\). However, the association of shortness of breath – the main symptom of the disease – and cigarette smoking does sound familiar to many people.

The definition of the disease is formulated as follows by the Global initiative for chronic Obstructive Lung Disease (GOLD)\(^3\):

"Chronic Obstructive Pulmonary Disease (COPD) is a common preventable and treatable disease, characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients."

The noxious particles and gases mentioned in the definition originate from cigarette smoking, occupational exposure to harmful gases and particulate matter and exposure to indoor and outdoor pollution\(^2,4,5\). COPD develops after cumulative exposures to these injurious substances over decades, but only in susceptible individuals\(^2,3\). It is estimated that at least 20% of all smokers develop COPD\(^4,6\). Several large-scale studies have demonstrated that genetic variation accounts for differences in susceptibility\(^7-10\). Other contributing factors are intra-uterine growth retardation and low birth weight, history of pulmonary infections during childhood, history of pulmonary tuberculosis, chronic asthma, poor nourishment, poor socio-economic status and importantly, aging\(^5,11,12\). Some of these additional risk factors may in turn be genetically and epigenetically regulated\(^10,13\).

In most patients, COPD is progressive, leading to severe disability. The only intervention that slows down the progression of the disease, is the removal of initiating factors, for instance smoking cessation. However, even after smoking cessation, the disease persists\(^4\). Treatments are mainly focussed on symptom relief\(^2\). Currently, there is no cure. Further investigation into the pathogenesis of this disabilitating disease will advance the search for better therapies\(^14,15\).
The global burden of COPD is substantial. The World Health Organisation (WHO) states that COPD is the third leading cause of death, responsible for approximately 3.1 million deaths worldwide in 2012. In Europe, around 2010, COPD was the cause of death of approximately 0.15 million people (Figure 1). In addition, an average of 1690 thousand disability-adjusted-life-years and a total economic cost of 141.4 billion euros per year can be accounted to this disease (Figure 1) 16.

![Figure 1: The burden of COPD. A) Burden of COPD in older adults in Europe around 2010. B) Distribution of economic costs per category for COPD in Europe. #: Global Initiative for Chronic Obstructive Lung Diseases stage II-IV. Figure adapted from The European Lung White Book Respiratory Health and Disease in Europe. Published 2013, ISBN 978-1-84984-042-2 16.](image)

Worldwide, approximately 1 billion people smoke tobacco and 3 billion people are exposed to smoke from biomass fuel 5,17. In addition, numerous people are exposed to air pollution or occupational hazardous substances, increasing the number of people at risk even further.

It is estimated that 9 - 10 % of the global population aged 40 years or older suffers from moderate to very severe COPD 15. Afonso and colleagues studied the same age-group in the Netherlands and reported a prevalence of 3 % of physician-diagnosed COPD and the incidence of physician-diagnosed COPD was 2.92/1000 person-years 18.

Although it is generally assumed that in Western Countries cigarette smoking is the most important cause of COPD, the prevalence in never-smokers in the USA is estimated at 6.6%, resulting in a quarter of COPD patients being never-smokers 19. This has been corroborated by studies in the UK and Spain 5.
1.3 Clinical manifestations of COPD and diagnosis

1.3.1 Main symptoms and diagnosis

Gradually progressing and persistent breathlessness is the main symptom of COPD. This may be accompanied by cough and/or sputum of varying severity \(^2,^3\).

The diagnosis of COPD is based on anamnesis - in particular exposure to cigarette smoke (CS) and other risk factors - and symptoms of dyspnea, cough and sputum and requires spirometry to confirm \(^2\). Spirometry is a lung function test which measures, among other values, the forced expiratory volume in 1 second (FEV\(_1\)) and the forced vital capacity (FVC). A ratio of FEV\(_1\)/FVC < 0.70 determines the presence of airflow limitation according to GOLD standards, whereas a FEV\(_1\)/FVC < lower limit of normal is required for diagnosing COPD according to the Global Lung function Initiative. If the airflow limitation persists after inhalation of a bronchodilator (usually salbutamol), the diagnosis of COPD can be made. Disease severity is currently assessed based on the degree to which the FEV\(_1\) is different from the predicted value for a person of that specific age, height and weight (Table 1). Recently, new guidelines are published which take in account airflow limitation as well as symptoms (based on the COPD Assessment Test or the Modified Medical Research Council Dyspnea Scale) and exacerbation risk. Based on these 3 criteria patients are divided into 4 groups, A to D) \(^3\). Additional tests include measurements of diffusing capacity of the lungs and computed tomography (CT) which contribute to the assessment of lung function and severity of emphysema.

<table>
<thead>
<tr>
<th>GOLD STAGE</th>
<th>COPD</th>
<th>FEV(_1)/FVC</th>
<th>FEV(_1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOLD I</td>
<td>Mild</td>
<td>&lt; 0.70</td>
<td>and (\geq 80%) predicted</td>
</tr>
<tr>
<td>GOLD II</td>
<td>Moderate</td>
<td>&lt; 0.70</td>
<td>and (50% \leq FEV(_1) &lt; 80%) predicted</td>
</tr>
<tr>
<td>GOLD III</td>
<td>Severe</td>
<td>&lt; 0.70</td>
<td>and (30% \leq FEV(_1) &lt; 50%) predicted</td>
</tr>
<tr>
<td>GOLD IV</td>
<td>Very severe</td>
<td>&lt; 0.70</td>
<td>and (&lt; 30%) predicted</td>
</tr>
</tbody>
</table>

Table 1: COPD classification according to the Global Initiative for Chronic Obstructive Lung diseases (GOLD) Adapted from Vestbo J. et al., Am J of Respir Crit Care Med 2013;187(4):347-365 \(^2\).

1.3.2 COPD Phenotypes

The heterogeneity in clinical presentation and progression of COPD is substantial. Furthermore, the response to therapy is highly variable. This has led to subdividing patients according to similar traits or similar response to therapy in so called ‘phenotypes’ \(^20\). Since phenotyping can be a rather confusing term,
Meilan Han and colleagues proposed to define a COPD phenotype as: “a single or combination of disease attributes that describe differences between individuals with COPD as they relate to clinically meaningful outcomes (symptoms, exacerbations, response to therapy, rate of disease progression, or death)” 21. Several phenotypes have been proposed (Table 2), however, only four are validated: α1 anti-trypsin deficiency, frequent exacerbators, emphysema and chronic bronchitis 14.

The monogenic disorder α1 anti-trypsin deficiency is an autosomal recessive inherited defect in the SERPINE1 gene. The product of this gene is a serine protease inhibitor which neutralizes proteases such as neutrophil elastase. It affects approximately 1 in 2000-5000 persons in Europe 16, 22. Pulmonary pathology is characterized by severe early-onset emphysema and is often accompanied by liver disease 22, 23.

An exacerbation of COPD is defined as “an event in the natural course of the disease characterized by a change in the patient’s baseline dyspnea, cough, and/or sputum that is beyond normal day-to-day variations, is acute in onset, an may warrant a change in regular medication in a patient with underlying COPD”. Exacerbations can be elicited by respiratory infections and increased environmental pollution. Patients with 2 or more exacerbations per year have a worse quality of life and an accelerated lung function decline 24-26.

The emphysema phenotype has been known as the ‘pink puffer’ phenotype and is associated with significant dyspnea, hyperinflation and weight loss, but with adequate oxygenation 27. One subgroup, patients with severe upper lobe emphysema and low exercise capacity at baseline, are a separate phenotype based on a survival advantage after lung-volume-reduction surgery compared to patients with severe emphysema with another craniocaudal distribution or with high exercise capacity at baseline 28.

The chronic bronchitis phenotype will be thoroughly discussed in Chapter 3.

Other phenotypes may exist but need to be validated. Importantly, patients can also express more than one phenotype 29, 30.

---

**Table 2: Proposed COPD phenotypes.**


<table>
<thead>
<tr>
<th>Proposed COPD phenotypes</th>
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<tbody>
<tr>
<td>α1-antitrypsin deficiency</td>
</tr>
<tr>
<td>Frequent exacerbators</td>
</tr>
<tr>
<td>Emphysema</td>
</tr>
<tr>
<td>Chronic Bronchitis</td>
</tr>
<tr>
<td>Severe hypoxia</td>
</tr>
<tr>
<td>Disproportionate symptoms</td>
</tr>
<tr>
<td>Persistent systemic inflammation</td>
</tr>
<tr>
<td>Chronic airway bacterial colonisation</td>
</tr>
<tr>
<td>Asthma/COPD overlap syndrome</td>
</tr>
<tr>
<td>Early severe airflow limitation</td>
</tr>
<tr>
<td>Extreme pulmonary hypertension</td>
</tr>
<tr>
<td>COPD in never-smokers</td>
</tr>
</tbody>
</table>

The 4 patient groups proposed by GOLD
1.3.3  SYSTEMIC MANIFESTATIONS AND CO-MORBIDITIES

Pulmonary symptoms in patients with COPD are often accompanied by systemic effects and comorbidities \(^{14,15,31,32}\). It is hypothesized that systemic effects occur due to ‘spill-over’ of pulmonary inflammatory mediators into the systemic circulation. However, other factors such as smoking, aging and physical inactivity may also contribute \(^{4,33}\). In stable COPD and even more so during exacerbations, increased levels of cytokines, chemokines, acute phase proteins and leukocytes are measured \(^{33}\). Alongside systemic inflammation, the compromised gas exchange also has systemic consequences. Cachexia and skeletal muscle wasting are common systemic manifestations in patients with COPD. Comorbidities include cardiovascular disease, diabetes, depression, normocytic anemia and importantly, lung cancer \(^{2,31,33}\). Furthermore, in the Rotterdam Study – a large population-based cohort study of subjects aged 45 years or older – COPD was found to be associated with sudden cardiac death, carotid artery wall thickening, stroke and frailty \(^{34-37}\).
1.4 **Pathology**

The pathological changes underlying the symptoms in patients with COPD are chronic bronchitis, obstructive bronchiolitis and emphysema (*Figure 2*)\(^7\), \(^{38}\). Vascular remodelling, both in the pulmonary compartment and systemically, is also a pathological feature of the disease \(^{39}\).

The chronically inflamed large airways (> 4 mm in internal diameter) are subjected to goblet cell metaplasia and enlargement of the submucosal glands, resulting in excessive mucus production \(^{38}\). The structure and function of the epithelial cilia that propel the mucus towards the larynx are compromised by CS \(^{40}\)-\(^{42}\). Further contributing to impairment of mucociliary clearance, is the detrimental effect of CS on epithelial ion channels that regulate the hydration of the mucus layer \(^{40}\). Finally, infiltration of inflammatory cells in the subepithelial area and deposition of connective tissue in the bronchial walls further adds to the pathological feature of chronic bronchitis \(^{38}\), \(^{43}\).

The constant supply of noxious gases and particles inflicts inflammation in the small airways (< 2 mm in internal diameter) as well. The inflammatory infiltrate is characterized by neutrophils, macrophages, B and T lymphocytes \(^{44}\). Chronic inflammation induces goblet cell metaplasia and mucus plugging \(^{45}\). The resistance in the small airways is further increased by smooth muscle hypertrophy and peribronchiolar fibrosis \(^{38}\). Finally, in severe COPD, lymphoid follicles contribute to an increased resistance by increasing the wall thickness \(^{38}\), \(^{44}\). The end result is obstructive bronchiolitis and air trapping.

Products of the inflammatory response to CS, such as proteases, break down lung tissue \(^{43}\). Subsequently, repair and remodelling processes are initiated. The result of this cascade is destruction of terminal and respiratory bronchioli, leading to centrilobular lesions and emphysema \(^{46}\). Loss of terminal bronchioli precedes emphysematous damage which is characterized by destruction of alveolar walls leading to bullous lesions \(^{46}\), \(^{47}\). Emphysema can also be generated through increased apoptosis \(^{48}\). The outcome is loss of elastic recoil and static hyperinflation.
Figure 2: COPD pathology: A) Healthy large airway. B) Large airway of a patient with COPD: goblet cell metaplasia and submucosal gland hypertrophy, resulting in a thick mucus layer. C) Healthy small airway and alveoli. D) Small airway and alveoli of a patient with COPD: a thick mucus layer and infiltration of inflammatory cells in the airway lumen, goblet cell metaplasia, lymphoid follicle formation, subepithelial fibrosis and destruction of alveolar walls (emphysema).
1.5 Pathogenesis

Lung inflammation is present in all smokers. However, not all smokers develop COPD. In those who do, the inflammatory response is abnormal. The difference in response to tobacco smoke can be contributed to genetic variations and early-life lung development. Since COPD is generally caused by CS in Western Countries, the pathogenesis of CS-induced COPD will be described. Importantly, CS is a mixture of thousands of toxic particles and fumes. It is estimated that each cigarette puff contains over 2000 xenobiotic compounds and $10^{14}$ free radicals.

1.5.1 Genetics

Twin-studies have suggested a strong genetic component in the risk of developing COPD and several Genome Wide Association Studies (GWAS) have identified genetic variants associated with lung function or risk of developing COPD. Hunninghake et al. have demonstrated that the minor allele of a single nucleotide polymorphism in the gene encoding for MMP12 reduced the risk of developing COPD, and more importantly, showed that the outcome of this genetic variation altered with different environments. Moreover, genome-wide studies also reveal genetic variations at the base of smoking behavior. Recently, the UK BiLEVE study demonstrated variability in the underlying genetic framework of both smoking behavior and lung health. Nevertheless, these variations only account for a small part of the phenotypic differences. In the last decade, the importance of epigenetic modulation has become apparent. Several microRNAs have already been described in association with COPD. And evidence linking other epigenetic mechanisms (long non-coding RNAs, DNA methylation and histone modifications) to the pathogenesis of COPD is rapidly emerging.

1.5.2 Early-life Lung Development

Although the importance of early-life lung development has been emphasised by paediatricians and was already implied by Fletcher and Peto in the 1970s, only fairly recent this issue has caught a spotlight in COPD research. The crucial years to be able to reach the maximal lung function in young adulthood, are the first 4 to 6 years of life. Even compromised lung development in utero – for instance due to maternal smoking during pregnancy - increases the risk for reduced lung function in adulthood. Other ‘childhood disadvantage factors’, such as maternal asthma, paternal asthma, childhood asthma and childhood respiratory infections, also contribute to an impaired lung function in adulthood and significantly increase the risk of developing COPD. The effect of having 2 or more early-life
disadvantages is estimated to be as important as heavy smoking for development of COPD. Recently, a large-scale study in 3 independent cohorts has confirmed that half of the patients with COPD displayed a low FEV1 in early adulthood and a subsequent moderate decline instead of the usual trajectory of a rapid decline in FEV1 starting from a normal lung capacity (Figure 3).

**Figure 3: Adapted Fletcher and Peto Curve.** The effect of impaired lung development and genetic susceptibility on lung function decline. Adapted from Brusselle GG., *N Engl J Med*, 2009;361(27):2664-2665.

### 1.5.3 Abnormal Inflammatory Response to Cigarette Smoke in COPD

Damage to airway epithelial cells is key in the initiation of pulmonary inflammation (Figure 4). The multitude of toxic substances in CS injure the epithelium directly or by inducing oxidative stress and tissue hypoxia, leading to cell death and release of damage-associated molecular patterns (DAMPs). In addition, CS exposure induces the release of DAMPs by macrophages and neutrophils as well. In broncho-alveolar lavage (BAL) fluid of patients with COPD, there is an increased concentration of DAMPs, such as high-mobility group box 1 (HMGB1) and extra-cellular ATP, compared to controls. DAMPs are recognised by pattern recognition receptors on innate immune cells and structural cells. In response to the release of DAMPs, airway epithelial cells and immune cells secrete an array of cytokines, chemokines and growth factors. As result, the capillary endothelium is activated to allow leukocyte adhesion and migration and leukocytes are attracted to the site of injury.
In response to the chemoattractant CXCL8, neutrophils infiltrate the airway lumen \(^{68,74}\), the bronchial wall and submucosal glands \(^{44,75,76}\) and airway smooth muscle bundles \(^{77}\). The substantial increase in neutrophils is associated with airflow limitation and peripheral airway dysfunction in patients with COPD \(^{75,78}\). In accordance, neutrophil-derived products are consistently shown to be elevated in airways of patients with COPD compared to controls and correlate with disease severity \(^{79,80}\). Neutrophils and their derivatives are implicated in several pathogenic mechanisms leading to COPD. First, they release a wide range of pro- and anti-inflammatory cytokines, chemokines and growth factors contributing to the activation and regulation of the immune response \(^{81}\). Second, neutrophils produce a variety of proteases. Neutrophil elastase, cathapsin-G, proteinase-3 and neutrophil-produced matrix metalloproteases (MMP) are important factors in degradation of tissue, propagating the development of emphysema \(^{4,82,83}\). Third, neutrophils are a major source of reactive oxygen intermediates (ROI), thus contributing to oxidative stress and tissue damage – directly and indirectly by inactivating anti-proteases \(^{81,84}\). Fourth, in COPD, the phagocytic activity of neutrophils is impaired leading to accumulation of apoptotic cells and secondary necrosis with release of danger signals \(^{85}\). Fifth, neutrophils play a significant role in the overproduction of mucins at least in part through its product neutrophil elastase \(^{83}\). And finally, neutrophils also produce neutrophil extracellular traps - fibrillary networks composed from nuclear material and pattern recognition molecules, such as pentraxin-3. These neutrophil extracellular traps are intended to work as a mesh to capture pathogens \(^{81}\). However, both pentraxin-3 and neutrophil extracellular traps have been associated with COPD \(^{86-88}\).

In the pathogenesis of COPD, macrophages play a pivotal role as well. Alveolar macrophages are present in homeostatic circumstances, but their numbers increase significantly upon CS exposure both through local proliferation and attraction of monocytes from the peripheral blood \(^{89,90}\). Interstitial macrophages, a second tissue-resident macrophage population, reside in the lung parenchyma and represent less than 10% of the lung macrophages \(^{90,91}\). The inflammatory response is characterised by a 5- to 10-fold increase in macrophages in airways, lung parenchyma, BAL and sputum of patients with COPD compared to controls \(^{4,38,75,83}\). The severity of COPD is positively correlated with macrophage numbers \(^{75,92}\). Furthermore, depletion of macrophages in a short-term mouse model of COPD resulted in protection against the development of COPD-like pathology, underlining their crucial role in the disease \(^{93}\). The release of anti-and pro-inflammatory cytokines, chemokines and growth factors, ROI and several proteases – including MMP2, MMP9, MMP12 and cathepsins K, L and S – by macrophages drives several elements in the pathogenesis of COPD \(^{52,83,90}\). Importantly, macrophages are also impaired in their ability to phagocytose apoptotic cells, resulting in secondary necrosis and amplification of inflammation \(^{89,94}\). Alveolar
macrophages exhibit an enormous plasticity to adapt to the ever changing microenvironment in the airways and alveoli. Exposure to CS drives the development of both pro-inflammatory (M1) macrophages and anti-inflammatory (M2) macrophages \(^ {90, 95}\). However, transcriptional profiling revealed a downregulation of M1-associated genes and an induction of M2-associated genes involved in immunoregulation and tissue remodelling \(^ {96}\).

Alongside increased numbers of macrophages and neutrophils, other innate immune cells infiltrate the lungs and airways of patients with COPD. Increased numbers of natural killer cells have been associated with disease severity \(^ {75}\). Mast cells are significantly increased in airway tissues of patients with COPD, especially in patients with centrolobular emphysema \(^ {65, 97}\). Interestingly, innate lymphoid cells type 3 (ILC3) and more specifically, the natural cytotoxicity receptor-negative ILC3, tend to be increased in patients with COPD \(^ {98}\).

**Figure 4: COPD pathogenesis.** Cigarette smoke damages airway epithelial cells and alveolar macrophages which release damage associated molecular patterns (DAMPs). Subsequently, inflammatory cells are attracted to the airways and dendritic cells migrate to the draining lymph nodes. In chronic inflammation, constant release of proteases by the inflammatory cells causes alveolar wall destruction. In addition, mucus plugging, airway wall remodeling and the formation of lymphoid follicles contribute to the small airway obstruction.
At the crossroad between innate and adaptive immunity, dendritic cells (DC) play a crucial role in the immune response to CS. Following encounter with an immunogenic antigen, these antigen presenting cells migrate towards the draining lymph nodes and display antigen in the T cell-rich zone. Langerin+ DCs accumulate in small airways of patients with COPD and their increase is associated with disease severity. Furthermore, increased expression of costimulatory molecules on several DC subsets is correlated with disease severity as well.

Where innate immunity is essential in the initiation phase of the immune response to CS, the adaptive immune system is important in the progression and consolidation phases of the COPD pathogenesis. Following antigen presentation by DCs, T cells are activated and recruited to the lung through binding of the chemoattractant CXCL10 – produced by structural cells in the lung – to its receptor CXCR3 on activated T cells. Characteristic for airways and lungs of patients with COPD are increased numbers of CD8+ cytotoxic T cells which correlate with disease severity. Importantly, CD8+ T cells in patients with COPD possess enhanced cytotoxicity compared to healthy smokers and non-smokers. In contrast to CD8+ cells which recognise antigen presented by major histocompatibility class 1 (MHCI) molecules on most cells, CD4+ T cells require antigen presented by MHCII on professional antigen-presenting cells. These cells are not cytotoxic themselves, but exert their function through release of cytokines which guide the responses of other cells. The percentage of airways containing CD4+ T cells significantly increases with COPD severity. However, this increase does not necessarily result in an increased immunological protection since the Th1 response to gram-negative bacteria is impaired in COPD. Th17 cells, which are important in immunological responses against extracellular pathogens and in auto-immune responses, are increased in bronchial mucosa and peripheral blood of patients with COPD. Interestingly, recent evidence shows that IL-17, which is primarily produced by Th17 cells, seems to be implicated in the pathogenesis of COPD.

In the last decade, B cells have gained importance in the pathogenesis of COPD. Although the increase in B cells in patients with COPD was already reported in the early nineties, it was not until the landmark paper of Hogg et al. that interest in this cell type rekindled. Not only isolated B cells are more numerous, B cells are the main cell type of tertiary lymphoid follicles and the percentage of airways associated with lymphoid follicles is significantly higher in patients with severe COPD compared to non-COPD control subjects and patients in the earlier stages of the disease. B cells, lymphoid follicles and their importance in the pathogenesis of COPD will be further discussed in Chapter 4.
1.5.4 **Mechanisms Driving the Pathogenesis of COPD**

Central in the pathogenesis of COPD is an excess of oxidants, both from exogenous and endogenous origin, which exceeds the amount of anti-oxidants present. Endogenous oxidants derive from innate immune cells and airway epithelial cells following injury. Markers for oxidative stress have been detected in exhaled breath condensate of patients with COPD and increase further during exacerbations. Increase in several oxidative stress markers is correlated with lung function decline in COPD. Oxidative stress contributes to many of the pathogenic events in COPD. Neutrophils and macrophages produce large quantities of proteases such as neutrophil elastase and MMP12. Oxidative stress adds to the imbalance between proteases and anti-proteases by inactivating the latter. Furthermore, oxidative stress enhances lung inflammation by inducing the release of chemokines which attract more inflammatory cells to the airways. These inflammatory cells induce fibrosis and remodelling of the airway wall and the proteases they release, cause tissue destruction. In addition, oxidants generate upregulation of nuclear factor κB (NF-κB) and activating protein 1 (AP-1), thereby inducing inflammation. Moreover, oxidants such as reactive oxygen species (ROS) lead to activation of the NLRP3 inflammasome, suggesting a role for this inflammasome in the pathogenesis of COPD. This large signalling complex controls the activation of IL-1β and IL-18, which are elevated in lungs of patients with COPD.

Although research has elucidated parts of the pathogenesis, there are still important questions to be raised. Recently, several exciting new hypotheses are being investigated to discover the missing links in the pathogenesis of COPD. For instance, autophagy, either defective or excessive, is observed in patients with COPD. Since autophagy, lung inflammation and oxidative stress are all witnessed in normal aging as well, it has been hypothesized that the mechanism driving COPD is accelerated aging of the lung. Furthermore, it is now recognised that interactions between host and microbiome are able to steer immune responses in the host. The microbiome in patients with COPD is less diverse and characterised by an expansion of the Proteobacteria phylum. In a murine model of COPD, lack of microbiota significantly attenuated both inflammation and decline of lung function. Moreover, in patients with COPD, decline in microbial diversity is associated with emphysema and remodelling. It is hypothesized that these interactions can account for the chronicity in COPD. This thought fits perfectly with the concept of innate immune training; a concept that can enlighten the self-perpetuating nature of the inflammatory response in COPD.

Finally, in advanced COPD, it is speculated that auto-immunity may contribute to the pathogenesis. Arguments for and against this hypothesis will be discussed in Chapter 4.
1.6 Treatment

COPD is treatable but not curable. Smoking cessation is the most effective intervention to slow down disease progression. To enhance the success rate of long-term smoking cessation, counselling is often supplemented with nicotine replacement products, such as nicotine gum or patches, and varenicline, bupropion or nortriptyline. Therapy is primarily based on symptom relief and prevention of exacerbations.

The degree of airflow limitation, the exacerbation frequency and the symptoms the patients experience determine the choice of bronchodilator and whether or not corticosteroids will be prescribed. Patients with mild to moderate airflow limitation and maximum 1 exacerbation per year, are treated with inhaled bronchodilators (either anticholinergics or β2-agonists). The choice for short-acting versus long-acting bronchodilators is based on the degree to which patients suffer from symptoms. Patients with severe or very severe airflow limitation and 2 or more exacerbations per year are treated with inhaled corticosteroids and long-acting bronchodilators. When these patients suffer from symptoms, roflumilast, a phosphodiesterase-4 inhibitor can be added to the treatment.

Aside from the pharmacological treatment, patients are encouraged to be physical active and when necessary, to pursue pulmonary rehabilitation. Very severe airflow limitation can warrant for oxygen therapy. To prevent exacerbations prophylactic measures, such as influenza vaccination, are advised.

The treatment of exacerbations depends on the cause of the exacerbation (non-infectious, bacterial or viral).

Currently, many new molecules are tested for safety and efficacy in clinical trials. Most of these new molecules induce bronchodilation. Some molecules exert an antagonistic function on muscarinic receptors while having an agonistic effect on β2-adrenoceptors. Triple therapy, combining a long-acting anticholinergic and a long-acting β2-agonist with a corticosteroid in 1 device, is also currently tested. This triple therapy could enhance therapy compliance.

Other candidate-drugs for COPD are new phosphodiesterase inhibitors, mitogen-activated protein (MAP) kinase inhibitors, andolast (modulator of calcium-activated potassium channels), ivacaftor (cystic fibrosis transmembrane conductance regulator (CFTR) enhancer) and several monoclonal antibodies. Of these monoclonal antibodies anti-IL-5 (mepoluzimab), anti-IL-5R (benralizumab), anti-IL-13 (lebrikizumab) and anti-IL-8 (ABX-IL8) are currently in phases 2 or 3 of the clinical trials. Others such as anti-IL-6, anti-17(R), anti-CD22 and anti-BAFF(R), are promising but are still in the first phases of testing.
PART I: INTRODUCTION

CHAPTER 2: MUCOCILIARY CLEARANCE IN COPD
2.1 Mucociliary clearance: introduction

Breathing air inevitably means that air-borne particles, toxins, gases, fumes and infectious agents enter the respiratory tract. Therefore, a mucus layer, which lies on top of the airway epithelium, delineates the respiratory tract and captures these inhaled noxious substances. The airway epithelium is largely ciliated and unidirectional beating of these cilia in combination with coughing propels the mucus, loaded with trapped particles and gases, towards the pharynx. This process of mucociliary clearance provides an essential first line of defence in respiratory immunity.

Dysfunction of the mucociliary clearance system can arise from excess mucus production, abnormal mucus hydration or cilia impairment. CS induces all three these defects, resulting in chronic bronchitis in a subpopulation of smokers and patients with COPD. In addition, independent of the presence of chronic bronchitis, mucus exudates cause obstruction of the small airways in patients with COPD and contribute to the narrowing of small airways. Moreover, progression of COPD is associated with the degree to which the lumen of small airways is filled with mucus exudates. Importantly, mucus stasis predisposes for bacterial infections in airways.

2.2 Mucociliary clearance in health

The airway epithelium fulfils many functions in the mucociliary clearance system. Not only does it provide cilia which propel mucus towards the pharynx, it also regulates airway surface liquid hydration, the secretion of immunoglobulins and other anti-microbial substances and provides mucins which constitute a major component of the mucus layer. Furthermore, epithelial cells also provide anti-inflammatory signals preventing patrolling immune cells in the airway lumen of overreacting in case of an encounter with a harmless antigen. The airway epithelium harbours ciliated cells, secretory cells, basal cells and a variety of less frequent cells, such as neuroendocrine cells. The relative contribution of these cell types depends on the airway level. In addition to secretory cells in the epithelium, large airways contain submucosal glands which secrete mucins, proteoglycans and numerous antimicrobial proteins into the airway lumen.

A large proportion of airway epithelial cells is ciliated. The cilia on these airway cells are motile and are typically constructed in a 9 + 2 arrangement of microtubules with dynein arms and radial spokes. Each ciliated epithelial cell generally possesses about 200 cilia at the apical plasma membrane and the cilia beat at a frequency of 10 to 15 Hz. Mucus is unidirectionally propelled towards the pharynx by a vertical movement.
effective stroke of the cilia, while the recovery stroke takes place in the periciliary layer in a horizontal plane 126,128. Ciliary beat frequency is regulated by changes in phosphorylation state of ciliary components such as dynein subunits, changes in intracellular pH and Ca\(^{2+}\) 129.

In healthy lungs, the epithelial cilia are surrounded by an approximately 7 µm high peri-ciliary layer (PCL) (Figure 5). This PCL gel-layer is covered by the mucus layer which is more visco-elastic than the PCL and variable in height. Together, these two layers constitute the airway surface liquid (ASL), which can be regarded as a movable physical barrier 40, 124, 130.

*Figure 5: Airway surface liquid and mucins.* The periciliary layer (PCL) and the mucus layer together constitute the airway surface liquid (ASL). Cell-tethered mucins (MUC1, MUC4, MUC16 and MUC20) attach to the microvilli and cilia of the airway epithelial cells and provide the gel structure of the PCL. MUC5B and MUC5AC are secreted mucins and form a mucus layer on top of the PCL. Mucus cells in the submucosal glands contribute to the production of MUC5B and MUC16.

In healthy individuals, the mucus layer exists of only 1.5 to 3 % solids, which include mucins, non-mucin proteins, anti-microbial substances, lipids, ions and cellular debris 40,131. Much of the properties of the mucus layer are mediated by the large glycoproteins, known as mucins 130. The predominant mucins in the mucus layer are MUC5AC and MUC5B, both heavily glycosylated proteins with at least one region of amino-acids rich in serine and threonine residues where carbohydrate structures are linked. The rich
diversity of carbohydrates aids in trapping bacteria by interaction with microbial sugar-binding proteins. Furthermore, by oligomerization at the 3’ and 5’ termini, mucins form a polymer network that provides structure to the mucus gel \(^{40, 130, 132}\). Recent research shows that globular proteins are important contributors to this framework and mucin-protein interactions are crucial for the biophysical properties of mucus \(^{133}\). Of note, these globular proteins constitute about 60 to 70 % of the solids in the mucus layer \(^{40, 133}\).

Mucins are secreted continuously at a baseline level \(^{40}\). Submucosal glands release mucins in a circadian rhythm, most likely regulated by the vagal nerve \(^{132}\). In contrast, goblet cells, which are a minority population of about 5 % of the epithelial cells in healthy individuals, secrete mucins due to stimulation of P2Y2 purinergic receptors by the low amount of ATP that is present in the ASL \(^{40, 131}\). Before secretion, mucins are packed in vesicles in dehydrated condition. Their negative charges are shielded by Ca\(^{2+}\) and H\(^{+}\). Upon release, mucins dramatically expand and bind water. Therefore, the mucus layer is able to function as a water reservoir for the underlying PCL \(^{124}\). Interestingly, MUC5B is thought to be predominantly secreted from the submucosal glands, whereas goblet cells secrete MUC5AC \(^{130}\).

In contrast to former believes, the PCL is not a watery layer but a gel-based structure, composed of several different mucins and proteins \(^{134}\). Cell-tethered mucins attach to the apical membrane and microvilli (MUC1), to the cilia (MUC4 and MUC20) or to goblet cells (MUC 16) of the airway epithelium (Figure 5) \(^{135}\). They possess a similar structure as secreted mucins and are heavily glycosylated as well, which causes them to extend far from the cell surface with the carbohydrates branching out from the axis. This conformation resembles that of a bottle brush \(^{136}\). This characteristic look of tethered mucins leads to shielding of extra-cellular receptors and hence protection against adhesion of microbial organisms \(^{137}\). The cell-tethered mucins and large mucopolysaccharides create a mesh with selective barrier functions \(^{135, 138}\). This relatively new concept has elegantly been described as a ‘gel-on-brush’ structure and explains why secreted mucins do not enter the PCL \(^{138}\).

For efficient mucociliary clearance, the ASL must be adequately hydrated \(^{124}\). The ‘gel-on-brush’ model explains that the highly concentrated PCL, with an osmotic pressure higher than that of the mucus layer, can withdraw water from the mucus layer up to the point where the concentration of the latter becomes so high that the osmotic pressure exceeds that of the PCL. At that point the PCL collapses and mucus stasis is established \(^{138}\).

ASL hydration basically means a balance between cation absorption and anion secretion. For a large part, water follows passively via the paracellular pathway. Cl\(^-\) secretion is mediated by CFTR (Cystic Fibrosis Transmembrane conductance Regulator) and Na\(^+\) absorption by ENaC (Epithelial Natrium Channel). In
mice, Ca$^{2+}$-activated Cl$^-$ channels (CaCC) additionally secrete Cl$^-$ into the lumen. Importantly, in addition to Cl$^-$, HCO$_3^-$ is secreted in smaller concentrations by CFTR and has a buffering effect on ASL pH. CFTR is a member of the adenine nucleotide-binding cassette transporter subfamily and works as an cAMP-dependent anion channel. ENaC is a heterotrimer with a α-, β- and γ-subunit. The α- and γ-subunit need to be proteolytically cleaved for activation. Volume sensors, such as nucleotides (ATP, UTP and UDP), adenosine and short palate lung and nasal epithelial clone 1 (SPLUNC1), regulate ASL hydration. Nucleotides and adenosine do so by interacting with purinergic receptors, initiating Cl$^-$ secretion. SPLUNC1 binds directly to ENaC, causing internalisation of this Na$^+$ channel and thus, its inactivation. Interestingly, cilia, beating in the mucus layer, sense alterations in mechanical strain which reflect alterations in hydration state of the mucus layer and subsequently regulate ATP release. Regulation of the ion channels also depends on whether or not they are co-expressed. CFTR seems to have a suppressing effect on the activation of ENaC. However, the interactions between both ion channels are not well understood.
2.3 Mucociliary clearance in COPD

2.3.1 Ciliary beating in COPD

CS impairs ciliogenesis, leads to shortened cilia, reduces ciliary beat frequency and increases the number of ciliary microtubule abnormalities. Cilia length was measured in endobronchial biopsies from healthy smokers and non-smokers and a significant 15% reduction in cilia length was detected in healthy smokers. It was recently determined that CS induces upregulation of histone deacetylase 6 activity which results in removal of ciliary proteins and subsequent ciliary shortening. Furthermore, chronic CS exposure of wild-type mice resulted in loss of ciliated cells and decreased ciliary beat frequency mediated by activation of protein kinase C. All these effects on ciliary function will have repercussions on the efficiency of mucociliary clearance, although coughing provides a back-up mechanism for mucus clearance.

2.3.2 Mucus production and secretion in COPD

The chronic bronchitis phenotype in COPD is characterized by excess production of mucus and is associated with current smoking. In addition, small airway obstruction due to mucus plugging is present in patients with severe COPD, irrespective of the presence of chronic bronchitis, and correlates to disease severity. Both glandular hyperplasia and goblet cell hyperplasia/metaplasia contribute to this overabundance of mucins. In smokers with airflow obstruction, goblet cell numbers and size in the large airways correlate with disease severity. The transition of ciliated cells into goblet cells, is most likely regulated via EGFR (epidermal growth factor receptor) and IL-13. EGFR activation inhibits apoptosis and IL-13 mediates activation of ERK1/2 and STAT6, which promotes the transdifferentiation to goblet cells. A transdifferentiation of Clara cells into goblet cells may also occur. In patients with COPD and in current smokers, EGFR and MUC5AC expression is increased. Takeyama et al. showed, both in vitro and in vivo, that CS induces activation of EGFR and subsequent upregulation of Muc5AC and goblet cell numbers. Upregulation of MUC5AC expression can be mediated by many stimuli, among which CS components, microbial agents, neutrophil elastase, MMP9, oxidants and several cytokines. Signalling through one of at least 5 different pathways that have been implicated in the upregulation of Muc5AC, leads to rapid production of the protein within 2 hours. The regulation of MUC5B is less understood. Mucin secretion is regulated separately from mucin production and is in goblet cells predominantly mediated via ATP binding to P2Y2 receptors. Mucin release from submucosal glands is neurologically regulated.
Mucus hypersecretion is driven by an enormous increase in MUC5AC, whereas the relative contribution of MUC5B is unclear. Interestingly, MUC5B is thought to be necessary for airway defence. It forms non-covalent heterotypic complexes with many anti-microbial agents, among which secretory IgA (sIgA) and amylase. Of note, glycosylation of the secretory component of sIgA is necessary for retention in bronchial mucus.

Mucin hypersecretion leads to a substantial increase in the percentage of solids within the mucus layer, causing a relative dehydration. While the mucus layer comprises a maximum of 3% solids in healthy individuals, in smokers, the percentage of solids can increase up to 10%. Anderson et al. observed that the osmotic pressure of the mucus layer causes compression of the PCL when the percentage of solids is higher than 3%. Furthermore, the percentage of solids is inversely correlated with mucus clearance in patients with chronic bronchitis. Both mucus clearance and the percentage of solids in sputum are negatively associated with airflow limitation in patients with chronic bronchitis. Developing pre-clinical animal models of chronic bronchitis is difficult since the definition of chronic bronchitis is a clinical one. Furthermore, there is substantial variation between species with regard to the occurrence and localisation of both submucosal glands and goblet cells. In response to CS, mice do not readily develop goblet cell metaplasia. In rats, goblet cell metaplasia following CS exposure is restricted to the proximal airways. In contrast, guinea pigs do develop goblet cell metaplasia in the small airways as well. However, using guinea pigs involves a number of disadvantages such as a greater cost, and a lack of tools (antibodies and gene sequences) to study these animals. Aside from CS, other inducing agents have been used to evoke chronic bronchitis in animals. The most frequently used agents are sulphur dioxide, lipopolysaccharide and proteases. The downside of these agents is that the relevance to mechanisms that occur with CS exposure is unclear. Finally, goblet cell metaplasia can be induced in genetically engineered mice, for instance by overexpression of Th2 cytokines. However, this approach induces other elements (such as eosinophilia) which do not comply with COPD pathogenesis.

2.3.3 Airway surface liquid hydration state in COPD

Aside from the effects of mucin hypersecretion and increase in percentage of solids in the mucus layer of patients with COPD, ASL is also compromised by direct and indirect effects of CS on the ion channels responsible for hydration.

The prototype disease of airway surface dehydration and subsequent mucociliary clearance dysfunction is cystic fibrosis (CF). This disease is caused by mutations of the Cftr gene and is recessive inheritable. Patients suffer from severe neutrophilic airway inflammation and mucus stasis from birth onwards and their life
expectancy is reduced due to recurrent bacterial pulmonary infections. The function of the CFTR protein is often completely diminished and as a result there is no active Cl\(^{-}\) and HCO\(_3\)\(^{-}\) secretion to the airway lumen through the CFTR channel. Mucus stasis predisposes for bacterial colonization and neutrophilic inflammation. The proteases which are released from the inflammatory cells further alter the function of ion channels and aggravate the airway surface dehydration. Lack of HCO\(_3\)\(^{-}\) leads to an acidic pH which inhibits effective bacterial killing \(^{159,160}\).

Although both COPD and CF result in obstructive lung disease in which airway surface dehydration contributes to the pathogenesis, there are several difference between both diseases. In contrast to CF, cigarette smoking induces an acquired dysfunction of the CFTR channel and the function can be restored after smoking cessation. The dysfunction is generally not complete; the CFTR channel remains active for some part \(^{159,160}\).

In cigarette smokers with and without COPD, the CFTR function is suppressed in both upper and lower airways \(^{161,162}\). Moreover, loss of CFTR function correlates with the presence of chronic bronchitis and dyspnea \(^{162}\). In chronic smokers, CFTR function is decreased by 55 to 70\%. This acquired dysfunction is thought to be due to internalisation of CFTR in a Ca\(^{2+}\)-dependent way (Figure 6) \(^{124,157}\). Protein misfolding of CFTR due to CS has been suggested as a potential cause for this internalisation \(^{124}\). The function of CFTR is also systemically impaired in cigarette smokers and acrolein has been identified as an agent conveying the deleterious effects of CS on CFTR \(^{163}\). Furthermore, oxidative stress and heavy metals, both present in lungs of patients with COPD, reduce the activity of CFTR \(^{124,164}\). Pharmacological intervention with ivacaftor, a CFTR potentiator, or roflumilast, a phosphodiesterase inhibitor, improved activation of CFTR and thereby airway surface dehydration \(^{165,166}\). Interestingly, CFTR dysfunction results in loss of HCO\(_3\)\(^{-}\) secretion and acidification of ASL, leading to impaired bacterial killing and increased infection risk \(^{139}\).

**Figure 6: CFTR and ENaC.** In healthy airways, the presence of CFTR inhibits the function of ENaC. Following cigarette smoke exposure, CFTR is internalized, resulting in loss of Cl\(^{-}\} and HCO\(_3\)\(^{-}\) secretion and activation of ENaC. Adapted from Ghosh et al. Cell Mol Life Sci. 2015; 72: 3637-3652 \(^{121}\).
The role of ENaC dysfunction in COPD is less clear. Downs et al. showed that ROS and H$_2$O$_2$, both oxidants present in CS, activate ENaC in alveolar epithelial cells $^{167,168}$. It was also reported that hypoxia inhibits ENaC activation $^{169}$. In lung tissue of COPD patients, Zhao et al. observed that the expression of α- and β-ENaC was increased and inversely correlated with lung function. They also showed that CFTR expression was decreased and this also correlated with lung function $^{170}$. Since CFTR has a suppressive effect on ENaC activity, it can be argued that loss of CFTR might result in activation of ENaC. Furthermore, proteases such as neutrophil elastase and MMP9 are abundantly present in airways of patients with COPD and are potent activators of ENaC by performing the necessary cleaving of the α- and γ-subunit $^{124,125,160}$. Nevertheless, these speculations need scientific proof. Of note, regardless of the importance of ENaC hyperactivation in the pathogenesis of COPD, pharmacological targeting of ENaC seems a promising approach for improving airway surface dehydration $^{171,172}$. 
2.4 β-ENaC transgenic mice: a murine model of airway surface dehydration

Research into the role of airway surface dehydration in the pathogenesis of COPD is a challenging quest. In health, airways of mice harbor only few goblet cells and exposure to CS does not substantially influence this feature. Furthermore, mice only possess submucosal glands near the first few tracheal rings in contrast to humans which possess submucosal glands in all the large airways (> 2 mm). As a result of these characteristics of mice, mimicking airway surface dehydration through increased mucus secretion is not evident. In addition, cftr⁻/⁻ mice do not exhibit a lung phenotype. Recently, Shah et al. demonstrated that this absence of phenotype in mice is caused by a lack of H⁺/K⁺ adenosine triphosphatase (ATP12A), which causes secretion of H⁺ into the lumen. In humans and pigs with CFTR dysfunction, this H⁺ secretion acidifies the ASL, resulting in impairment of host defenses and increase in airway bacteria.

![Figure 7: βENaC-transgenic mice.](image)

In wild-type mice, CFTR (cystic fibrosis transmembrane conductance regulator) regulates the function of ENaC (epithelial natrium channel), balancing Na⁺ absorption and Cl⁻ secretion. Normal hydration of the airway surface liquid results in a periciliary layer (PCL) of approximately 7 µm which allows the cilia to beat optimally. In βENaC-transgenic mice, overexpression of βENaC leads to Na⁺ hyperabsorption and a subsequent imbalance between Na⁺ absorption and Cl⁻ secretion. The result is dehydration of the airway surface liquid, compression of the PCL and impaired mucociliary clearance.

Adapted with permission from Mall MA et al., Nat Med, 2004;10:487-493.
To obtain airway surface dehydration, Mall and colleagues created transgenic mice that overexpress the β-subunit of ENaC (Scnn1b) in an airway specific way by coupling to a Clara cell specific promoter (CCSP). This overexpression is responsible for hyperabsorption of Na⁺ and subsequent dehydration of the ASL (Figure 7). In contrast to cftr⁻/⁻ mice, βENaC-transgenic (βENaC-Tg) mice display a lung phenotype resembling cystic fibrosis with reduced mucus transport, airway mucus obstruction and pulmonary death 176. The severity of the induced lung disease is demonstrated by the high neonatal death due to mucus plugging and subsequent asphyxia. Surviving mice develop remarkable goblet cell metaplasia and airway inflammation, followed by substantial emphysema at early age, features resembling COPD 177. By backcrossing to a C57BL/6 wild-type background, Johannesson et al. created βENaC-Tg mice with significantly less mucus plugging and neonatal mortality, resulting in a phenotype more relevant for COPD research 178.
PART I: INTRODUCTION

CHAPTER 3: THE CHRONIC BRONCHITIS PHENOTYPE IN COPD
3.1 Definition

Chronic bronchitis (CB) is a disease entity on its own and is most commonly described as: \(^{147}\)

"chronic cough and sputum production for at least 3 months per year for two consecutive years".

Chronic bronchitis, affecting 3.4 – 22.0 % of the general population, has long been linked to tobacco smoking \(^{179}\). Other risk factors include exposure to environmental tobacco smoke, mineral dust and high exposure to noxious gases and fumes. In CB patients without COPD, male gender involves an increased risk for developing CB \(^{180}\).

The chronic bronchitis phenotype of COPD describes patients with COPD and concomitant CB, with or without emphysema. In this population of subjects with both COPD and CB, Dijkstra, et al. did not find an association with occupational exposures to gases, fumes or mineral dust; nor with male gender \(^{180}\). In contrast, the COPDGene study did report an association between the presence of chronic cough and phlegm and occupational exposure to dust and fumes \(^{181}\). Furthermore, COPD patients with chronic cough and phlegm are more likely to suffer from allergic upper airway symptoms \(^{182}\). The underlying pathological features of chronic bronchitis in COPD have been briefly discussed in Chapter 1.

3.2 Epidemiology

The prevalence of CB in patients with COPD ranges from 7.4 % up to 74 % \(^{147, 183}\). This wide dispersion results from the use of different CB definitions and differences in the studied populations, such as ethnicity, sex and disease severity \(^{147}\). Ethnicity may play a role in the prevalence of CB in COPD patients, with a higher prevalence in Caucasian patients with COPD. Gender differences have been an issue of debate. All three possible outcomes (no difference in prevalence between men and women, more prevalent in men and more prevalent in women) have been reported repeatedly \(^{180, 181, 184}\). It must be taken into account that cultural issues can influence this outcome, underlining the importance of geographical background of studies \(^{179}\). Furthermore, the highest prevalence of CB was found in a cohort of COPD patients with predominantly severe COPD, recruited from university hospitals in France, whereas the lowest prevalence was described in a population-based cohort in South-America with subjects with predominantly mild-to-moderate COPD \(^{179, 183}\). This notion emphasises the difficulties in appointing a clear-cut prevalence percentage to this phenotype in patients with COPD.
3.3  CLINICAL MANIFESTATIONS AND IMPACT OF CHRONIC BRONCHITIS IN COPD

Linking clinical features to a COPD phenotype encloses several difficulties. First, the natural course of the disease or treatment of the disease may change the phenotype of patients over time. Second, patients can have more than one phenotype. And finally, patients can suffer from several diseases. However, making an abstraction of these difficulties, the CB phenotype in COPD has been associated with several clinical outcomes.

In the COPDGene study, COPD patients with CB exhibited worse respiratory symptoms. These symptoms included wheezing and awakening by cough or dyspnea. The ECLIPSE study, the PLATINO study and a population-based study in China all reported that COPD patients with CB had on average worse airflow limitation than those without CB. Moreover, in the Copenhagen City Heart Study, an excess decline in FEV$_1$ of 22.8 ml/year was detected in men with COPD and CB compared to male COPD patients without CB. There was no significant excess decline in FEV$_1$ in women with both COPD and CB. In this cohort, chronic mucus hypersecretion was also associated with hospitalization due to COPD.

The presence of chronic productive cough significantly affects health-related quality of life of patients with COPD. This was illustrated in the COPDGene and ECLIPSE studies by worse St. George’s Respiratory Questionnaire disease-specific quality of life scores and in the PLATINO study by more limitations due to physical health.

An important clinical outcome in COPD is the exacerbation rate, which has a significant impact on the progression of the disease. In the GOLD executive summary, an exacerbation is described as “an acute event characterized by a worsening of the patient’s respiratory symptoms that is beyond normal day-to-day variations and leads to a change in medication.” Reports regarding the association between the chronic bronchitis phenotype in COPD and COPD exacerbations render inconsistent data. In the ECLIPSE study, COPD patients with CB did not have a significantly increased risk for exacerbations. Additionally, in the PLATINO study, the number of exacerbations in the previous year did not differ significantly between COPD patients with and without CB, although the percentage of subjects with both COPD and CB which reported at least one exacerbation, was significantly higher than in COPD subjects without CB. In contrast, in the COPDGene study, patients with both COPD and CB reported significantly more exacerbations than COPD patients without CB. Moreover, COPD patients with CB experienced more severe exacerbations in the previous year compared to those without CB. Results of other patient-based studies support the association between the presence of CB and COPD exacerbations.
even a productive cough which has not been present for two consecutive years, is associated with increased exacerbation risk and increased mortality risk \(^{190}\).

Whether or not COPD patients with CB have an increased mortality is under discussion as well. Data from the Copenhagen City Heart study show that the presence of chronic mucus hypersecretion entails a relative risk (RR) of death from COPD with pulmonary infection of 3.5 (95 % confidence interval (CI) 1.8-7.1). However this study did not indicate that COPD patients with CB have an increased mortality risk in absence of pulmonary infection \(^{191}\). In a study incorporating two rural Finnish cohorts, Pelkonen et al. determined that the presence of CB increased the hazard ratio for all-cause mortality in addition to mortality due to respiratory and cardiovascular diseases \(^{192}\). However, in the NHANES study, only a trend towards increased all-cause mortality risk was registered (RR: 1.2 (95 % CI: 0.97-1.4)) \(^{193}\). These conflicting data suggest the need for further epidemiological research.

3.4 Treatment

Smoking cessation is the most important intervention and can improve cough and mucociliary function. Mucus clearance can be improved by chest physiotherapy. Pharmaceuticals improving symptoms of chronic bronchitis include short-acting β-adrenergic receptor agonists, methylxanthines, glucocorticoids and antioxidants \(^{147, 182}\). A meta-analysis reviewing the effect of mucoactive drugs, revealed that the use of N-acetylcysteine or carbocysteine reduced the number of exacerbations \(^{194}\). Furthermore, the use of low dose macrolides reduced the number of exacerbations as well; however, there is no additional benefit for COPD patients with CB \(^{182}\).
### 3.5 Overview of Epidemiological Studies Regarding the Chronic Bronchitis Phenotype in COPD

<table>
<thead>
<tr>
<th>Study (ref)</th>
<th>Subjects</th>
<th>Follow-up</th>
<th>Prevalence in COPD</th>
<th>Main observations regarding COPD patients with CB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Copenhagen City Heart Study</strong>&lt;sup&gt;a&lt;/sup&gt;&lt;br&gt;Population-based Longitudinal</td>
<td>14223 subjects</td>
<td>10 years</td>
<td>ND</td>
<td>• Association with FEV&lt;sub&gt;1&lt;/sub&gt; decline&lt;br&gt;• Association with hospitalization due to COPD&lt;br&gt;• Association with COPD-related mortality with pulmonary infection&lt;br&gt;• No association with COPD-related mortality without pulmonary infection</td>
</tr>
<tr>
<td><strong>ECLIPSE Study</strong>&lt;sup&gt;a&lt;/sup&gt;&lt;br&gt;Population-based Longitudinal</td>
<td>2138 patients GOLD II-IV</td>
<td>3 years</td>
<td>34.66 % at baseline</td>
<td>• Lower mean FEV&lt;sub&gt;1&lt;/sub&gt; at baseline but not a more rapid decline&lt;br&gt;• Association with lower health-related quality of life&lt;br&gt;• No association with number of exacerbations</td>
</tr>
<tr>
<td><strong>Population-based Study in Finland</strong>&lt;sup&gt;a&lt;/sup&gt;&lt;br&gt;Longitudinal</td>
<td>1711 men 40-59 years</td>
<td>40 years</td>
<td>10.37 % at baseline</td>
<td>• Cumulative incidence of COPD + CB = 19%&lt;br&gt;• Association with lower FEV&lt;sub&gt;1&lt;/sub&gt;&lt;br&gt;• Association with all-cause mortality&lt;br&gt;• Association with mortality by respiratory and cardiovascular diseases</td>
</tr>
<tr>
<td><strong>COPDGene Study</strong>&lt;sup&gt;a&lt;/sup&gt;&lt;br&gt;Patient-based Cross-sectional</td>
<td>1061 patients GOLD II-IV</td>
<td></td>
<td>27.33 %</td>
<td>• Association with respiratory symptoms&lt;br&gt;• Association with lower health-related quality of life (SGRQ and mMRC)&lt;br&gt;• Association with number of exacerbations in the previous year (including severe exacerbations)</td>
</tr>
<tr>
<td><strong>PLATINO Study</strong>&lt;sup&gt;a&lt;/sup&gt;&lt;br&gt;Population-based Cross-sectional</td>
<td>5314 subjects (759 with COPD)</td>
<td></td>
<td>7.4 %</td>
<td>• Association with lower FEV&lt;sub&gt;1&lt;/sub&gt;&lt;br&gt;• Association with respiratory symptoms&lt;br&gt;• Association with lower quality of life (short form-12 physical score, limitation due to physical health)&lt;br&gt;• No significant association with number of exacerbations</td>
</tr>
<tr>
<td><strong>Patient-based study in Spain</strong>&lt;sup&gt;a&lt;/sup&gt;&lt;br&gt;Cross-sectional</td>
<td>331 patients GOLD I-IV</td>
<td></td>
<td>44.7 %</td>
<td>• No association with exacerbations&lt;br&gt;• Association with arterial hypertension, dyslipidemia, sleep apnea syndrome and diabetes</td>
</tr>
<tr>
<td><strong>Population-based Study in China</strong>&lt;sup&gt;a&lt;/sup&gt;&lt;br&gt;Cross-sectional</td>
<td>20245 subjects (1668 with COPD)</td>
<td></td>
<td>29.98 %</td>
<td>• Association with current smoking and male gender&lt;br&gt;• Association with FEV&lt;sub&gt;1&lt;/sub&gt;/FVC (%) and dyspnoea severity (mMRC)</td>
</tr>
<tr>
<td><strong>Patient-based study in France</strong>&lt;sup&gt;a&lt;/sup&gt;&lt;br&gt;Cross-sectional</td>
<td>433 patients GOLD I-IV (&gt; 50% III-IV)</td>
<td></td>
<td>74.1 %</td>
<td>• Association with frequent exacerbations, (moderate and severe exacerbations)&lt;br&gt;• Number of exacerbations in the previous year = independent factor linked to CB</td>
</tr>
<tr>
<td><strong>Patient-based study in Sweden</strong>&lt;sup&gt;a&lt;/sup&gt;&lt;br&gt;Cross-sectional</td>
<td>373 patients GOLD stage III-IV</td>
<td></td>
<td>36.97 %</td>
<td>• Association with current smoking&lt;br&gt;• Association with frequent exacerbations&lt;br&gt;• Association with musculoskeletal symptoms</td>
</tr>
</tbody>
</table>

ND: not determined
PART I: INTRODUCTION

CHAPTER 4: B CELLS, ECTOPIC LYMPHOID FOLLICLES AND B CELL-ACTIVATING FACTOR IN COPD

Based on:
4.1 B CELLS, ECTOPIC LYMPHOID FOLLICLES AND B CELL-ACTIVATING FACTOR: INTRODUCTION

4.1.1 B CELL DEVELOPMENT

B cells develop from common lymphoid progenitor cells through a highly controlled process. The early developmental stages take place in specialized niches in bone marrow (Figure 8)\(^\text{198}\). The differentiation from pro-B cells into immature B cells depends on rearrangement of immunoglobulin heavy-chain genes, followed by rearrangement of light-chain loci. Strongly self-reactive immature B cells follow the path of either receptor editing or clonal deletion\(^\text{199}\). Weakly or non-self-reactive B cells develop into transitional B cell stages and further into mature B cells; a process that is predominantly completed in the spleen. At this point B cells have become either marginal zone B cells or follicular B cells, depending on the strength of the B cell receptor (BCR) signal and the presence of B cell-activating factor (BAFF)\(^\text{199, 200}\). Marginal zone B cells are located in the marginal sinus of the spleen, have the capacity to self-renew and are primarily responsive to blood-borne pathogens\(^\text{200}\). Follicular B cells – the fate chosen by the majority of B cells – recirculate through the bloodstream.

When passing through lymph nodes, a chemotactic gradient of CXC-chemokine ligand 13 (CXCL13), which binds to collagen IV in the basal lamina of the high endothelial venules and the surrounding reticular network, entice B cells to migrate through the endothelial wall into B cell follicles\(^\text{201-203}\). This process is mediated by the interaction of CXCL13 with its receptor CXCR5, which is expressed by B cells and T follicular helper (T\(_{\text{fh}}\)) cells. In lymph nodes, an encounter with cognate antigen is mainly facilitated through antigen presentation by follicular dendritic cells (FDC), macrophages and dendritic cells (DC)\(^\text{201}\). Subsequent to antigen-recognition, B cells can differentiate into extrafollicular plasma cells, germinal center cells or early memory cells, depending largely on the strength of the BCR antigen recognition. High affinity will induce the formation of plasma cells which then produce high affinity immunoglobulins (Ig). In case of low to moderate affinity of the BCR for the antigen, B cells will enter the germinal center reaction, which is defined by affinity maturation, somatic hypermutation and class switch recombination. Mutated BCRs with high affinity are positively selected via interaction with FDC and become either long-lived memory cells or plasma cells\(^\text{200, 204, 205}\).
Figure 8: B cell development. B cell development begins in the bone marrow from common lymphoid progenitor cells (1). Via a pro-B cell (2) and pre-B cell stage (3), B cells develop into an immature B cell which possesses a mature B cell receptor (BCR) (4). Maturation proceeds via two transitional stage (5) and is completed in the spleen (6). At this point a fate decision is made whether B cells become marginal zone B cells or follicular B cells. The decision is dependent on the strength of the BCR signal, the presence of BAFF, Notch2 and NF-κB activation. Marginal zone B cells reside in the marginal sinus in the spleen, whereas follicular B cells recirculate and enter follicles in secondary lymphoid organs in search of their cognate antigen. When antigen is encountered and co-stimulatory signals are provided by T cells at the B cell/T cell border, B cells can enter the germinal center (GC) to increase the affinity of their BCR for the antigen. In the dark zone of the germinal center, B cells undergo somatic hypermutation (SHM) and proliferation. Next, B cells enter the light zone of the germinal center. Follicular dendritic cells (FDC) present antigen and the affinity of the mutated BCRs for the antigen is tested. Low affinity results in apoptosis. B cells with high affinity compete for T follicular helper cell (Tfh cell) help. Finally, B cells can either re-enter the dark zone for further affinity maturation, become a memory B cell or plasma cell.

4.1.2 Ectopic lymphoid follicle formation in lung

In health, mucosal immunity in the lung does not seem to incorporate organized lymphoid tissue. The presence of organized bronchus-associated lymphoid tissue (BALT) in healthy adults is debatable \(^{206, 207}\). The formation of BALT requires a specialized epithelium above the organized lymphoid follicles and this lymphoepithelium has only rarely been observed \(^{208}\). In contrast, ectopic lymphoid follicles which show varying degrees of organization, have been described in healthy controls \(^{110, 209, 210}\). This organization includes the presence of B- and T cell areas, follicular dendritic cells, high endothelial venules, germinal center formation and efferent lymphatic vessels \(^{208, 211}\). Fully organized, these lymphoid follicles are often called inducible BALT (iBALT) or tertiary lymphoid organs (TLO), indicating their similarity with secondary lymphoid organs such as lymph nodes \(^{206, 211}\).

The process responsible for the formation of ectopic lymphoid follicles is called lymphoid neogenesis. It is initiated by repeated or chronic inflammation in non-lymphoid organs \(^{206, 212}\). Much like lymph node organogenesis, the basic ingredients of lymphoid neogenesis are stromal cells, haematopoietic cells, homeostatic chemokines and their receptors, adhesion molecules and cytokines (Figure 9) \(^{206, 213}\).

**Figure 9: Lymphoid neogenesis.** Lymphoid follicle formation begins with the expression of lymphotoxin α1β2 (LT α1β2) by inducing cells, such as B cells, and the expression of lymphotoxin β receptor (LTβR) by stromal cells, such as fibroblasts. Interaction of LTαβ2 with LTβR leads to the release of homeostatic chemokines which attract dendritic cells, T cells and B cells.
Briefly, in lymph node organogenesis, lymphoid tissue inducer cells express lymphotoxin α_1β_2 (LTα1β2) which interacts with the LTβ-receptor (LTβR) on lymphoid tissue organizer cells. This induces the expression of adhesion molecules and homeostatic chemokines, resulting in accumulation and organization of lymphocytes and dendritic cells into lymph nodes. In this process, CC-chemokine ligand (CCL)19, CCL21, CXC-chemokine ligand (CXCL)12 and CXCL13 attract DC, T and B cells via the receptors CCR7 and CXCR5. The cells and molecules involved in this process are not necessarily identical for lymphoid neogenesis and several murine models of COPD have been used to investigate both the similarities and the discrepancies between both (see 4.2.3 Lymphoid neogenesis in murine models of COPD).

4.1.3 **B cell-activating factor**

The tightly controlled process of B cell development does not only rely on BCR editing but requires the presence of several survival factors as well. One of these factors is B cell-activating factor from the tumor necrosis factor (TNF) family, also known as BAFF, which was discovered in 1999. BAFF is a type II transmembrane homotrimer protein and signals through three receptors: BAFF-receptor (BAFFR), transmembrane activator and cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA). BAFF itself is produced by stromal cells, innate immune cells, activated T cells and B cells, whereas the receptors are predominantly located on lymphocyte subsets (Figure 10). A proliferation-inducing ligand (APRIL), a family member of BAFF, also interacts with TACI and BCMA. B cell homeostasis requires processed soluble BAFF through proteolytic cleavage; however, membrane-bound BAFF is active as well.

BAFF functions primarily as a necessary survival and maturation factor for B cells through interaction with BAFFR. Both BAFF^−/− and BAFFR^−/− mice exhibit a limited number of peripheral B cells past transitional stage 1. Pro- and pre-B cells in the bone marrow do not seem to rely on BAFF signaling. BAFF interaction with BAFFR induces activation of the non-canonical NF-κB pathway via de-repression of NF-κB inducing kinase (NIK), resulting in upregulation of anti-apoptotic proteins. In contrast, BAFF signaling through TACI activates the classical NF-κB pathway and negatively regulates the B cell pool. Furthermore, TACI is necessary for T cell-independent responses and class-switch recombination. BAFF signaling through BCMA activates classical NF-κB as well and results in promotion of long-lived plasma cell maintenance in bone marrow.
Systemic BAFF production is thought to be relatively constant and Gorelik et al. demonstrated that radio-resistant non-hematopoietic cells are capable of sustaining B cell homeostasis 225, 226. For instance, in secondary lymphoid organs, fibroblastic reticular cells are responsible for the production of BAFF that is necessary for B cell homeostasis and organization of follicles 227. However, within germinal centers, T follicular helper (Tfh) cell-derived BAFF is indispensable for efficient selection of high affinity B cell clones 228. BAFF derived from other immune cells is also thought to be most important during inflammation 229.

Alongside the non-redundant role in B cell homeostasis, other immune cells are affected by BAFF as well. BAFF promotes T helper type 1 (Th1)-mediated and inhibits Th2-mediated responses and promotes the development of Th17 cells 229, 230. In addition, BAFF induces survival of monocytes and differentiation of monocytes into macrophage-like cells 231. Furthermore, DC activation and maturation is stimulated by BAFF 232.

Soon after its discovery, BAFF has been implicated in several auto-immune diseases. Belimumab, a monoclonal antibody against BAFF, is already approved for use in patients with systemic lupus erythematosus (SLE). Aside from SLE, rheumatoid arthritis, multiple sclerosis and primary Sjögren syndrome have repeatedly been associated with excessive levels of BAFF as well 233. In addition, abnormal BAFF levels have also been detected in other diseases such as graft-versus-host disease, common variable immunodeficiency, several infections, allergy and hematological and lymphoid cancers 224.

Figure 10: B cell-activating factor (BAFF) and its receptors. BAFF is produced by innate immune cells, stromal cells, activated T cells and B cells. BAFF interacts with the highest affinity to BAFF-receptor (BAFFR) which is located on B cells and T cell subsets. BAFF also interacts with Transmembrane Activator and Cyclophilin ligand Interactor (TACI). BAFF binds with the least affinity to B cell maturation antigen (BCMA) situated on plasma cells.
4.2 B CELLS, ECTOPIC LYMPHOID FOLLICLES AND B CELL-ACTIVATING FACTOR IN COPD

4.2.1 ISOLATED B CELLS IN THE LUNGS IN COPD

Under homeostatic conditions, a limited number of B cells forms an integrated part of the mucosal immunity in lungs. Memory B cells, locally generated during infection, remain in the lung in peri-bronchial infiltrates and can rapidly differentiate into plasma cells when activated. Upon reinfection, these tissue-resident memory B cells produce not only more immunoglobulins but also cross-reactive immunoglobulins important in response to viral escape. Brandsma and colleagues observed a significant increase in memory B cells in lungs of patients with COPD compared to non-COPD controls, but no significant difference was noticed in total CD20+ B cell numbers between both groups. In contrast, Bosken et al. and Hogg et al. did detect a significant raise in total B cell numbers in peri-bronchiolar regions and additionally, a correlation between the accumulated volume of B cells and COPD severity. The number of B cells was also significantly expanded in central airways of patients with COPD and this correlated with disease severity as well. Alongside CD20+ B cells, plasma cells are located in subepithelial and periglandular regions of the airways and produce predominantly polymeric IgA, a crucial component of the mucociliary clearance system of the airways. Smokers with chronic bronchitis and airflow obstruction tend to harbor increased numbers of plasma cells in these peri-bronchial regions. Moreover, the majority of the peri-glandular plasma cells produce IL-4 and/or IL-5, suggesting a role in mucus hypersecretion.

4.2.2 ECTOPIC LYMPHOID FOLLICLES IN COPD

In COPD, B cells are the dominant cell type in ectopic lymphoid follicles and these follicles are located in peri-bronchiolar and peri-vascular regions and in lung parenchyma. The landmark paper of Hogg et al. demonstrated an association between disease severity and the percentage of small airways containing lymphoid follicles. Several other studies have since corroborated an increase in number and even size of lymphoid follicles in patients with severe COPD compared to never-smokers and smokers without airflow limitation. Lymphoid follicles in patients with COPD seem to possess some characteristic features. In order to organize lymphoid follicles, lymphocytes need to be recruited to the lung. In patients with COPD, there is an increased expression of CXCL13 which attracts B cells. In addition, lymphocytes within lymphoid follicles express CXCR3 and the percentage of CXCR3+ cells correlates inversely with the degree of airflow limitation. Furthermore, an increase in expression of CXCL9, CXCL10 and CXCL11 – ligands of CXCR3 – was measured in macrophages, DC and airway epithelium in the
vicinity of the lymphoid follicles, suggesting a role for CXCR3 in lymphocyte recruitment and/or retention in lymphoid follicles in patients with COPD. Recently, Roos et al. measured an elevated level of IL-17A in peripheral lung tissue of patients with COPD compared to non-COPD controls. They found that IL-17A contributes to the formation of lymphoid follicles in end-stage COPD by inducing CXCL12 expression.

Few differences are reported in cellular composition of lymphoid follicles in patients with and without COPD. Plumb et al. observed an increased number of regulatory T cells (Tregs) in lymphoid follicles in patients with COPD compared to control subjects. No differences were detected in Treg count in subepithelial areas and unorganized clusters of lymphocytes, indicating that this augmented Treg count is specific for lymphoid follicles. A possible function of these Tregs in the lymphoid follicles in COPD is mediation of B cell-activity; however, their exact role must yet be elucidated. Lymphoid follicles of patients with COPD also harbor an increased number of CD57+ cells. The function of these cells within the follicles needs to be unraveled as well. Although cellular composition does not substantially alter, two types of lymphoid follicles are described based on the density of apoptotic B cells and BAFF-producing B cells. Compared to mild to moderate COPD, lymphoid follicles in severe COPD harbor less apoptotic B cells and more BAFF-producing B cells.

Although crucial to determine whether these lymphoid follicles are beneficial or harmful in the pathogenesis of COPD, the inducing antigens are still unknown. However, some advances have been made that can help to narrow down the possible suspects. First, using 3D-reconstruction, Mori et al. found that the vast majority of lymphoid follicles are in contact with alveolar epithelium and that this epithelium changes towards a columnar type in severe COPD. At the interface between lymphoid follicles and changed alveolar epithelium, they observed an accumulation CD207+ DC, which are responsible for antigen-capturing in the alveoli. Furthermore, lymphoid follicles are in contact with a vast network of lymphatic vessels. In patients with severe COPD, an increased number of lymphatic vessels are detected in the alveolar parenchyma when compared to never-smokers. Van der Strate et al. described that in patients with emphysema, B cells in lymphoid follicles engage in an oligoclonal, antigen-specific proliferation and the authors did not detect viral nor bacterial nucleic acids. This oligoclonality was recently corroborated by Baraldo et al. who reported this feature in COPD patients with and without α1-antitrypsin deficiency. Finally, a network analysis of lung transcriptomics recently showed a distinct B cell signature and lymphoid follicle formation in COPD patients with emphysema but not in those with obstructive bronchiolitis.
4.2.3 **LYMPHOID NEOGENESIS IN MURINE MODELS OF COPD**

To unravel the inducing factors and cellular and molecular requirements of lymphoid follicle formation in COPD, murine models of the disease are of great use. Hundreds of genetically manipulated strains are available in which a single gene is either over-expressed or knocked-out, making it possible to study the specific role of these genes and their proteins.\(^{251, 252}\)

Exposing wild-type mice chronically (i.e. 3 to 6 months) to CS leads to lymphoid follicle formation in the lungs. The number and size of the follicles increase as the duration of the exposure increases.\(^{242}\) As in patients with COPD, these follicles are situated in peri-bronchial, peri-vascular and parenchymal areas. Using this model, Demoor et al. showed that LTα – although essential in the formation of secondary lymphoid organs - is not necessary for the development of lymphoid follicles in the lung. However, deficiency in LTα resulted in very low IgA levels in bronchoalveolar lavage (BAL) fluid, suggesting a lack of germinal center formation and isotype-switching in absence of LTα. In addition, this paper indicated that stimulation of LTβR in lung fibroblasts resulted in enhanced mRNA expression of CXCL13 and CCL19, supporting the presumption that fibroblasts take on the role of organizer cells in the formation of ectopic follicles.\(^{253-255}\)

Other studies showed that deletion of CCR5 or CCR6 attenuated the formation of CS-induced follicles, whereas CS-exposure of CCR7\(^{-/-}\) mice unexpectedly demonstrated that CCR7 was not required for this process, again in contrast to lymph node organogenesis.\(^{256-258}\) On the other hand, neutralization of CXCL13 by administering a monoclonal anti-CXCL13 antibody to CS-exposed wild-type mice, almost completely abolished formation of organized follicles without substantially affecting the presence of unorganized lymphoid aggregates. As a result, airway inflammation and emphysematous destruction of lung parenchyma were significantly attenuated.\(^{246}\) Finally, a recent study using the CS-induced COPD model, demonstrated a crucial role of interleukin (IL)-17 in CS-induced lymphoid follicle formation. Deletion of IL-17 led to unorganized lymphoid clusters of B cells as opposed to highly organized lymphoid follicles in wild-type mice. This was accompanied by a reduction in the number of CXCL12\(^+\) cells, but did not have an influence on CXCL13 expression.\(^{108}\)

Patients with COPD are more vulnerable to respiratory infections and are often chronically colonized by pathogens, hence murine models using pathogens or pathogen-derived products are highly relevant to study lymphoid follicle formation in COPD.\(^{15, 206}\) Using both intra-nasal instillation with lipopolysaccharide (LPS) and influenza infection, Rangel-Moreno et al. showed that lymphoid follicles can be induced in lungs.
in absence of ILC3-type lymphoid tissue inducer cells. Furthermore, they demonstrated a crucial role for IL-17 in the initiation of lymphoid follicle formation \(^{259}\). Fleige et al. also concluded that IL-17 is important in the development of lymphoid follicles by stimulating stromal cells to produce CXCL12. Additionally, they showed that the induction of lymphoid follicles is pathogen-dependent \(^{260}\). Recently, in a murine model using LPS/elastase treatment, it was illustrated that chronic inflammation results in an altered microbiota in the lungs. Moreover, this paper showed that microbiota enhance inflammation and induce the production of IL-17A by T cells. Neutralizing IL-17A reduced lung compliance, airway inflammation and lymphoid follicle formation \(^{109}\). Finally, although lymphocytes constitute the majority of lymphoid follicles, two different infection models have demonstrated the indispensable role of DC in maintenance of lymphoid follicles \(^{261,262}\).

### 4.2.4 **B cell-activating factor in COPD**

In 2010, Polverino and colleagues reported that BAFF expression was increased in alveolar macrophages and lymphoid follicles of patients with COPD compared to non-COPD controls. Moreover, the number of BAFF-positive macrophages correlated inversely to airflow limitation in patients with COPD \(^{263}\). Recently, this group added that the number of BAFF-positive B cells in blood and in BAL was also significantly higher in patients with COPD compared to control subjects. Furthermore, they observed more lymphoid follicles containing a high percentage BAFF-positive B cells in patients with severe COPD compared to patients with mild/moderate COPD \(^{219}\). BAFF mRNA expression is significantly higher in bronchial epithelium of patients with COPD versus non-COPD subjects as well. In addition, co-culture of healthy B cells with airway epithelium from patients with COPD induced upregulation of TACI in B cells. Conversely, airway epithelium from healthy controls did not cause this effect in B cells \(^{264}\). Evidence of a role for BAFF in COPD was also illustrated in the recent network analysis of lung transcriptomics of patients with COPD, which revealed that BAFF protein was specifically upregulated in lung tissue of patients with emphysema but not in patients with obstructive bronchiolitis \(^{250}\).

![Figure 11: Confocal microscopy image of a BAFF-staining of a lymphoid follicle (COPD patient GOLD stage III). Blue: DAPI. Red: BAFF.](image)
4.3 FUNCTION OF B CELLS, ECTOPIC LYMPHOID FOLLICLES AND B CELL-ACTIVATING FACTOR IN COPD

The respiratory system is in constant contact with the outside world and utilizes mucociliary clearance as a first line defense \(^{40}\). An important component in this system is secretory IgA (sIgA), produced by local plasma cells in the subepithelial areas of airways \(^{265}\). Moyron-Quiroz et al. demonstrated that ectopic lymphoid follicles are capable of mounting a primary immune response against influenza in mice who lack secondary lymphoid organs (SLO), thus driving local Ig production \(^{266}\). Moreover, lymphoid follicles in the lung were shown to be a niche for maintenance of memory cells. This was demonstrated by an effective secondary immune response to influenza in absence of SLOs \(^{267}\). In lungs of patients with COPD, there is an increase in plasma cell number and along with disease severity, lymphoid follicles develop \(^{44, 241}\). As expected, an elevated IgA synthesis is detected in lungs of COPD patients compared to non-COPD subjects \(^{264}\). Interestingly, this does not translate in an increased mucosal IgA immunity due to downregulation of the receptor responsible for translocation of polymeric immunoglobulins to the airway lumen (pIgR) \(^{268}\). Downregulation of pIgR and decrease of sIgA in BAL both correlate with disease severity \(^{268, 269}\).

The role of lymphoid follicles and B cells in COPD is not yet unraveled. It is possible that lymphoid follicles arise from pulmonary infections and that they are essential to generate high affinity antibodies against pathogens. COPD patients with CB are more vulnerable for chronic pulmonary infections. As such, lymphoid follicles might develop as a consequence of CB. On the other hand they might produce antibodies against self-antigens or CS-derived neo-antigens as well. The oligoclonal nature of B cell proliferation in lymphoid follicles in COPD has repeatedly been observed \(^{242, 249}\). Moreover, several reports have described the presence of auto-antibodies in patients with COPD \(^{270-272}\). Interestingly, in a murine model of COPD, the presence of microbiota significantly increased the amount of auto-antibodies \(^{109}\). These studies suggest that lymphoid follicles might well be important in the persistence and progression of inflammation in COPD. In this light it is proposed that the enhanced BAFF production by B cells creates a self-perpetuating loop ensuring survival of B cells and expansion of lymphoid follicles \(^{219}\).

Aside from their essential role in humoral immunity, B cells are important regulators of T cell mediated immunity (Figure 12) \(^{273, 274}\). They do so by presenting antigen to T cells and are in this capacity especially useful when the amount of antigen is limited \(^{204, 274}\). Furthermore, upon stimulation by TLR-ligands or interaction with T cells, B cells produce cytokines that modulate the immune response as well \(^{275}\). Alongside modulation of the T cell response, polarization of macrophages is influenced by cytokine-producing B cells \(^{276, 277}\). In vitro stimulation with CS extract resulted in release of IL-10 by B cells and this
IL-10 stimulated macrophages to produce MMP12. Reports of the importance of B cell-derived cytokines in the pathogenesis of COPD are scarce, therefore requiring further investigation.

Figure 12: B cell function. Aside from antibody production, B cells produce a wide range of cytokines which regulate the immune response. Regulation of immune response includes dendritic cell and T cell regulation and lymphoid follicle formation. Furthermore, B cells are important professional antigen-presenting cells.
PART I: INTRODUCTION

CHAPTER 5: TRANSLATIONAL RESEARCH IN COPD: METHODS
5.1 Translational Research in COPD

Translating research from bench-to-bedside to expedite the search for new therapies is the ultimate goal of translational research (Figure 13). Using genetically manipulated mouse models, it is possible to research the importance of a single gene or molecule in vivo. In wild-type mice, administration of antibodies against a specific molecule can reveal specific functions of that molecule in a disease model. In our translational laboratory, a model has been optimized in which mice are exposed to CS to provoke the development of CS-induced hallmarks of COPD. Results obtained in this murine model are confirmed by ex vivo studies on human lung samples.

Alongside the traditional from bench-to-bedside translation, a third research approach is used to study COPD: population-based research. Here, we use data from a large, prospective cohort study, the Rotterdam Study, to investigate the contribution of symptoms or co-morbidities to clinically important outcomes in subjects with COPD. Furthermore, the impact of having COPD on the vulnerability to other diseases is also studied.

Figure 13: Translational research: the intersection of laboratory research with clinical research (from bench-to-bedside) and the intersection with population-based research.
5.2  **Murine model of COPD**

5.2.1  **Protocol**

In order to elicit the hallmarks of COPD, mice are exposed to CS 4 times a day, with 30 minute smoke-free intervals and for 5 days a week. To administer the CS, mice are placed in a plexiglass chamber which is connected to a smoking apparatus and are subjected to the mainstream smoke of 5 simultaneously lit cigarettes. The plexiglass chamber is supplied with pressured air to obtain a smoke/air ratio of 1/6.

In this dissertation, exposure periods of 4, 8 and 24 weeks were used. Chronic CS exposure of 24 weeks allows investigation of lymphoid follicles and structural changes such as emphysema and airway wall remodeling. In experiments with shorter CS exposure terms, the main goal is to study inflammation.

Experiment endpoints are performed 24 hours after the last CS exposure. Mice are weighed and sacrificed with an overdose of pentobarbital. Next, bronchoalveolar lavage (BAL) is performed and blood and lung tissue are collected. All *in vivo* manipulations were approved by the local Ethics Committee for animal experimentation of the Faculty of Medicine and Health Sciences.

5.2.2  **Advantages and limitations of the CS-induced murine model of COPD**

A major advantage of CS-exposed mice as a model for COPD, is the use of the same etiologic agent as in the human disease. This adds to the relevance of this model compared to animal models which elicit COPD-like symptoms by instillation of 1 agent, for instance elastase and lipopolysaccharide. Furthermore, the use of mice has several advantages compared to other animals, in particular the relative low cost, the existence of hundreds of genetically modified strains and the availability of a substantial number of antibodies and gene sequences. As in patients with COPD, CS induces a neutrophilic inflammation in the airways of mice. Macrophages and CD8+ T cells are also increased in BAL and lung tissue of CS-exposed mice. As in humans, chronic CS exposure induces emphysematous lesions in lung parenchyma and remodeling of small airways in mice.

The most important limitations are the differences in anatomy (less bronchial generations, lack of respiratory bronchioli, submucosal glands are only present near the proximal tracheal rings and few to no goblet cells are present in the lower airways) and the lack of chronic bronchitis symptoms in response to CS. In addition, even after chronic CS exposure (6-8 months or longer), mice do not develop a disease resembling severe or very severe COPD. Finally, CS-exposed mice do not experience acute exacerbations which are a very important aspect of the human disease.
5.2.3 Inflammation

By performing BAL, intra-luminal and alveolar inflammatory cells can be isolated. BAL cells are processed, resulting in cytopsins and cells labelled for flow cytometric analyses. Cytopsins are cytocentrifuged preparations which are stained with May-Grünwald-Giemsa staining (Figure 14). At least 400 cells are counted to obtain a differential cell count.

Figure 14: Cytopsins.
A) Cytopsin of cells isolated via bronchoalveolar lavage (BAL) of an air-exposed mouse. B) Cytopsin of BAL cells of a CS-exposed mouse.
1 = macrophage, 2 = neutrophil, 3 = lymphocyte

By flow cytometric analyses of BAL cells, the percentage of macrophages, neutrophils, dendritic cells, B-lymphocytes and CD4+ and CD8+ T-lymphocytes is assessed. BAL supernatants is used for protein measurements by ELISA or Cytometric Bead Array.

Pulmonary inflammation is assessed by multiple methods. First, a single cell suspension is made of one lobe of the right lung. This single cell suspension is labelled to determine inflammatory cell percentages by flow cytometry (Figure 15). Second, a lobe of the right lung is suspended in RNA later and stored at -80°C to be processed for quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Third, the left lung is embedded in paraffin and subsequently fixed in formalin. Sections of the left lung are used for immunohistochemical stainings of several proteins. Additionally, mediastinal lymph nodes are processed to obtain single cell suspensions and are subsequently labeled for flow cytometric analyses.
Figure 15: Gating strategy for the characterisation of inflammatory cells (lung tissue). A) Selection of single cells. B) FVD = Fixable Viability Dye; cells negative for this dye are alive. C) Selection of CD45+ cells (leukocytes). D) Eosinophils are characterised as SiglecF-positive and CD11c-negative. The rest fraction is further analysed. E) Dendritic cells are defined as CD45+, SiglecF-, MHCII+ and CD11c+. The MHCII- and CD11c- fraction is further analysed. F) Selection of the CD11b-positive cells. G) Neutrophils are characterized as CD45+, SiglecF-, MHCII-, CD11c-, CD11b+, Ly6c± and Ly6G+. Monocytes are CD45+, SiglecF-, MHCII-, CD11c-, CD11b+, Ly6G- and Ly6C+. 
5.2.3 LYMPHOID FOLLICLE FORMATION

Localization and quantification of lymphoid follicles is based on immunohistochemical staining of CD3 (T cell marker)/B220 (B cell marker) on sections of the formalin-fixed, paraffin-embedded left lung (Figure 16). All lymphoid infiltrates in the proximity of airways and in the parenchyma are counted. Dense accumulations of at least 50 cells are defined as lymphoid follicles, whereas accumulations of less than 50 cells are defined as lymphoid aggregates. The number of infiltrates in the proximity of airways is normalized for the number of airways per lung section. Counts of infiltrates in the parenchyma are normalized for the area of lung parenchyma. All measurements are performed in a blinded fashion.

5.2.4 GOBLET CELL METAPLASIA

Goblet cell metaplasia is assessed by staining sections with Periodic acid-Shiff (PAS) (Figure 17). The number of goblet cells are counted with Axiovision software (Zeiss) and are expressed as the number of cells per millimeter basal membrane. Both major mucins in the airways, Muc5ac and Muc5b, have also been stained immunohistochemically on airways and trachea respectively (Figure 17). The gene expression of Muc5ac and Muc5b is quantified by qRT-PCR. All measurements are performed in a blinded fashion.
5.2.5 Emphysema

Airspace enlargement is determined on haematoxylin and eosin stained sections of the left lung by measuring the mean linear intercept (Lm) (Figure 18). Using a Zeiss microscope, photomicrographs of the lung parenchyma are captured. Only images without artefacts, compression, blood vessels and airways are assessed. To measure the Lm, a 100 x 100 µm grid is placed upon the photomicrographs of the parenchyma. Each intercept of alveolar wall with a line of the grid is counted and the total length of each line of the grid is divided by the total number of intercepts on that line. This results in an average distance between two alveolar walls, i.e. the Lm.

Figure 18: Mean linear intercept (Lm). Using Image J software, a grid is placed over photomicrographs. Each intersection of an alveolar wall with a grid line is counted. The length of the grid lines divided by the number of intersections is the mean distance between alveolar walls.

In addition, using the same photomicrographs, destruction of alveolar tissue is measured by the Destructive Index (DI) (Figure 19). For measurement of the DI a grid with 42 points is placed over the lung sections. For each point of the grid it is determined whether the structure underneath the point is normal (N) or destroyed (D). The DI is calculated as follows: DI = D/(D+N) x 100. All measurements are performed in a blinded fashion.

Figure 19: Destructive Index (DI). Using Image J software, a grid of 42 points is placed over the photomicrographs. N = normal, D = destroyed. DI = D/(D+N) x 100
5.2.6 Airway wall remodeling

Airway wall remodeling is assessed by measuring the deposition of collagen and fibronectin. Collagen is stained with Sirius Red, whereas fibronectin is stained immunohistochemically with anti-fibronectin antibody. By using the Axiovision software, the area between the basal membrane and the adventitial perimeter that stains positive for collagen or fibronectin is calculated and normalized for the length of the basal membrane (Figure 20). All airways with a basal membrane length less than 2000 µm and cut in reasonable cross sections are included in the analyses. All measurements are performed in a blinded fashion.

5.2.7 Lung function measurements

Lung function measurements are assessed using the Flexivent System (SCIREQ, Montreal, Canada). For baseline lung function measurements the ‘snapshot’ perturbation, the forced oscillation (Quick Prime 3) perturbation and pressure-volume loops are performed, so that static and dynamic compliance, resistance, elastance, total lung capacity and hysteresis are determined. Lung function measurements are performed on anaesthetized and tracheostomised mice, which received a neuromuscular blocker (pancuronium bromide (1mg/kg)).
5.3 Human Studies

Ex vivo studies are performed on human lung tissue from patients undergoing lung resection surgery at the Ghent University Hospital. Diagnosis of COPD and severity assessment is made based on pre-operative spirometry according to the GOLD classification. All participants provide a written informed consent according to protocols approved by the medical ethical committee of the Ghent University Hospital. Exclusion criteria for participation are treatment with neo-adjuvant chemotherapy, tuberculosis, recent COPD exacerbations and recent pulmonary infections. All participants are interviewed on their smoking habits and medication use. Ex-smokers are defined as having quit for at least one year.

Lung tissue is collected by a pathologist at maximum distance from the pulmonary lesions and without signs of retro-obstructive pneumonia or tumor invasion. Small pieces of the resection samples are submersed in RNA-later and stored at -80°C until RNA extraction and subsequent qRT-PCR analyses. In addition, tissue blocks from the resection samples are snap-frozen in liquid nitrogen and stored at -80°C. This tissue is used for protein detection by ELISA. A final set of tissue blocks are fixed in paraformaldehyde and embedded in paraffin. Sections of these blocks are used for immunohistochemical staining (Figure 21).

Figure 21: Protocol for the collection of human lung tissue. Lung tissue from resection surgery is collected by a pathologist. This tissue is used for immunohistochemical analyses of proteins, qRT-PCR analyses of mRNA expression and protein measurement via ELISA. Adapted from F. Verhamme.
5.4 The Rotterdam Study

In order to investigate the impact of chronic bronchitis on clinically important outcomes in subjects with COPD, we analyzed data from the Rotterdam Study (Figure 22). This is a large, prospective, population-based study, situated in the city of Rotterdam, the Netherlands. The study started in 1990 when all residents from the district of Ommoord, aged 55 or older, were invited to participate (first cohort). In 2000 and in 2006, residents who had become 55 and 45 years respectively, were invited to join the study (second and third cohort). Follow-up examinations occur approximately every 4 to 5 years. Data is collected from home interviews and a set of physical examinations performed is a designated research building. In addition, medical and pharmacy records are linked to the study.

Figure 22: The Rotterdam Study. The green blocks represent examination cycles of the first cohort which enrolled in 1989-1993. The second cohort was asked to participate in 2000 and the examination cycles of this cohort is represented by yellow blocks. A third cohort was enrolled in 2006 and they have been invited for an examination twice, represented by the two purple blocks. Adapted from Hofman et al. Eur J Epidemiol. 2015;30(8):661-708.
PART II: RESEARCH WORK

CHAPTER 6: RESEARCH OBJECTIVES
RESEARCH OBJECTIVES

Worldwide, COPD affects more than 200 million people, imposing a huge burden of disease. Noxious particles and gases entering the airways cause the characteristic airway inflammation, mucus hypersecretion, airway wall remodelling and emphysema in susceptible individuals. The pathogenesis of COPD is multifaceted and not fully understood. Bearing in mind that there is currently no cure for COPD, an improved comprehension of the pathogenesis could aid in the search for new therapeutic targets. In this research we have applied a translational approach combining functional studies in murine models with ex vivo studies on human lung resection tissue and population-based research embedded in the Rotterdam Study.

We aimed to investigate the role of mucociliary dysfunction in COPD in two separate studies. Mucociliary impairment arises primarily from dehydration of the airway surface, originating either from excess production of mucins and non-mucin proteins, dysfunction of ion channels or both.

First, the role of airway surface dehydration in the pathogenesis of COPD has not been determined in vivo. Therefore, we studied the effect of airway surface dehydration on the hallmarks of COPD in βENaC-Tg mice, a mouse model for airway surface dehydration, and exposed these mice and their wild-type littermates to air or CS.

Research question 1: Does airway surface dehydration aggravate cigarette smoke-induced inflammation and structural changes in a mouse model of COPD?

Next, airway surface dehydration is associated with chronic bronchitis and increases the risk for airway infections. In addition, airway infections are a major cause of COPD exacerbations. Therefore, we wondered whether subjects with COPD and concomitant chronic bronchitis exhibit an increased risk for exacerbations and mortality, two clinically relevant outcomes. Since literature renders inconsistent reports on this subject, we aimed to investigate the exacerbation and mortality risk in COPD subjects with and without chronic bronchitis in a large prospective population-based cohort, the Rotterdam Study.

Research question 2: Does the presence of chronic bronchitis affect the exacerbation and mortality risk in subjects with COPD?
We also used a translational approach to continue our research on the role of B cell-rich lymphoid follicles in COPD, by investigating the role of B cell activating factor (BAFF) in the pathogenesis of COPD. In COPD, an increased number of B cells and airways with lymphoid follicles are associated with disease severity. In addition, the level of BAFF in these lymphoid follicles is associated with disease severity as well. We hypothesised that the presence of BAFF and B cells contributes to the pathological hallmarks of COPD. For this research, we investigated the functional role of BAFF in a mouse model of CS-induced pathology. Furthermore, we aimed to investigate the expression of BAFF in patients with COPD compared to control patients and the localisation of BAFF within lymphoid follicles in patients with COPD.

**Research question 3:** Does antagonizing B cell activating factor attenuate the hallmarks of cigarette smoke-induced pulmonary disease in a murine model of COPD?
PART II: RESEARCH WORK

CHAPTER 7: AIRWAY SURFACE DEHYDRATION AGGRAVATES CIGARETTE SMOKE-INDUCED HALLMARKS OF COPD IN MICE

Imbalance of ion secretion and absorption contributes to airway surface dehydration which leads to mucociliary dysfunction. Research shows that airway surface dehydration is present in smokers with and without COPD. However, the role of airway surface dehydration in the pathogenesis of COPD has not been studied in vivo.

**ABSTRACT**

**Introduction:** Airway surface dehydration, caused by an imbalance between secretion and absorption of ions and fluid across the epithelium and/or increased epithelial mucin secretion, impairs mucociliary clearance. Recent evidence suggests that this mechanism may be implicated in chronic obstructive pulmonary disease (COPD). However, the role of airway surface dehydration in the pathogenesis of cigarette smoke (CS)-induced COPD remains unknown.

**Objective:** We aimed to investigate *in vivo* the effect of airway surface dehydration on several CS-induced hallmarks of COPD in mice with airway-specific overexpression of the β-subunit of the epithelial Na⁺ channel (βENaC).

**Methods:** βENaC-Tg mice and wild-type (WT) littermates were exposed to air or CS for 4 or 8 weeks. Pathological hallmarks of COPD, including goblet cell metaplasia, mucin expression, pulmonary inflammation, lymphoid follicles, emphysema and airway wall remodelling were determined and lung function was measured.

**Results:** Airway surface dehydration in βENaC-Tg mice aggravated CS-induced airway inflammation, mucin expression and destruction of alveolar walls and accelerated the formation of pulmonary lymphoid follicles. Moreover, lung function measurements demonstrated an increased compliance and total lung capacity and a lower resistance and hysteresis in βENaC-Tg mice, compared to WT mice. CS exposure further altered lung function measurements.

**Conclusions:** We conclude that airway surface dehydration is a risk factor that aggravates CS-induced hallmarks of COPD.
INTRODUCTION

Efficient mucociliary clearance is an essential innate defence mechanism of the lung. Although ciliary activity and mucus secretion play an important role in airway mucus clearance, evidence from biophysical studies indicates that the hydration state of the airway surface is the key determinant. While airway surface dehydration is a well-established disease mechanism in cystic fibrosis, recent research suggests that this abnormality may also play a role in chronic obstructive pulmonary disease (COPD). Pathologically, COPD is mainly caused by cigarette smoking and characterized by mucus obstruction of the small airways, chronic pulmonary inflammation, obstructive bronchiolitis and emphysema.

Several studies demonstrated that cigarette smoke (CS) has detrimental effects on the hydration of airway surfaces. First, it was shown that CS affects ion channels in the apical membrane of airway epithelial cells, thereby disturbing the balance between Na⁺ absorption and Cl⁻ secretion and leading to airway surface dehydration. Most notably, CS induces an acquired deficiency of the cystic fibrosis transmembrane conductance regulator (CFTR), a crucial cAMP-dependent Cl⁻ channel that is mutated in cystic fibrosis. In chronic smokers, CFTR function is reduced to ~45% of normal and mucus is hyperconcentrated in vivo. This acquired CFTR dysfunction contributes to inadequate mucociliary transport and is associated with chronic bronchitis and dyspnoea in smokers with and without COPD. Furthermore, exposure to CS extract enhances the activity of the epithelial Na⁺ channel (ENaC) in alveolar type I and type II cells, suggesting that CS exposure results in a hyposecretory/hyperabsorptive ion transport phenotype. Along these lines, recent studies on the protein levels of CFTR and ENaC in lung tissue of COPD patients demonstrated that CFTR levels were positively correlated with lung function, whereas levels of α- and βENaC showed a negative correlation with lung function. In mice, an imbalance between Na⁺ absorption and Cl⁻ secretion, has been achieved in βENaC transgenic (βENaC-Tg) mice. In these mice, airway-specific overexpression of βENaC causes constitutive airway surface dehydration and spontaneous chronic obstructive lung disease characterized by airway mucus obstruction, neutrophilic inflammation and development of emphysema early in life.

A second mechanism by which CS can contribute to airway surface dehydration, is the CS-induced mucin hypersecretion. The two major secreted mucins in airways, MUC5AC and MUC5B, are both increased in patients with COPD. These mucin macromolecules are secreted in a dry form and thus increase the concentration of the mucus gel layer if the availability of airway surface fluid is limited. In their gel-on-brush model, Button et al. recently showed that an increased concentration of mucins
causes an increase in the osmotic pressure of the mucus gel layer and, above a certain threshold, this causes a compression of the subjacent periciliary layer, leading to a collapse of the cilia and mucostasis. Since observational evidence indicates that there is a degree of airway surface dehydration in patients with COPD, this study aimed to investigate the in vivo effect of airway surface dehydration on several pathological hallmarks of COPD and on lung function. To achieve this goal, we exposed βENaC-Tg mice and wild-type (WT) littermates to air or CS for 4 or 8 weeks and determined mucin expression, goblet cell metaplasia, pulmonary inflammation, lymphoid follicles, pulmonary emphysema and airway wall remodelling, and measured lung function.
METHODS

Details of materials and methods used can be found in the online supplement.

Primary tracheal epithelial cultures
For each experiment, freshly excised tracheae were collected and pooled from 10 mice per group. Epithelial cells were isolated and cultured on membranes (T-Col, Costar, Cambridge, MA) under air-liquid interface conditions as described previously 290, and cultures were studied after reaching confluence (14 days).

Measurement of airway surface liquid height
Primary tracheal epithelial cultures were washed with PBS, and 20 µl of PBS containing 2 mg/ml Rhodamine dextran (10 kDa; Molecular Probes) was added to the lumen to visualize the airway surface liquid layer. To avoid evaporation of the ASL, 80 µl of immiscible perfluorocarbon (Fluorinert-77, Sigma-Aldrich) was added to the airway surface following the addition of the labeling dye. Images of the Rhodamine-labeled airway surface liquid were acquired by confocal microscopy (Leica TCS SP8, Leica Microsystems, Mannheim, Germany). The height of the airway surface liquid was measured by averaging the heights obtained from xz scans of sixteen predetermined positions on the culture as previously described 290. Airway surface liquid height was measured 5 min following the addition of the Rhodamine dextran and at designated time points over a period of 24 h in primary tracheal epithelial cultures from βENaC-Tg mice and WT littermates.

Animals
Male βENaC-Tg mice, backcrossed onto the C57Bl/6 background 178, were mated with female C57Bl/6Cr1 wild-type (WT) mice (Charles River). All mice were bred in the animal facility of the Ghent University Hospital and housed in filtertop cages in standard conditions under a 12 h light-dark cycle and provided with a standard diet (Pavan, Brussels, Belgium) and chlorinated tap water ad libitum. Mice were euthanized with an overdose of pentobarbital (Sanofi, Libourn, France). All in vivo manipulations were approved by the local Ethics Committee for animal experimentation of the Faculty of Medicine and Health Sciences (Ghent University).

Cigarette smoke exposure
Groups of 8 to 11 βENaC-Tg mice and WT littermates were exposed whole body to cigarette smoke as described before (a total of 120 mice was used) 257. In short, mice were exposed 5 days a week to the mainstream cigarette smoke of 5 cigarettes (Reference cigarette 3R4F without filter, University of
Kentucky, Lexington, KY, USA), 4 times a day with a 30 minute smoke free interval between exposures. A standard smoking apparatus was used with the smoking chamber adapted for a group of mice. A smoke/air ratio of 1/6 was obtained. Control mice were exposed to room air. CS exposure started at an age of 7-8 weeks and the exposure period was either 4 or 8 weeks.

**βENaC immunohistochemistry**

Lung sections were evaluated for overexpression of the β-subunit of ENaC, using a rabbit polyclonal anti-βENaC antibody.

**Goblet cell analysis and mucin gene expression**

Transversal sections were made from the paraffin-embedded left lung and were stained with Periodic acid-Schiff (PAS). Goblet cells were counted using Axiosvision software (Zeiss) and were expressed as number of cells per millimetre basal membrane. Expression of Muc5ac and Muc5b was quantified by quantitative real-time polymerase chain reaction (qRT-PCR).

**Pulmonary inflammation**

Bronchoalveolar lavage (BAL) fluid was collected via a tracheal cannula. Differential cell counts of the lavage fluid were obtained by cytocentrifuged preparations after May-Grünwald-Giemsa staining. Flow cytometric analysis was used for quantifying inflammatory cell types in BAL fluid and single cell suspensions of lung tissue. qRT-PCR was used to evaluate the expression of several chemokines. The protein levels of Cxcl1 and Ccl20 in BAL fluid supernatant of mice were determined with an ELISA kit (R&D systems).

**Lymphoid follicles**

In order to quantify lymphoid follicles, defined as dense accumulations of at least 50 lymphocytes, paraffin-embedded sections of the left lung were immunohistochemically stained with anti-CD3 (Dako) and anti-B220 (BD biosciences). The number of follicles was normalized for the total area of parenchyma that was scored.

**Emphysema**

In order to evaluate pulmonary emphysema, two complementary methods were used, the mean linear intercept (Lm) and the destructive index (DI). The Lm is a measurement of alveolar space enlargement whereas the DI is a calculation of the percentage of destroyed alveolar walls. Both analyses were performed using the Image J software on haematoxylin and eosin (H&E) stained lung sections.
Airway wall remodelling

To evaluate the deposition of fibronectin and collagen, paraffin-embedded sections of the left lung were used for immunohistological staining. Fibronectin was stained with mouse anti-fibronectin (Thermo Scientific). Collagen was stained chemically with Sirius Red. The amount of collagen and fibronectin in the airway wall was quantified using the Axiovision software (Zeiss).

Lung function measurements

Using the Flexivent System (SCIREQ, Montreal, Canada), baseline lung function was examined invasively in tracheostomised anaesthetized mice. The jugular vein was used to administer pancuronium bromide (1 mg/kg) (Inresa, Freiburg, Germany), which induces a neuromuscular blockade. The mice were ventilated with an average breathing frequency of 150 breaths/minute. Once the mice were stable, resistance (R) and dynamic compliance (C\text{dyn}) were measured using a ‘snapshot perturbation’ manoeuvre. The forced oscillation perturbation (Quick Prime 3) was applied to assess the tissue damping (G). Pressure-volume (PV) loops were generated to measure the static compliance (C\text{stat}), total lung capacity (TLC) and hysteresis.

Statistical analyses

Sigma Stat Software (SPSS 21.0, Chicago, IL, USA) was used to perform non-parametric tests (Kruskall-Wallis and Mann-Whitney-U). Reported values are expressed as mean ± SEM. P-values < 0.05 were considered to be significant.
RESULTS

Overexpression of βENaC and reduced airway surface liquid height in βENaC-Tg mice

To confirm the overexpression of the β-subunit of ENaC in βENaC-Tg mice, we performed an immunohistochemical staining for βENaC on lung tissue sections from WT and βENaC-Tg mice. βENaC-positive cells were readily detected in conducting airways and alveoli in lungs of WT mice (Fig. 1 A). The intensity of the immunoreactive signal was substantially stronger in airways from βENaC-Tg compared to WT mice, consistent with a marked increase in βENaC protein in epithelial cells (Fig. 1 A).

Figure 1. Overexpression of βENaC and reduced airway surface liquid height in βENaC-Tg mice.
Immunolocalization of βENaC in airways from WT and βENaC-Tg mice. (A) Representative images of βENaC immunostaining of lung sections from WT and βENaC-Tg mice that were exposed to air or CS for 8 weeks. n = 5 per group. Dysregulation of steady state airway surface liquid (ASL) height on airway epithelia from βENaC-Tg mice under thin film conditions. Representative confocal images (B) and summary of measurements of airway surface liquid height (C) at t = 0, 2, 4, 8 and 24h after mucosal addition of 20 µl of PBS containing Rhodamine dextran to primary tracheal epithelial cultures from βENaC-Tg mice and WT littermates. Scale bar, 7 µm. n = 4 experiments per group.*p<0.001 compared to βENaC-Tg; §p<0.001 for t = 0h compared to all other time points within the same genotype; †p<0.05 for t = 24h wild-type compared to t = 2h wild-type; ‡p<0.005 for t = 24h wild-type compared to t = 4h and 8h wild-type.
To determine the effects of βENaC overexpression on the regulation of airway surface liquid, primary tracheal epithelial cell cultures of WT and βENaC-Tg mice grown at an air-liquid-interface were challenged with a small volume of liquid added to the luminal compartment. Airway surface liquid height was monitored sequentially by confocal microscopy over a period of 24h. Within 2h after the initial volume challenge, the airway surface liquid was absorbed to a height of ~4.5 µm in both WT and βENaC-Tg mice (Fig. 1 B-C). However, at 24h, airway surface liquid height increased to ~6.3 µm in airway cultures from WT mice, whereas it remained significantly reduced in βENaC-Tg mice (Fig. 1 B-C). Consistent with previous studies, these results demonstrate that βENaC-Tg airway epithelia fail to regulate airway surface liquid to normal levels, and that steady state airway surface liquid is reduced in βENaC-Tg compared to WT mice.

Cigarette smoke-induced mucin expression is aggravated in βENaC-Tg mice

The expression of Muc5ac and Muc5b was quantified in total lung tissue by qRT-PCR. The airway surface dehydration in air-exposed βENaC-Tg mice resulted in a higher expression of both Muc5ac and Muc5b, compared to WT mice (Fig. 2 A-B). Four weeks of CS exposure significantly increased the expression of Muc5ac in βENaC-Tg mice, but not in WT mice (Fig. 2 A). In contrast, CS exposure did not induce a significant upregulation of Muc5b expression in lung, neither in βENaC-Tg mice nor in WT mice (Fig. 2 B). Similar results were obtained after 8 weeks of CS exposure (Suppl. Fig. S1).

Figure 2. Cigarette smoke-induced mucin expression is increased in βENaC-Tg mice.

mRNA expression of Muc5ac (A) and Muc5b (B) in total lung tissue upon 4 weeks of air or CS exposure. mRNA expression data were normalized for 3 reference genes (Hprt1, Gapdh, Tfrc). n = 6/group
*p<0.05, **p<0.01, ***p<0.001

Goblet cell metaplasia was assessed by quantifying the number of periodic-acid-Schiff positive (PAS+) cells in the bronchial epithelium. Air-exposed βENaC-Tg mice had higher numbers of PAS+ cells compared to WT mice. However, 4 weeks of CS exposure did not induce an increase in PAS+ cells, neither in WT nor in
\(\beta\text{ENaC-Tg mice (Fig. 3 A-C).}\) In a subgroup of animals (n=3/group), PAS+ mucus content was measured in the airway lumen of non-lavaged mice following 4 weeks of air or CS exposure. Similar to goblet cell metaplasia, there was more mucus present in the airway lumen of \(\beta\text{ENaC-Tg mice, compared to WT controls independent of CS exposure (Fig. 3 D-F).}\) Quantification of goblet cell metaplasia after 8 weeks of CS exposure, resulted in a similar outcome (Suppl. Fig. S1).

*Figure 3. Goblet cell metaplasia and mucus secretion upon air or CS exposure.*

(A) Goblet cell count upon 4 weeks of air or CS exposure. n=8/group. Representative images of goblet cells in airways of CS-exposed WT mice (B) and CS-exposed \(\beta\text{ENaC-Tg mice (C) upon 4 weeks of CS exposure. Arrows indicate goblet cells. (D) Quantification of PAS+ mucus content in lumen of airways of non-lavaged mice upon 4 weeks of air or CS exposure n=3/group. Representative image of PAS+ mucus content in airways of CS-exposed WT mice (E) and CS-exposed \(\beta\text{ENaC-Tg mice (F).}\) *\(p<0.05, **p<0.01, ***p<0.001\)*

*Cigarette smoke-induced pulmonary inflammation is aggravated in \(\beta\text{ENaC-Tg mice}*

Following air exposure, the numbers of inflammatory cells in BAL fluid were significantly increased in \(\beta\text{ENaC-Tg compared to WT mice. Four weeks of CS exposure led to a significant increase in the number of total BAL cells, macrophages, neutrophils and lymphocytes, both in WT and \(\beta\text{ENaC-Tg mice (Fig. 4 A-D).}\) Importantly, this increase in innate and adaptive immune cells was significantly higher in \(\beta\text{ENaC-Tg mice, compared to WT mice (Fig. 4 A-D). Additionally, 4 weeks of CS exposure significantly increased the number*
of macrophages in the lungs of βENaC-Tg and WT mice (Fig. 4 E), but had no effect on the number of neutrophils, dendritic cells and CD4+ and CD8+ T-lymphocytes (data not shown). Interestingly, the CS-induced increase in macrophages in lung tissue was aggravated in βENaC-Tg mice, compared to WT controls (Fig. 4 E).

Similar results were obtained after 8 weeks of CS exposure (Suppl. Fig. S2). Following 8 weeks of CS exposure, additional cell types were quantified. While the CS-induced increase in dendritic cells and CD4+ T-lymphocytes in BAL did not differ in WT and β-ENaC (Fig. 4 F-H), the increase in CD8+ T-lymphocytes was significantly aggravated in CS-exposed βENaC-Tg mice, compared to WT littermates (Fig. 4 H). Quantification of inflammatory chemokines revealed higher mRNA expression of Cxcl1 and Ccl20 in lung tissue of air-exposed βENaC-Tg mice, compared to WT littermates (Fig. 5 A-D). The CS-induced increase in
pulmonary Ccl20 mRNA expression was aggravated in βENaC-Tg mice, especially upon 8 weeks of CS exposure (Fig. 5 A-D). Protein levels of Cxcl1 and Ccl20 in BAL fluid were significantly higher in βENaC-Tg mice, compared to WT littermates (Fig. 5 E-H). Moreover, the CS-induced increase in Cxcl1 protein levels in BAL was significantly aggravated in βENaC-Tg mice (Fig. 5 E).

Figure 5. mRNA expression and protein levels of chemokines upon air or CS exposure. mRNA expression of Cxcl1 in total lung tissue upon 4 weeks (A) and 8 weeks (C) air or CS exposure. mRNA expression of Ccl20 in total lung tissue upon 4 weeks (B) and 8 weeks (D) air or CS exposure. mRNA expression data were normalized for 3 reference genes (Hprt1, Gapdh, Tfrc). n = 6-8/group. Protein levels of Cxcl1 in BAL fluid upon 4 weeks (E) and 8 weeks (G) air or CS exposure. Protein levels of Ccl20 in BAL fluid upon 4 weeks (B) and 8 weeks (D) air or CS exposure. Protein levels were measured by ELISA. n = 8-11/group. *p<0.05, **p<0.01, ***p<0.001

Cigarette smoke-induced formation of lymphoid follicles is accelerated in βENaC-Tg mice

Eight weeks of CS exposure did not induce lymphoid follicles in WT mice. In contrast, after 8 weeks of CS exposure, the formation of lymphoid follicles was already detected in βENaC-Tg mice (Fig. 6). Whereas lymphoid follicle formation upon chronic CS exposure (i.e. 6 months) was shown to be associated with
elevated expression of Cxcl13 in WT mice, transcript levels of this chemokine were not increased following 8 weeks of CS-exposure in βENaC-Tg mice (data not shown).

**Figure 6. Cigarette smoke-induced lymphoid follicle formation in βENaC-tg, but not in WT mice after 8 weeks of CS exposure.**

(A) Quantification of lymphoid follicles normalized for the area of parenchyma (mm²) upon 8 weeks of air or CS exposure. n = 8-11/group. (B-C) Representative images of lymphoid follicles found in CS-exposed βENaC-Tg mice. *p<0.05, **p<0.01, ***p<0.001

**Cigarette smoke-induced alveolar destruction is aggravated in βENaC-Tg mice**

Emphysema was measured by two complementary methods. The mean linear intercept (Lm), a measure for alveolar airspace enlargement, was significantly enlarged in the βENaC-Tg mice compared to WT littermates (Fig. 7 A). However, 4 weeks of CS exposure did not further increase the Lm, neither in the βENaC-Tg mice nor in the WT controls (Fig. 7 A). Emphysema was also quantified by determining the destructive index (DI). This measure quantifies the percentage of destruction of the alveolar walls. The DI tended to be higher in air-exposed βENaC-Tg compared to WT mice (Fig. 7 D). Importantly, 4 weeks of CS exposure did not result in an increased DI in WT mice, but did induce a significantly increased level of destruction in βENaC-Tg mice. (Fig. 7 D). Similar results were obtained after 8 weeks of CS exposure (Suppl. Fig. S3). Interestingly, Mmp12 mRNA expression was higher in lungs of βENaC-Tg mice compared to WT littermates (Fig. 7 G).

**Cigarette smoke does not induce airway wall remodelling in βENaC-Tg mice**

We investigated airway wall remodelling in mice by measuring the amount of fibronectin and collagen deposited in the airway walls. Fibronectin and collagen deposition in the airway walls did not differ between WT and βENaC-Tg mice and was not affected by 4 or 8 weeks of CS exposure (Suppl. Fig. S4).
Cigarette smoke-induced changes in lung function in WT and βENaC-Tg mice

We assessed the pulmonary function of WT and βENaC-Tg mice after 4 weeks of exposure to air or CS. Air-exposed βENaC-Tg mice exhibited a lower total pulmonary resistance, compared to air-exposed WT mice (Fig. 8 A). CS exposure did not influence the total pulmonary resistance, neither in WT nor in βENaC-Tg mice (Fig. 8 A). However, CS exposure significantly decreased the tissue damping in βENaC-Tg mice, whereas CS exposure of WT mice resulted in an increased tissue damping (Fig. 8 B). This parameter is used to assess the tissue resistance. In addition, the tissue elasticity was significantly lower in CS-exposed βENaC-Tg mice, compared to CS-exposed WT mice (Fig. 8 C).

A significantly increased static and dynamic compliance was registered in βENaC-Tg mice compared to WT mice, consistent with the increase in mean linear intercept measured in βENaC-Tg mice (Fig. 8 A). Whereas CS exposure induced a significant decrease of both compliances in WT mice, CS exposure had no significant
effect on the elevated static and dynamic compliance in the βENaC-Tg mice (Fig. 8 D-E). The total lung capacity (TLC) was significantly higher in βENaC-Tg compared to WT mice. CS exposure induced a decrease in TLC in WT mice, but did not influence this parameter in βENaC-Tg mice (Fig. 8 F). Analysis of the PV-loops demonstrated that the hysteresis, i.e. area between inflating and deflating part of the PV-loop, was significantly decreased in air-exposed βENaC-Tg mice compared to WT mice (Fig. 9 A-C). Exposing mice to CS, decreased the hysteresis both in WT and in βENaC-Tg mice. Of note, hysteresis was significantly lower in CS-exposed βENaC-Tg mice compared to CS-exposed WT mice (Fig. 9 C).

Figure 8. Effect of CS exposure on lung function in WT and βENaC-Tg mice.
Lung function was determined in WT and βENaC-Tg mice after exposure to air or CS for 4 weeks. (A) Resistance (R) of the entire compartment (airways, tissue and chest wall). (B) Tissue damping (G), related to tissue resistance. (C) Tissue elasticity (H). (D) Static compliance (C_{stat}). (E) Dynamic compliance (C_{dyn}). (F) Total lung capacity (TLC). *p<0.05, **p<0.01, ***p<0.001
Figure 9. Pressure-Volume loops of air- and CS-exposed mice.
(A) Representative PV loops of air-exposed WT (black) and βENaC-Tg (red) mice. (B) Representative PV loops of CS-exposed WT (black) and βENaC-Tg (red) mice. (C) Area of PV loop or hysteresis.
*p<0.05, **p<0.01, ***p<0.001
DISCUSSION

There is increasing evidence of airway surface dehydration in smokers and patients with COPD. This study demonstrates that airway surface dehydration in βENaC-Tg mice aggravates CS-induced airway inflammation, mucin expression and destruction of alveolar walls and accelerates the formation of pulmonary lymphoid follicles. In contrast, CS exposure did not induce airway wall remodelling and had no effect on goblet cell metaplasia in βENaC-Tg mice.

It has been demonstrated that CS suppresses the function of the CFTR channel, with airway surface dehydration and decreased mucociliary clearance as consequences. Moreover, Dransfield et al. showed that a diminished CFTR function correlates with the presence of chronic bronchitis and the degree of dyspnoea. Since Cftr-deficient mice do not exhibit imbalanced airway ion transport in the lower airways and do not display cystic fibrosis-like disease, we used βENaC-Tg mice, backcrossed onto a C57Bl/6 background, to test the hypothesis whether the presence of airway surface dehydration has an impact on CS-induced pathology and pathophysiology in vivo. βENaC-Tg mice overexpress the β-subunit of ENaC through an airway-specific cub cell secretory protein (CCSP) promoter. The constitutive hyperabsorption of Na+ leads to airway surface dehydration and decreased mucociliary clearance.

Besides an imbalance of epithelial ion transport, mucus hypersecretion may contribute to airway surface dehydration and mucociliary dysfunction. As Button et al. elegantly showed in their gel-on-brush model, a hyperconcentrated mucus gel layer with > 8% solids can create sufficient osmotic pressure to cause a collapse of the cilia and mucostasis. Clunes et al. found that mucus of chronic cigarette smokers contained approximately 10% solids, which makes it likely that osmotic pressure of the hyperconcentrated mucus layer contributes to mucociliary dysfunction in smokers. The predominant secreted mucins in airway mucus are MUC5AC and MUC5B and both are upregulated in COPD. Consistent with previous studies, we observed higher transcript levels of Muc5ac and Muc5b and a significant higher number of goblet cells in βENaC-Tg mice compared to WT mice. Although the Muc5b mRNA expression tended to be increased after 4 weeks of CS exposure, this increase did not reach significance. In contrast, CS induced a significant increase of Muc5ac expression in the βENaC-Tg mice, both after 4 and 8 weeks of CS exposure. Since mice do not readily develop goblet cell metaplasia upon CS exposure, we did not observe an increase in goblet cell metaplasia after CS exposure, neither in WT nor in βENaC-Tg mice. However, substantial mucin hypersecretion has been detected without increase in goblet cell numbers. It has been suggested that Clara cells produce and secrete Muc5ac without being subjected to metaplastic changes, including the formation of PAS positive storage granules. Taken together, our results are
consistent with the observation that Muc5ac is highly inducible by noxious stimuli and suggest that hypersecretion of Muc5ac in response to CS exposure may contribute to mucus hyperconcentration and airway surface dehydration.

Airway surface dehydration produces chronic airway inflammation in BAL in βENaC-Tg mice, with higher numbers of macrophages, neutrophils and lymphocytes compared to WT littermates. Our study confirms and extends these findings by showing that BAL of βENaC-Tg mice also exhibits more dendritic cells and CD8+ T-lymphocytes. By exposing both WT littermates and βENaC-Tg mice to CS, we demonstrated that airway surface dehydration was associated with an aggravated CS-induced inflammatory response in βENaC-Tg mice. In BAL, CS exposure induced a significantly higher increase in macrophages, neutrophils and CD8+ T-lymphocytes, key players in COPD pathophysiology, in βENaC-Tg mice than in WT mice. Interestingly, this exaggerated response already occurred after a subacute CS exposure, i.e. 4 weeks.

In lung tissue, the CS-induced inflammatory response was limited to an increase in macrophages. Along with this increase in macrophage numbers, we observed an increase in matrix metalloproteinase 12 (Mmp12), a macrophage-derived protease important in emphysema development in WT and βENaC-Tg mice.

Severe COPD is associated with increased numbers of airways containing lymphoid follicles. Moreover, the presence of lymphoid follicles has also been demonstrated in the lung parenchyma of patients with COPD. In WT mice, lymphoid follicles are usually detected after 6 months of CS exposure. In this study, lymphoid follicles were already detected in βENaC-Tg mice after 8 weeks of CS exposure, suggesting that airway surface dehydration accelerates the CS-induced formation of lymphoid follicles. Since we did not observe a dominance of B cells in these lymphoid follicles, nor an upregulation in total lung tissue of Cxcl13 transcript levels, a chemokine involved in lymphoid follicle formation upon chronic CS exposure, we speculate that we observed early stages of follicle formation. Importantly, we did not find evidence of pulmonary infection.

In COPD patients, emphysema develops after many years of cigarette smoking and it consistently requires several months of CS exposure, i.e. 6 months, to evoke emphysema in WT mice. In contrast, βENaC-Tg mice develop severe emphysema early in life. Similar elements that lead to the onset of emphysema, can be found in both βENaC-Tg mice and patients with COPD. First, in COPD, a proteinase/antiproteinase imbalance plays a key role in the development of emphysema. Recently, it has been shown that Mmp12 and neutrophil elastase also mediate emphysema in βENaC-Tg mice. Second, McDonough et al. showed that small airway obstruction precedes emphysematous destruction in
COPD patients. In βENaC-Tg mice, airway surface dehydration leads to mucus obstruction in the first days of life and preceding the onset of emphysema, constituting another similarity to COPD patients. In this study, we confirmed severe emphysema in air-exposed βENaC-Tg mice, as quantified by the mean linear intercept (Lm). However, we did not find a further increase in Lm in the βENaC-Tg mice following CS exposure. Given the severity of the constitutive emphysema in adult βENaC-Tg mice, it can be questioned whether it is at all possible to establish further enlargement of the Lm upon CS exposure. In contrast, quantifying the destructive index (DI), a measure for alveolar destruction, clearly showed that 4 weeks of CS exposure already induced significant destruction of alveolar tissue in βENaC-Tg mice, while there was not yet an effect in WT mice. We also performed lung function measurements and observed a decreased resistance and increased compliance and TLC, indicating that the lung function is dominated by the severe spontaneous emphysema in βENaC-Tg mice. Interestingly, following CS exposure, the increased compliance measured in βENaC-Tg mice was accompanied by a decreased tissue damping and tissue elasticity. Together with the morphometric analysis of the DI, these data demonstrate that airway surface dehydration aggravated CS-induced emphysema, even after short term exposure, suggesting that this mechanism may contribute to emphysema formation in smokers with COPD.

This study suggests that therapeutic targeting of airway surface dehydration may be beneficial in patients with COPD, although extrapolation of our experimental findings in mice after (sub)acute cigarette smoke-exposure to the complex chronic disease COPD in humans should be performed with caution. It has been shown in vitro that the CFTR potentiator ivacaftor can partially rescue the CS-induced CFTR deficiency, thereby restoring the airway surface dehydration and mucociliary clearance in non-CF epithelial cells. Moreover, the phosphodiesterase inhibitor roflumilast has beneficial effects on the CS-induced dehydration of the airway surface of epithelial cell lines through elevation of intracellular cAMP levels and activation of CFTR. These proof-of-concept studies may facilitate future clinical trials that will be required to determine therapeutic effects improving CFTR function and airway surface hydration on mucus obstruction, airway inflammation and emphysema in patients with COPD.

Of note, our results also suggest that CS exposure of βENaC-Tg mice can be used as a time-saving model for COPD-like pathology. Within a short time frame of 4 to 8 weeks, these mice already develop a strong pulmonary inflammation, with the formation of lymphoid follicles, and destruction of alveolar tissue, whereas these pathologies in WT mice can only be observed following 6 months of CS exposure. In addition, βENaC-Tg mice also possess more characteristics of chronic bronchitis, including goblet cell metaplasia and intraluminal mucus content, than WT mice.
In summary, acquired CFTR malfunction and mucin hypersecretion, both leading to mucus hyperconcentration, have been demonstrated in smokers with and without COPD, implicating that airway surface dehydration is present in these patients \textsuperscript{157,161}. In our study, we have shown that the presence of airway surface dehydration in βENaC-Tg mice significantly aggravates the CS-induced pathological hallmarks of COPD, including mucin expression, pulmonary inflammation, formation of lymphoid follicles and destruction of alveolar walls. We speculate that the impaired mucociliary clearance caused by airway surface dehydration results in a retention and concentration of CS in the airways, thus leading to exaggerated host responses, such as mucus hypersecretion and enhanced inflammation. Proteases, originating from macrophages and neutrophils, can then lead to destruction of alveolar tissue and emphysema. The results of our study identify airway surface dehydration as a novel risk factor for CS-induced pathology.
The chronic bronchitis phenotype in COPD is a validated phenotype with important impact on clinical outcomes. However, there are inconsistent reports on the importance of chronic bronchitis on exacerbation rate and mortality risk in patients with COPD. Therefore, we aimed to investigate the epidemiology of chronic bronchitis in COPD in the Rotterdam Study.

ABSTRACT

BACKGROUND: Research on the association between chronic bronchitis and Chronic Obstructive Pulmonary Disease (COPD)-exacerbations has led to discordant results. Furthermore, the impact of chronic bronchitis on mortality in COPD subjects is unclear.

METHODS: This study is embedded within the Rotterdam Study, a population-based cohort study among subjects aged 45 years and older with long-term follow-up. Chronic bronchitis was defined as having a productive cough for at least three months a year for two consecutive years. Cross-sectional and longitudinal analyses were performed with logistic regression and Cox proportional hazard models respectively.

RESULTS: Of 972 COPD subjects included in the study, 752 COPD subjects had no chronic phlegm production (CB-) and 220 subjects had chronic phlegm production of whom 172 (78%) met the definition of chronic bronchitis (CB+). CB+ subjects were older, more frequently current smokers and had a higher amount of pack years of cigarette smoking than CB- subjects. Adjusted for age, sex and pack years, COPD subjects with CB had an increased risk of frequent exacerbations (OR 4.0 95%CI 2.7-5.9). During follow-up, CB+ subjects had significantly larger decline in lung function (-38 ml per year, 95%CI -61.7;-14.6). Survival was worse in especially female COPD subjects with CB. Regarding cause-specific mortality, CB+ COPD subjects had a significantly increased risk of respiratory mortality (HR 2.16, 95%CI 1.12-4.17, p=0.022 adjusted for age, sex and pack years).

CONCLUSIONS: COPD subjects with chronic bronchitis have an increased risk of exacerbations and (respiratory) mortality compared to COPD subjects without chronic phlegm production.
INTRODUCTION

Progressive, not completely reversible airflow limitation is the main characteristic of chronic obstructive pulmonary disease (COPD). Patients with COPD typically suffer from chronic cough, expectoration of phlegm, dyspnoea and a variety of extra-pulmonary symptoms. Clinical presentation, response to therapy and disease progression are very heterogeneous among patients with COPD. Identifying and studying subgroups of patients with similar traits within the COPD syndrome can aid in guiding more individualised therapy and assessment of prognosis. Several COPD phenotypes have been proposed, but only a few have been validated. One of these COPD phenotypes is the chronic bronchitis (CB) phenotype which is generally defined as patients with COPD which experience chronic cough and sputum production for at least three months per year for two consecutive years.

Epidemiological studies linking the CB phenotype in COPD to outcomes, have generated inconsistent results, especially regarding exacerbations and mortality. Both outcomes are crucial since they have a substantial impact on the burden of disease. Although COPD is a chronic disease, acute exacerbations and the rate at which they occur, determine the progression of the disease. Because COPD exacerbations have been associated with worse quality of life, accelerated deterioration of lung function, an increased risk for hospital admission and an increase in mortality, they have an important impact on the health care-related costs. Two large-scale, patient-based observational studies have reported on exacerbation rates in COPD patients with CB, with opposing results. The Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points (ECLIPSE)-study did not find an association between chronic bronchitis and COPD exacerbations, whereas the COPDGene study detected a strong association. Other patient-based studies seem to contribute to these discordant results. In contrast, observations from population-based studies are scarce. The effect of the presence of CB in COPD patients on mortality risk is unclear as well. Although several papers observed an increased mortality risk in COPD patients with CB, it is not clear whether this subgroup of COPD patients is more prone to all-cause mortality or specifically to COPD-related respiratory mortality. Furthermore, several studies found different effects of the presence of CB on mortality in men and women. Since previous studies on the impact of chronic bronchitis on the course of COPD have provided discordant results, we aimed to investigate the exacerbation rate and mortality risk of COPD patients with or without chronic bronchitis in a prospective population-based cohort study with long-term follow-up, the Rotterdam Study.
METHODS

Study design
The present study is embedded within the Rotterdam Study (RS), a population based cohort study comprising almost 15,000 participants aged ≥45 aiming at assessing the occurrence of, and risk factors for chronic diseases in the elderly. The study started in 1989 and all participants are invited every 3 to 4 years to the research centre for follow-up examinations, including spirometry. All participants with prevalent COPD and completed questionnaires on chronic bronchitis during the examination rounds performed from 2001 up to 2008, were included. Follow-up time was defined as the time period between the subject’s visit to the examination round, and death or end of study period (e.g. January 1st, 2013 for the validation of (cause-specific) death), whichever came first. The medical ethics committee of the Erasmus Medical Centre, Rotterdam, and the review board of The Netherlands Ministry of Health, Welfare and Sports, approved the study. Participants gave written informed consent.

Assessment of COPD
The diagnosis of COPD was based on an obstructive spirometry examination according to the modified Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (proportion of the forced vital capacity exhaled in the first second (FEV₁/FVC) < 0.7) or in absence of an interpretable spirometry at the research center, based on the diagnosis by a respiratory physician or general practitioner combining clinical history, physical examination and spirometry. Spirometry was performed between 2001 and 2008 using a SpiroPro® portable spirometer (Erich Jaeger GmbH, Hoechberg, Germany) and from 2009 onwards using a Master Screen® PFT Pro (CareFusion, San Diego, CA) by trained paramedical personnel according to the ATS/ERS guidelines. The incident COPD date was defined as the date of the first obstructive lung function or the date of COPD diagnosis in the medical reports or the date of the first prescription of COPD medication, whichever came first.

Assessment of chronic bronchitis
Chronic bronchitis was assessed by questionnaire and defined as having a productive cough for at least three months a year during the last two years. More specifically, all subjects were questioned (1) ‘Did you cough nearly daily for three consecutive months during the last two years?’ and (2) ‘Did you cough up phlegm nearly daily for three consecutive months during the last two years?’. Subjects answering no on both occasions or no to the second question, were defined as having no chronic phlegm production (CB-). Subjects answering yes twice were defined as having chronic bronchitis (CB+).
Assessment of exacerbations

All COPD subjects were prospectively followed for the onset of exacerbations from their examination visit until death or end of study period (e.g. January 1st, 2011 for the validation of exacerbations). Moderate COPD exacerbations were defined as acute episodes of worsening symptoms needing a course of steroids and/or antibiotics. Complete information on all filled prescriptions on a day-to-day basis was obtained in automated format from the pharmacies. Severe COPD exacerbations were defined as exacerbations requiring a hospitalization due to COPD. All hospital admissions were continuously registered in The Dutch Medical Registry (LMR). The exacerbation rate was defined as the ratio of the total number of exacerbations over the total follow-up time. COPD subjects with frequent exacerbations were determined as subjects who had at least two (rounded) moderate or severe exacerbations on average per year during follow-up.

Assessment of quality of life and mortality

Quality of life was measured by questionnaire and summarized as the weighted average of scores given to questions about mobility, ability and activity, self-care, pain and anxiety. Information on vital status was obtained from general practitioners and from municipal records. Causes of death during follow-up were coded according to the International Classification Of Diseases (ICD)-10}\(^\text{313}\). The following categories were applied: pulmonary mortality (ICD-10: J15-J44), cardiac mortality (ICD-10: I21-I73, R96), death from bronchial carcinoma (ICD-10: C34), death from other malignancies (ICD-10: C15-C96 except C34) and other causes of death (ICD-10: all other used codes).

Statistical analyses

Differences between subjects with and without chronic bronchitis were studied using Mann-Whitney U test for continuous variables and Chi-Square test for categorical variables. Logistic regression models were used to calculate the risk of frequent exacerbations. Cox hazard regression models were used to calculate the time to (cause-specific) death. All models were adjusted for age, sex and pack-years of cigarette smoking. Statistical analyses were performed using SPSS, version 23.0 for Windows (IBM, North Castle, NY).
RESULTS

Patient population

Of 972 COPD subjects included in the cohort study, 752 subjects with COPD had no chronic phlegm production (CB-) and 220 subjects had chronic phlegm production of whom 172 (78%) met the strict criteria of chronic bronchitis (CB+) (Figure 1). CB+ COPD subjects were older, more frequently current smokers and had a higher amount of pack years of cigarette smoking (Table 1). Furthermore, COPD subjects with CB had a worse lung function at baseline than COPD subjects without CB (Table 1). In addition, quality of life was significantly worse in COPD subjects suffering from chronic bronchitis (Table 1).

![Figure 1: Study flow chart]
<table>
<thead>
<tr>
<th></th>
<th>COPD, CB- (n=752)</th>
<th>COPD, CB+ (n=172)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>70.5 (15.2)</td>
<td>74.1 (13.6)</td>
<td>0.004</td>
</tr>
<tr>
<td>Males</td>
<td>380 (50.5%)</td>
<td>96 (55.8%)</td>
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<td>Smoking status</td>
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<td>Never smoker</td>
<td>134 (17.8%)</td>
<td>17 (9.9%)</td>
<td></td>
</tr>
<tr>
<td>Former smoker</td>
<td>407 (54.1%)</td>
<td>89 (51.7%)</td>
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<td>Current smoker</td>
<td>211 (28.1%)</td>
<td>66 (38.4%)</td>
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<tr>
<td>Pack-years cigarette smoking</td>
<td>23.0 (41.2)</td>
<td>30.6 (35.1)</td>
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</tr>
<tr>
<td>Height (m)</td>
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<td>1.68 (0.12)</td>
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<td>Weight (kg)</td>
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<td>2.9 (1.4)</td>
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<td>FEV₁ (% pred)</td>
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<td>70.5 (27.8)</td>
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<td>FVC (l)</td>
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<td>&lt;0.001</td>
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<td>91.9 (33.0)</td>
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<td>FEV₁/FVC (%)</td>
<td>65.4 (7.0)</td>
<td>61.0 (10.0)</td>
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<td>Quality of life (%)</td>
<td>80.0 (21.9)</td>
<td>71.9 (31.3)</td>
<td>&lt;0.001</td>
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</table>

Table 1: Baseline characteristics of COPD subjects without chronic bronchitis and with chronic bronchitis. Categorical variables are expressed as numbers (percentage). Values of continuous variables are expressed as median (interquartile range). Pack years were missing in 46 subjects, height/weight/BMI in 43 subjects, interpretable baseline lung function measurement was missing in 330 subjects, lung function decline over time in 653 subjects and quality of life in 12 subjects.

Abbreviations: BMI = body mass index; CB = chronic bronchitis; COPD = Chronic Obstructive Pulmonary Disease; FEV₁ = forced expiratory volume in one second; FVC = forced vital capacity

Figure
Impact of chronic bronchitis on lung function and exacerbation rate

281 COPD subjects had an interpretable lung function measurement between 2002-2008 and a second between 2009-2014. During this follow-up, COPD subjects with CB had a -38.2 ml per year larger decline in lung function than COPD subjects without CB (95%CI -61.7;-14.6) adjusted for age, sex and pack years of cigarette smoking (Table 2). The adjusted decline in lung function was -42.8 ml per year for male COPD subjects with CB and -36.1 ml per year for female COPD subjects with CB compared to male and female COPD subjects without CB respectively (Table 2).

<table>
<thead>
<tr>
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<th>Model 1 (n=270)</th>
<th>Model 2 (n=259)</th>
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<td></td>
<td>β</td>
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<td>COPD with chronic bronchitis</td>
<td>-35.2</td>
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<td>Male COPD with chronic bronchitis</td>
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<tr>
<td>Female COPD with chronic bronchitis</td>
<td>-29.4</td>
<td>-58.7;-0.05</td>
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</tbody>
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Table 2: Association between chronic bronchitis and lung function decline (ml/yr)
Model 1: age and sex* adjusted  
Model 2: adjusted for age, sex* and pack years of cigarette smoking.  
Abbreviations: β= unstandardized bèta-coefficient of millilitres lung function decline per year; CI= Confidence Interval; COPD= Chronic Obstructive Pulmonary Disease  
*only in the unstratified analyses.

During follow-up until January 1st, 2011, COPD subjects with CB had a three times higher median rate of moderate to severe exacerbations per year (0,33 exacerbations per year (IQR 0,85) in the CB-COPD group versus 0,99 (IQR 1,78) in the CB+ COPD group; p ≤ 0.001). The percentage of COPD subjects whom experienced at least one severe exacerbation during follow-up was also higher for COPD subjects with CB compared to COPD subjects without CB (20.3% versus 7.0%, p<0.001). Adjusted for age, sex and pack years of cigarette smoking, COPD subjects with CB had a fourfold increased risk (OR 4.0 95%CI 2.7-5.9) of frequent exacerbations (≥2 moderate or severe exacerbations per year) (Table 3). The odds ratio on frequent exacerbations was 3.12 for male COPD subjects with CB and 5.10 for female COPD subjects with CB compared to male and female COPD subjects without CB respectively (Table 3). Similar results on lung function decline and exacerbation rate were observed when the group of 172 subjects meeting the strict definition was extended to the 220 subjects with chronic phlegm production.
<table>
<thead>
<tr>
<th></th>
<th>Model 1 (n=924)</th>
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<th>Model 2 (n=878)</th>
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<td>p-value</td>
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<td>COPD with chronic bronchitis</td>
<td>4.13</td>
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<td>Male COPD with chronic bronchitis</td>
<td>3.21</td>
<td>1.90-5.42</td>
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<td>Female COPD with chronic bronchitis</td>
<td>5.16</td>
<td>2.94-9.05</td>
<td>&lt;0.001</td>
<td>5.10</td>
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</table>

Table 3: Association between chronic bronchitis and the frequent exacerbator phenotype.
Model 1: age and sex* adjusted
Model 2: adjusted for age, sex* and pack years of cigarette smoking.
Abbreviations: CI= Confidence Interval; COPD= Chronic Obstructive Pulmonary Disease; OR= Odds Ratio *only in the unstratified analyses.

Impact of chronic bronchitis on all-cause and cause-specific mortality

During 5 856 person years of follow-up, 351 COPD subjects died: 92 (53.5%) among those with CB and 259 (34.4%) among those without CB. COPD subjects with CB had worse survival than COPD subjects without CB as illustrated in Figure 2. Compared to COPD subjects without CB, mortality risk was increased by 33% in COPD subjects with CB after adjustment for age, sex and pack years (Table 4, HR 1.33, 95%CI 1.04-1.71, p=0.024). Stratified for sex, female COPD subjects with CB had a significantly worse survival, while the effect was less pronounced in men and no longer significant after adjustment for pack years of cigarette smoking (Table 4).

Figure 2: Kaplan-Meier curve of all-cause mortality according to COPD without chronic bronchitis (CB-, n=752) or with chronic bronchitis (CB+, n=172).
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<td></td>
<td>HR</td>
<td>95% CI</td>
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<tr>
<td>COPD with chronic bronchitis</td>
<td>1.45</td>
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<tr>
<td>Male COPD with chronic bronchitis</td>
<td>1.32</td>
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<tr>
<td>Female COPD with chronic bronchitis</td>
<td>1.69</td>
<td>1.14-2.53</td>
</tr>
</tbody>
</table>

Table 4: Association between chronic bronchitis and the risk of all-cause death in COPD, additionally stratified according to sex.

**Model 1:** age and sex* adjusted

**Model 2:** adjusted for age, sex* and pack years of cigarette smoking.

**Abbreviations:** CI= Confidence Interval; COPD= Chronic Obstructive Pulmonary Disease; HR= Hazard Ratio, *only in the unstratified analyses.

The most frequent causes of death in COPD patients are shown in **Figure 3** and listed in more detail in **Table S2** of the online supplement. Compared to CB- COPD subjects, COPD subjects with CB died more frequently due to pulmonary complications of COPD including exacerbations, emphysema or pneumonia (**Figure 3**). COPD subjects with CB had a more than twofold increased risk of pulmonary mortality compared to COPD subjects without CB (HR 2.70, 95%CI 1.45-5.04, p=0.002 adjusted for age and sex; HR 2.16, 95%CI 1.12-4.17, p=0.022 adjusted for age, sex and pack years). Of note, mortality due to lung cancer (bronchial carcinoma) was numerically higher but did not significantly differ between COPD subjects with and without CB (14.1% versus 12.4%) (**Figure 3**).
Figure 2: Major causes of death according to COPD without (CB-; n=752) or with chronic bronchitis (CB+; n=172).
DISCUSSION

In this large, population-based cohort study, we demonstrate that COPD subjects with concomitant chronic bronchitis (CB+) have a worse lung function at baseline and a subsequent larger lung function decline during follow-up. In addition, CB+ COPD subjects experience significantly more exacerbations during follow-up than COPD subjects without CB. Moreover, CB+ COPD subjects have a fourfold increased risk of frequent exacerbations. Furthermore, this study shows that CB+ COPD subjects have a higher mortality risk, especially COPD-related pulmonary death. Importantly, the increased risk of frequent exacerbations and the increased risk of mortality is more pronounced in women than in men.

In our study, the prevalence of CB in subjects with COPD is 17.7%. Defining CB by the presence of chronic phlegm, with or without the presence of chronic cough, increased the prevalence in our study towards 22.6%. There is a considerable variability in the prevalence of CB among subjects with COPD reported in other studies, ranging from 7.4% \textsuperscript{183} up to 74% \textsuperscript{30}. Several variations of the classical definition have been used to determine CB. In the PLATINO Study, the prevalence doubled using a less stringent definition \textsuperscript{183}. Furthermore, a higher prevalence is reported in patient-based studies which generally do not include a high percentage of patients with mild COPD, whereas population-based studies, such as the Rotterdam Study, incorporate an unselected COPD population, which generally includes a larger proportion of subjects with mild COPD. Other factors that influence the prevalence of CB among COPD subjects are smoking history and geographical localisation \textsuperscript{180, 186}. Lu \textit{et al.} reported that living in a rural area was associated with an increased risk for the development of chronic bronchitis \textsuperscript{186}.

A remarkable observation is that during the follow-up period, COPD subjects with CB experienced an excess lung function decline of 38.2 ml/year compared to COPD subjects without CB. Considering that lung function decline was determined in a subgroup of the population that survived until the next examination round and that had two interpretable measurements, this difference in lung function decline is considerable. The association of CB with a more rapid decline in lung function remained significant with the less stringent definition of CB and was slightly more pronounced in male COPD subjects with CB. In line with our findings, the population-based Copenhagen City Heart Study reported an excess decline in FEV\textsubscript{1} of 22.8 ml/year for men and 12.6 ml/year for women during a 5 year follow-up in COPD subjects with CB, whereas the patient-based ECLIPSE Study did not detect a difference in lung function decline in COPD patients with or without CB during a three year follow-up, indicating the importance of a relevant follow-up period \textsuperscript{187, 195}. In addition, Allinson \textit{et al.} recently showed in a British cohort study with long-term follow-up, that the presence of CB was associated with an additional 4.5 ml/year decline in FEV\textsubscript{1}, irrespective of the presence of COPD \textsuperscript{314}. 
Conflicting results exist regarding the exacerbation rate in COPD patients with CB. In our study, we observed a significantly increased exacerbation rate and a fourfold increased risk of frequent exacerbations in CB+ COPD subjects compared to COPD subjects without CB. In line, an increased exacerbation rate and an increased risk of frequent exacerbations was also reported for COPD subjects with CB in the COPDGene Study and in a small study (n=70) in the UK. In contrast, the observations from the ECLIPSE study and a patient-based study in Spain did not significantly associate chronic bronchitis to COPD exacerbations. In the population-based PLATINO study, the number of exacerbations in the past year were not significantly different for CB+ compared to CB- subjects, but the percentage of subjects with at least one exacerbation in the past year was significantly higher in the COPD group with CB compared to the COPD group without CB.

Viral and bacterial infections account for the majority of exacerbations in COPD. Observations from the Copenhagen City Heart Study indicate that indeed subjects with chronic bronchitis are more vulnerable for pulmonary infections. This was also reported in the Lung Health Study where subjects with chronic bronchitis reported significantly more lower respiratory illness than those without chronic bronchitis. These reports strengthen the association between CB and COPD exacerbations.

Potentially due to the increased vulnerability to exacerbations, we observed that COPD subjects with CB are especially more prone to pulmonary-related death compared to CB- COPD subjects. Furthermore, in this population, we demonstrated that the presence of CB induced an increased all-cause mortality risk of 33% in COPD subjects. Our results of increased mortality in CB+ COPD subjects are in line with those of Pelkonen et al., reporting an even higher increase in both all-cause and respiratory-related mortality.

An important observation in this study is that the associated risk of having CB on frequent exacerbations and on mortality, is more pronounced in female than in male subjects with COPD. Analogue results were found in the Copenhagen City Heart Study, where women with CB experienced more COPD-related hospitalisations than men with CB. In addition, results from the US Six Cities Study also indicated that women with CB have an increased risk for COPD-related death, compared to men with CB. Gender differences have been described in the effect of cigarette smoking on lung function and hospitalisation for COPD, with women being more vulnerable for the effects of cigarette smoking. Since the presence of CB is strongly associated with current smoking, the higher vulnerability of women for the effects of cigarette smoking might add to the differences in the risk ratios we observed in this study.

The strengths of our study are the general population-based setting, allowing us to investigate associations in an unselected COPD population, the long-term follow-up and the prospective data collection. In the Rotterdam Study, moderate and severe exacerbations are indeed continuously and automatically
registered based on pharmacy filled prescription data and a national hospitalization register, which prevents recall bias compared to self-reporting of the exacerbation frequency during the previous year in retrospective studies. This also includes a limitation, since we have no data on the frequency of mild exacerbations, which are attended by an increase of the regular medication.

A second limitation of our study is the fact that lung function decline could only be determined in a subgroup of the COPD subjects since a substantial proportion of the subjects died before lung function could be measured a second time and not all subjects had two interpretable lung function tests. However, even in this smaller group, the difference in lung function decline between COPD subjects with and without CB was significant.

In conclusion, we have shown that subjects with both COPD and CB have more decline in lung function, experience more exacerbations and have a higher risk for pulmonary-related mortality. Moreover, the increased risk of frequent exacerbations and mortality is even more pronounced in female COPD subjects with CB. The results indicate that COPD patients with CB need optimal prophylaxis and close monitoring to prevent exacerbations. Furthermore, this study shows that there is a need for better therapies which target CB since these might reduce the exacerbation rate and mortality risk in patients with COPD.
In COPD, the number of airways with lymphoid follicles is associated with disease severity. In addition, the expression of B cell-Activating Factor (BAFF) in these lymphoid follicles in patients with COPD is also correlated with disease severity. In order to investigate the role of BAFF and lymphoid follicles in COPD, we localized BAFF expression in lymphoid follicles of patients with COPD and antagonized BAFF in a murine model of COPD.


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ABSTRACT

Rationale: B cell–activating factor (BAFF) plays a major role in activation of B cells and in adaptive humoral immune responses. In chronic obstructive pulmonary disease (COPD), lymphoid follicles have been associated with disease severity, and overexpression of BAFF has been demonstrated within lymphoid follicles of patients with severe COPD.

Objectives: To investigate expression and localization of BAFF in the lungs of patients with COPD and to study the role of BAFF in COPD by antagonizing BAFF in a mouse model of chronic cigarette smoke (CS) exposure.

Methods: We quantified and localized BAFF expression in lungs of never-smokers, smokers without COPD, and patients with COPD and in lungs of air- or CS-exposed mice by reverse-transcriptase polymerase chain reaction, ELISA, immunohistochemistry, and confocal imaging. Next, to investigate the role of BAFF in COPD, we antagonized BAFF by prophylactic or therapeutic administration of a soluble fusion protein of the BAFF-receptor, BAFFR-Fc, in mice exposed to air or CS for 24 weeks and evaluated several hallmarks of COPD and polarization of lung macrophages.

Measurements and Main Results: BAFF expression was significantly increased in lungs of patients with COPD and CS-exposed mice. BAFF staining in lymphoid follicles was observed around B cells, CD4+ cells, dendritic cells, follicular dendritic cells, and fibroblastic reticular cells. Prophylactic and therapeutic administration of BAFFR-Fc in mice reduced pulmonary B-cell numbers and prevented CS-induced formation of lymphoid follicles and increases in immunoglobulin levels. Interestingly, prophylactic BAFFR-Fc administration significantly attenuated pulmonary inflammation and destruction of alveolar walls. Moreover, antagonizing BAFF altered the phenotype of alveolar and interstitial macrophages.

Conclusions: BAFF is significantly increased in lungs of patients with COPD and is present around both immune and stromal cells within lymphoid follicles. Antagonizing BAFF in CS-exposed mice attenuates pulmonary inflammation and alveolar destruction.
INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is a progressive disease, characterized by pulmonary inflammation, emphysema and obstructive bronchiolitis. In Western countries the main cause of COPD is cigarette smoking. However, even after smoking cessation, the disease persists and the underlying mechanisms need to be elucidated.

Pulmonary inflammation in COPD is characterized by an innate immune response, alongside an important increase in adaptive immune cells. The innate immune response is thought to be primarily driven by recruited neutrophils as well as the most abundant resident cells in the lung, the alveolar and interstitial macrophages. Elevated numbers of CD4+ and CD8+ T lymphocytes and B lymphocytes have been observed in both large and peripheral airways of patients with COPD. In addition, the presence of lymphoid follicles is significantly increased in patients with severe COPD. B cells are the major cell population in these follicles and B cell proliferation within the germinal centers of the follicles has an oligoclonal, antigen-specific character. Importantly, the antigens responsible for the oligoclonal proliferation are not yet identified and could either have a microbial nature or could derive from smooth muscle, airway epithelium, pulmonary endothelium or degradation of extracellular matrix. As a result, the question remains whether these follicles are beneficial or harmful. In addition, the role of B lymphocytes in COPD remains unclear.

B lymphocytes rely on B cell activating factor (BAFF) for maturation, differentiation and survival. This cytokine is a type II transmembrane homotrimer protein of the tumor necrosis factor (TNF) family and is expressed by innate immune cells, T cells, activated B cells, stromal cells and airway epithelium. Processed soluble BAFF is necessary for B cell homeostasis, but BAFF is also expressed as a membrane-bound protein. Three receptors have been described for BAFF, namely BAFF receptor (BAFFR), transmembrane activator and cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA). All three receptors are located on subsets of lymphocytes.

A significantly higher level of BAFF expression was measured in lymphoid follicles and macrophages of patients with COPD compared to control smokers and non-smokers. However, the role of BAFF in the pathogenesis of COPD is not yet clear. Therefore, we stained BAFF in lungs and lymphoid follicles of patients with COPD and co-stained for dendritic cells (DC), B cells, T cells, follicular DC and fibroblastic reticular cells. Next, we antagonized BAFF in chronic cigarette smoke-exposed mice and investigated the effect on B cells, the occurrence of lymphoid follicles and finally the impact on pulmonary inflammation and emphysema. Furthermore, since macrophages contribute to inflammation in the lung, we investigated whether BAFFR-Fc administration altered the lung inflammatory state through changes in the polarization...
status of alveolar and interstitial macrophages. Some of the results of these studies have been previously reported in the form of an abstract 329.
METHODS

More details of methods and materials can be found in the online supplement.

Human study populations

Written informed consent was obtained from all subjects according to protocols approved by the medical ethical committee of the Ghent University Hospital. All tissue originated from lung resection specimens of patients diagnosed with solitary pulmonary tumors at Ghent University. Tissue was harvested from the resection specimen at maximum distance of the pulmonary lesions by a pathologist. Only tissue without signs of pneumonia or tumor invasion was collected. Study populations of 70 and 69 subjects were used respectively for RT-PCR analysis on total lung tissue and protein detection in lung homogenates (Table 1 and 2).

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Table 1: Characteristics of Study Subjects for Lung mRNA Analysis by Quantitative Real-Time Polymerase Chain Reactions

Definition of abbreviations: COPD = Chronic Obstructive Pulmonary Disease; ICS = inhaled corticosteroids; mRNA = messenger RNA; NA = not applicable
Data are presented as median (IQR)
* P<0.001, Fisher’s exact test
† P<0.001 versus never-smokers, Mann-Whitney U test
‡ P<0.05 vs smokers, Mann-Whitney U test
§ P<0.001 vs smokers, Mann-Whitney U test
Table 2: Characteristics of Study Subjects for Protein Analysis in Lung Homogenate by ELISA

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<th>Smokers</th>
<th>COPD II</th>
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</thead>
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<td>FEV₁/FVC (%)</td>
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<td>ICS (yes/no)</td>
<td>0/19#</td>
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<td>15/17#</td>
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Definition of abbreviations: COPD = Chronic Obstructive Pulmonary Disease; ICS = inhaled corticosteroids; mRNA = messenger RNA; NA = not applicable

Data are presented as median (IQR)

† P<0.001, Fisher’s exact test
‡ P<0.001 versus never-smokers, Mann-Whitney U test
§ P<0.05 vs smokers, Mann-Whitney U test
§§ P<0.001 vs smokers, Mann-Whitney U test

Mice

Male C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 6-8 weeks of age. The mice were kept under standard conditions with a 12h light-dark cycle and a standard diet (Pavan, Brussels, Belgium). Chlorinated tap water was provided at libitum. The local Ethics Committee for animal studies of the Faculty of Medicine and Health Sciences (Ghent University) approved all in vivo manipulations.

Cigarette smoke exposure

Mice were exposed to mainstream cigarette smoke (CS) as described previously. Four times a day, five days a week, mice were exposed whole body to the smoke of five cigarettes (Reference Cigarette 3R4F without filter, University of Kentucky, Lexington, KY). A smoke-free interval of 30 minutes was respected in between smoke exposures. The smoke/air ratio was 1/6. The control groups were exposed to room air.

Antagonizing BAFF – study protocol

Eight groups of 10 mice were exposed to air or CS for 24 weeks. Six groups of mice were treated twice weekly intraperitoneally (ip) with 200µl of either phosphate-buffered saline (PBS), mouse IgG₁ isotype (clone IA7) (1 µg/µl) (MedImmune, Gaithersburg, MD, USA) or BAFFR-Fc (1 µg/µl) (AstraZeneca, Alderley Park, UK) during the 24 weeks (i.e.prophylactic setup). In a therapeutic setup, two other groups received twice weekly ip treatment with either mouse IgG₁ isotype or BAFFR-Fc from halfway through the CS exposure period, i.e. starting at week 13 of the CS exposure period. (Suppl. Fig. E1). The injections were
given 1 hour before air or CS exposure. The antagonizing agent, BAFFR-Fc, is a fusion protein that exists of 
the extracellular domain of mouse BAFFR, fused at the C-terminus to a linker peptide and the Fc-portion 
of human IgG1.

We did not detect a meaningful difference between the PBS-treated and the isotype-treated control 
groups nor between the prophylactic and therapeutic isotype-treated groups. Therefore, for reasons of 
clarity, the PBS-treated groups and the therapeutic isotype-treated group will not be displayed.

**Quantification and detection of BAFF**

BAFF mRNA expression was measured in mice and human lung tissue using TaqMan Gene Expression 
Assays (Applied Biosystems, Foster City, CA) \(^{330}\). Immunofluorescence staining was performed on frozen 
sections of human lung tissue of patients with COPD. These sections were stained with anti-BAFF (Abcam), 
anti-BAFFR (Abcam), anti-CD19, biotinylated anti-CD11c, biotinylated anti-CD68, anti-CD4, anti-CD8, anti-
Ki-M4 and anti-ERTR-7. Immunohistochemical staining was performed with anti-BAFF antibody (Abcam) 
on lung tissue of mice. The protein levels of BAFF in BAL fluid supernatant of mice and in homogenate of 
human lung tissue were determined with an ELISA kit (R&D systems).

**Quantification of lymphoid follicles and aggregates**

Paraffin-embedded sections of the left lung of mice were stained immunohistochemically with anti-CD3 
(DAKO) and anti-B220 (BD biosciences), as described previously \(^{257}\). Dense accumulations of more than 50 
cells were counted as lymphoid follicles. Diffuse infiltrations of less than 50 cells were counted as lymphoid 
aggregates.

**Quantification of inflammation**

Flow cytometry (FACSCalibur, BD biosciences) was used to quantifiy the inflammatory cells in BAL fluid and 
lung tissue \(^{253,292,293}\). The flow cytometry data of the BAL samples were supplemented with differential cell 
counts via cytospin. In BAL fluid and lung homogenate, the protein levels of cytokines and chemokines 
were determined with a cytometric bead array (BD biosciences, San Diego, CA) \(^{279}\). Immunoglobulins were 
measured in BAL fluid and serum via commercially available ELISA kits (R&D systems). Reverse 
transcriptase-polymerase chain reaction (RT-PCR) with the Biomark System (Fluidigm, San Francisco, CA) 
was performed to establish the mRNA expression levels of 92 target genes.

**Quantitative image analysis of macrophage phenotypic markers**

Immunohistochemical staining with anti-F4/80, anti-iNOS and anti-SOCS3 antibody from Abcam, anti-
Arginase-1 antibody (Atlas Antibodies) and anti-CD163 antibody (Leica) was performed using an IntelliPath 
FLX automated staining machine. Computerized image analysis using Visiopharm Integrator System
software (version 4.6.3, Visiopharm, Horsholm, Denmark) was used. Results were expressed as area fractions.

Quantification of emphysema
Emphysema was measured by two complementary methods. By calculating the mean linear intercept (Lm), the enlargement of the alveolar airspaces was established. Destruction of the alveolar walls was quantified by determining the destructive index 246, 281.

Statistical analysis
The statistical analyses were calculated with Sigma Stat software (SPSS 21.0; Chicago, IL) using non-parametric tests (Kruskall-Wallis, Mann-Whitney-U). All reported values are expressed as mean ± SEM and p-values < 0.05 were considered significant.
RESULTS

Expression of BAFF in patients with COPD

The mRNA levels of BAFF were quantified in lung tissue of 70 subjects and were significantly upregulated in patients with COPD and smokers without COPD compared to never smokers (Fig. 1A). In agreement with the mRNA expression, quantification of BAFF protein levels in lung homogenates of 69 subjects, revealed significantly higher BAFF protein in patients with COPD and smokers without COPD, compared to never smokers (Fig. 1B). No significant differences were observed between smokers with or without COPD, neither on the mRNA nor on the protein level (Fig. 1A-B).

Figure 1: mRNA and protein levels of B cell activating factor.

BAFF mRNA levels in total lung tissue of 16 never-smokers, 24 smokers without airway obstruction and 30 patients with COPD GOLD stage II (A). mRNA expression levels in human lung tissue are relative to 7 reference genes (HPRT1, GAPDH, POP4, IPO8, PPIA, POLR2A, PSMC4). BAFF protein levels in lung homogenates of 19 never-smokers, 18 smokers without airway obstruction and 32 patients with COPD GOLD stage II, as measured by ELISA (B). BAFF mRNA levels in total lung tissue (C) and BAFF protein levels in bronchoalveolar lavage fluid (D) of mice exposed to 4 and 24 weeks of air or cigarette smoke (CS). mRNA expression levels in mice are relative to 3 reference genes (Hprt1, Gapdh, Tfrc). Immunohistochemical staining of BAFF on lung tissue of mice exposed to 24 weeks of CS: (E) isotype control staining, (F-G) BAFF staining in lymphoid follicles, airway epithelium, endothelium, macrophages and alveolar walls.

*p<0.05, **p<0.01, ***p<0.001
Figure 2: Confocal staining of B cell activating factor in pulmonary lymphoid follicles of patients with COPD

Immunofluorescence staining of conventional dendritic cells (CD11c) (A), B cells (CD19) (B), B cell activating factor (BAFF) (C) and the merge of the stainings (D).

Immunofluorescence staining of conventional dendritic cells (CD11c) (E), B cells (CD19) (F), BAFF receptor (BAFFR) (G) and the merge of the stainings (H).

Immunofluorescence staining of CD4+ cells (I), B cell activating factor (BAFF) (J) and the merge of the stainings (K). Immunofluorescence staining of CD8+ cells (L), B cell activating factor (BAFF) (M) and the merge of the stainings (N).

(O) Immunofluorescence staining of BAFF and follicular dendritic cells (identified using Ki-M4 as marker). (P) Immunofluorescence staining of BAFF and fibroblastic reticular cells (identified using ERTR7 as marker).
Confocal imaging of lung tissue of patients with COPD was performed to localize BAFF and BAFFR in lymphoid follicles. BAFF, B cells and conventional DC were co-stained in lymphoid follicles (Fig 2 A-D). Merged images showed that BAFF was present in the area closely surrounding DCs and parts of the B cell area. In a second immunofluorescence staining, BAFFR was co-stained with B cells and conventional DCs (Fig 2 E-H). These images showed that BAFFR was co-localized with B cells. Co-staining of BAFF with CD4 or CD8 revealed BAFF staining in the vicinity of CD4⁺, but not CD8⁺ cells (Fig. 2I-N). Co-staining of BAFF with follicular dendritic cells (identified using Ki-M4 as marker) and fibroblastic reticular cells (identified using ER-TR7 as marker) showed that BAFF was present in the vicinity of these stromal cell types in lymphoid follicles of patients with COPD (Fig. 2O-P).

**Expression of BAFF in mice following cigarette smoke exposure**

Analysis of the pulmonary BAFF mRNA levels after 4 and 24 weeks of air or CS exposure did not reveal significant differences (Fig 1C). Four weeks of CS exposure induced a significant increase in BAFF protein levels in BAL fluid. This increase in BAFF protein was even more pronounced after 24 weeks of CS exposure (Fig 1D). The localization of the BAFF protein in lung tissue of mice was established by immunohistochemistry. In mice exposed to 24 weeks of CS, the lymphoid follicles, airway epithelium, smooth muscle cells, macrophages and neutrophils were positive for BAFF (Fig 1E-G).

**Antagonizing BAFF reduces B cells in BAL, lung and mediastinal lymph nodes**

CS exposure induced an increase in the numbers of B cells in BAL, lung and mediastinal lymph nodes (MLN). To investigate the functional role of BAFF, we administered BAFFR-Fc to C57Bl/6 mice, either prophylactically (i.e. from the start of the CS exposure) or therapeutically (i.e. starting after 13 weeks of CS exposure) (Suppl. Fig. E1). Both prophylactic and therapeutic administration of BAFFR-Fc significantly reduced the number of B cells in all three compartments in air- and CS-exposed mice (Fig 3 A-C). In lung and MLN the B cell count was diminished to approximately a tenth of the number of B cells of the isotype-treated control groups (Fig 3 B-C). In BAL the B cell population was decreased to a greater extent (Fig 3 A). Moreover, mRNA expression levels of CD19 and CXCR5, both highly expressed in B cells, were significantly reduced in all BAFFR-Fc-administered groups (Suppl Table E1).

In addition, the mRNA transcript levels of BAFF, APRIL and their receptors BAFFR, TACI and BCMA (which are mainly expressed on B cells) were determined. CS exposure did not evoke a significant change in any of the transcript levels. On the other hand, BAFFR-Fc administration significantly decreased the transcript levels of APRIL, BAFFR, TACI and BCMA, but had no effect on the mRNA expression of BAFF (Fig. 3 D-H).
Figure 3: Antagonizing B cell activating factor reduces the number of B cells in bronchoalveolar lavage, lung and mediastinal lymph nodes.

B cell numbers in bronchoalveolar lavage (BAL) (A), lung (B) and mediastinal lymph nodes (MLN) (C).

mRNA expression of BAFFR (gene name Tnfrsf13c) (D), TACI (gene name Tnfrsf13b) (E), BCMA (gene name Tnfrsf17) (F), BAFF (gene name Tnfsf13b) (G) and APRIL (gene name Tnfsf13) (H) in total lung tissue relative to 3 reference genes (Hprt1, Gapdh, Tfrc).

*p<0.05, **p<0.01, ***p<0.001

However, quantitative scoring of immunohistochemical staining revealed reduced levels of BAFF protein in the airway epithelium of prophylactic BAFFR-Fc-treated mice compared to isotype-treated mice (Suppl Fig. E2). Additionally, macrophages were scored semi-quantitatively for BAFF protein, but no differences were detected between groups (data not shown).
Antagonizing BAFF prevents lymphoid follicle formation

Upon CS exposure, lymphoid follicles, defined as large, dense infiltrations of more than 50 cells, were generated in the isotype-treated group, both peribronchial and in the lung parenchyma (Fig 4 A-B). Conversely, CS exposure did not induce the formation of lymphoid follicles in groups treated both prophylactic and therapeutic with BAFFR-Fc. CS exposure also elicited lymphoid aggregates, defined as small-numbered, diffuse lymphoid infiltrations, in the isotype-treated group. BAFFR-Fc administration minimized the occurrence of both peribronchial and parenchymal aggregates (Fig 4 C-D). Moreover, the CS-induced upregulation of CXCL13, a chemokine important for lymphoid follicle formation, was significantly decreased in the prophylactic BAFFR-Fc-treated, CS-exposed group (Suppl. Table E1).

Figure 4: Antagonizing B cell activating factor prevents formation of lymphoid follicles and diminishes the number of lymphoid aggregates.

Quantification of peribronchial lymphoid follicles normalized for the number of airways (A) and parenchymal follicles normalized for the area of parenchyma (B).
Quantification of peribronchial aggregates normalized for the number of airways (C) and parenchymal aggregates normalized for the area of parenchyma (D).
(E) Immunohistochemical staining of a pulmonary lymphoid follicle with anti-cd3 (brown) and anti-B220 (blue) **p<0.01, ***p<0.001

Consistent with the reduction in the number of B cells in BAL, lung and MLN, and the absence of lymphoid follicles, antagonizing BAFF resulted in significantly lower levels of immunoglobulins in BAL and serum (Fig: 5 A-F). In BAL, IgA, IgG and IgM were reduced in the BAFFR-Fc-treated groups compared to the isotype-
treated groups (Fig 5 A-C). Furthermore, in serum, IgG and IgM levels were decreased in all BAFFR-Fc-treated groups, compared to the isotype-treated groups (Fig. 5 D-F).

Figure 5: Immunoglobulins in bronchoalveolar lavage and serum.
Immunoglobulin (Ig) levels in bronchoalveolar lavage (BAL): IgA(A), IgG (B) and IgM. (C) Immunoglobulin (Ig) levels in serum: IgA(D), IgG (E) and IgM (F).
*<p><0.05, **p<0.01, ***p<0.001

Antagonizing BAFF attenuates the immune response in BAL and lung
In BAL, CS exposure induced an increase in total cell count, macrophages, neutrophils, DC and lymphocytes in the isotype-treated group (Fig. 6). Prophylactic BAFFR-Fc administration significantly attenuated the CS-induced increase in total cells, macrophages, neutrophils and DC (Fig. 6 A-D), and tended to attenuate the increase in T lymphocytes (Fig. 6 E-G). Therapeutic BAFFR-Fc administration did not affect the CS-induced inflammation in BAL (Fig. 6 A-G).
Protein levels of chemokines and cytokines were measured in BAL supernatant. CS exposure induced an increase in KC, RANTES, MCP-1, TNF, IL-6, IFNγ and IL-17A in both isotype-treated and BAFFR-Fc-treated groups (Suppl. Table E2). However, no significant differences were detected between the CS-exposed groups.

In lung, CS exposure induced a significant increase in the number of DC, CD3+ T cells, CD4+CD69+ T cells, CD8+CD69+ Tcells and regulatory T cells (Tregs) in the isotype-treated group. Prophylactic BAFFR-Fc administration significantly attenuated the CS-induced increase in DCs, CD4+CD69+ T cells and Tregs, but not in CD8+CD69+ T cells (Fig. 7 A-E).

Figure 6: Prophylactic administration of BAFFR-Fc attenuates the immune response in bronchoalveolar lavage of chronic cigarette smoke-exposed mice.

Quantification of total bronchoalveolar lavage (BAL) cells (A), BAL macrophages (B), BAL neutrophils (C), BAL dendritic cells (D), BALT cells (E), BAL CD4+ T cells (F) and BAL CD8+ T cells (G).

*p<0.05, **p<0.01, ***p<0.001
mRNA expression analysis of 92 target genes on total lung tissue revealed primarily a downregulation of B cell-associated genes in BAFFR-Fc-treated groups, as well as a significant reduction in CTLA4 and IL-12a mRNA levels (Suppl. Table E1).

In addition, protein levels of chemokines and cytokines were measured in lung homogenates. CS exposure induced an increase in KC, MCP-1, MIG, GM-CSF, TNF, IFNγ, IL-17A, IL-4 and IL-10 in the isotype-treated group (Suppl. Table E3). The CS-induced increase in GM-CSF protein levels was significantly attenuated in groups treated prophylactically and therapeutically with BAFFR-Fc (Suppl. Table E3).

**Antagonizing BAFF alters the macrophage phenotype in the lung**

In lungs of patients with COPD, CD68-positive macrophages were detected in and around lymphoid follicles (Fig 8 A-B). In accordance, F4/80-positive macrophages were localized in lymphoid follicles of mice exposed to 24 weeks of CS (Fig. 8 C).

In order to determine the macrophage phenotype, cross-sections of the left lung of mice were stained with anti-F4/80, anti-iNOS, anti-SOCS3, anti-CD163 and anti-Aarginase-1 antibodies. Alveolar and interstitial macrophages stained equally positive for the pan-macrophage marker F4/80. Interestingly, alveolar
macrophages stained more positive for SOCS3, a marker of M(IFNγ)-like phenotype, compared to interstitial macrophages. In contrast, compared to alveolar macrophages, interstitial macrophages stained stronger for Arginase-1 and CD163, both markers of a M(IL-4)-like phenotype (Fig. 8 D-M).

In murine lung, CS induced a significant increase in F4/80-positive alveolar and interstitial macrophages, as well as a significant increase in the number of macrophages positive for the phenotypic markers iNOS and CD163 (Fig. 8 D-M). Antagonizing BAFF did not influence the numbers of F4/80-positive macrophages. However, prophylactic administration of BAFFR-Fc significantly enhanced the CS-induced increase of iNOS-positive alveolar macrophages. In contrast, both prophylactic and therapeutic administration of BAFFR-Fc significantly attenuated the CS-induced increase of CD163-positive interstitial macrophages (Fig. 8 D-M).
Antagonizing BAFF partially protects mice against alveolar wall destruction

Emphysema was determined by two complementary methods. The mean linear intercept (Lm) was determined in order to quantify alveolar airspace enlargement. Although the Lm was numerically increased upon CS exposure, no significant changes were observed between the study groups (Fig 9 A). Destruction of alveolar walls was quantified using the destructive index (DI). CS exposure significantly increased the alveolar wall destruction in isotype and BAFFR-Fc treated groups. However, prophylactic BAFFR-Fc administration significantly attenuated the CS-induced destruction of alveolar walls (Fig 9 B).

Figure 9: Prophylactic BAFFR-Fc administration reduces CS-induced alveolar wall destruction.
A: Mean linear intercept (Lm), B: Destructive index (DI).
Representative images of the parenchyma of isotype-treated, air-exposed mice (C), isotype-treated cigarette smoke-exposed (CS) mice (D), BAFFR-Fc-treated, air-exposed mice (E), prophylactic BAFFR-Fc-treated CS-exposed mice (F), and therapeutic BAFFR-Fc-treated, CS-exposed mice (G). *p<0.05, **p<0.01, ***p<0.001
DISCUSSION

In this translational study, we demonstrate elevated BAFF mRNA and protein levels in lung tissue of patients with COPD. Furthermore, confocal imaging illustrates that BAFF is present in the vicinity of both immune and stromal cells in lymphoid follicles of patients with COPD. In addition, immunohistochemical staining of the lung tissue illustrates that macrophages are present in and around lymphoid follicles. Experimentally, antagonizing BAFF in chronic CS-exposed mice dramatically reduced the number of B cells and immunoglobulin levels and completely prevented the formation of lymphoid follicles. Importantly, prophylactic inhibition of BAFF reduced the CS-induced inflammatory response in BAL fluid and lung tissue, reduced the expression of macrophage phenotypic markers and attenuated the CS-induced alveolar wall destruction.

B cell activating factor (BAFF) was discovered in 1999 as a key factor in B cell homeostasis. Soon thereafter, its role in autoimmunity became clear and recently the role of BAFF in several other diseases, such as infection, allergy and hematological and lymphoid cancers, has been described. Several reasons warrant an investigation into the role of BAFF in COPD. First, B cell numbers are increased in patients with COPD. Second, an increased number of ectopic lymphoid follicles consisting mainly of B cells, has been described in patients with severe COPD. And finally, the possibility has been raised that an autoimmune component contributes to the pathogenesis of COPD.

Previously, Polverino et al. reported higher protein levels of BAFF in lungs of patients with COPD compared to never smokers. We confirmed this finding in our study population both at mRNA and protein level. Interestingly, BAFF mRNA and protein levels were already elevated in smokers without airflow limitation. It should be noted that the gender distribution of our never-smoker and smoker populations are skewed towards respectively a female and male predominance. Immunofluorescence staining and confocal imaging illustrate that BAFF is present in pulmonary lymphoid follicles of patients with COPD, more specifically in the vicinity of B cells, CD4+ cells, conventional dendritic cells, follicular dendritic cells and fibroblastic reticular cells. However, since BAFF appears mainly as a soluble protein, we cannot be absolutely certain that these cells actually produce BAFF. Nevertheless, this is the first time BAFF has been localized in the close surroundings of stromal cells of lymphoid follicles in lungs of patients with COPD, indicating that, once a follicle is organized, a micro-environment is created in which B cell maturation and survival is promoted.

In mice, antagonizing BAFF with a soluble BAFFR-Fc complex resulted in a strong reduction in the number of B cells. This is in line with the fact that BAFF is a necessary survival factor for all mature B cell subsets except memory B cells and a fraction of the B1 B cells. Since B1 B cells are confined to specific
anatomic sites, we suggest that the few B cells that are still present in lungs and mediastinal lymph nodes after BAFFR-Fc administration, are memory B cells. As a consequence of the reduction in B cell numbers, the mRNA expression levels of all 3 BAFF receptors are significantly decreased since these receptors are mainly located on B cells. BAFFR-Fc administration also resulted in a downregulation of APRIL mRNA expression levels but did not influence the mRNA expression levels of BAFF. In all BAFFR-Fc-treated groups, the absence of B cells and lymphoid follicles resulted in significantly reduced levels of immunoglobulins, both in BAL fluid and in serum. Although lower immunoglobulin levels suggest an increased susceptibility to infections, this has not been detected in the phase III clinical trials of belimumab, a human monoclonal antibody against BAFF registered for treatment of systemic lupus erythematosus. However, the immunoglobulin levels were more severely decreased in our BAFFR-Fc-treated mice compared to the decrease in immunoglobulins reported in the clinical trials.

Next to the effect on B cells, BAFFR-Fc administration led to an attenuation of the CS-induced inflammatory response in BAL fluid and lung tissue. Especially in BAL, the inflammatory cell count was severely reduced after prophylactic BAFFR-Fc administration.

In recent years, it has become clear that the function of BAFF is not limited to B cell homeostasis but extends to influencing both adaptive and innate immunity. It is suggested that BAFF stimulates DCs in order to attract immune cells to sites of inflammation. In our experiment, antagonizing BAFF resulted in decreased numbers of DCs following CS exposure, both in BAL and lung. It has also been described that BAFF stimulates monocyte survival and differentiation into a macrophage phenotype. Prophylactic administration of BAFFR-Fc in CS exposed mice, decreased the numbers of macrophages in BAL, but not in lung, and reduced the levels of GM-CSF in lung homogenates. Macrophages play a central role in the pathogenesis of COPD. We have demonstrated that macrophages are present in and around lymphoid follicles in lungs of patients with COPD, underlining their ubiquitous presence in the lung. In order to investigate their function in COPD, several studies have reported on the phenotype of macrophages in the disease. However, the reports are ambiguous. In the murine lung, we have observed that both alveolar and interstitial macrophages express F4/80 to the same extent. In contrast, alveolar macrophages express more SOCS3, a marker of a pro-inflammatory (M1) macrophage phenotype, whereas interstitial macrophages express substantially more CD163 and Arginase-1, both markers of an immunoregulatory (M2) macrophage phenotype. This difference in expression pattern may relate to differences in location and function of the two types of tissue-resident macrophages. Exposing mice to CS resulted in higher numbers of F4/80-, iNOS-, CD163- and Arginase-1-positive macrophages. Interestingly, administration of BAFFR-Fc enhanced the CS-induced increase in iNOS-positive alveolar
macrophages, while it almost completely prevented the CS-induced increase in CD163-positive interstitial macrophages.

Several studies have explored the effect of BAFF on T cell function. Sutherland et al. observed that BAFF favors Th1 responses and suppresses Th2 responses. In addition, BAFF promotes Th17 and regulatory T cells. Although we did not determine the different T helper subsets, we did measure significantly fewer CD4+CD69+ T cells and regulatory T cells in the prophylactically BAFFR-Fc administered group following CS exposure. However, since BAFF exerts some of its effects on inflammation via a B cell dependent mechanism, it is difficult to distinguish between the direct effects of BAFF and the effects of B cell depletion.

In our experiment, CS-induced destruction of alveolar walls was significantly attenuated by prophylactic BAFFR-Fc administration. This was consistent with lower numbers of macrophages and neutrophils in BAL, two cell types that produce proteolytic enzymes. Importantly, the development of emphysema in C57BL/6 mice is rather mild, suggesting that the early stages of COPD have been evaluated in this experiment. This is different from human COPD, where accumulation of lymphoid follicles is observed in more severe stages of the disease.

Recently, it has been shown that B cell deficient mice are completely protected against CS-induced emphysema. Previously, we demonstrated that neutralization of CXCL13 in chronic CS-exposed mice resulted in the absence of lymphoid follicles without a significant reduction of B cell numbers in the lung. As demonstrated here, BAFF neutralization not only eliminated tertiary follicles, but also led to a significant reduction in B cell numbers in the lung. Interestingly, anti-CXCL13 treatment attenuated the CS-induced alveolar wall destruction, similar to what we observed with BAFF neutralization. Together, the data indicate that organized B cell lymphoid follicles, rather than the mere presence of B cells in lung tissue, contribute to the development of emphysema. As follicles are required for an efficient humoral response this suggests that (auto)antibodies, locally produced in the lungs of CS exposed mice, might contribute to alveolar wall destruction. Our observation that both BAFF and CXCL13 neutralization resulted in significant reductions in immunoglobulins, locally and systemically, may further support this notion.

In conclusion, we have demonstrated that BAFF mRNA and protein levels are increased in patients with COPD. We also illustrated that BAFF is present in lymphoid follicles of patients with COPD, in the vicinity of B cells, CD4+ cells, dendritic cells, follicular dendritic cells and fibroblastic reticular cells, suggesting that organized lymphoid follicles are niches in which B cell maturation and survival is promoted. By antagonizing BAFF with BAFFR-Fc in CS-exposed mice, we have demonstrated that the immune response to CS exposure is attenuated in absence of BAFF. Furthermore, the phenotype of pulmonary macrophages
is altered after BAFFR-Fc administration. Importantly, antagonizing BAFF partially protects against destruction of lung parenchyma. These novel findings may influence future therapeutic strategies for COPD.
PART II

CHAPTER 10: DISCUSSION AND FUTURE PERSPECTIVES
In this translational research work, the mucosal immune response to cigarette smoke is the central theme. We have studied the innate defense response by investigating the effect of mucociliary dysfunction on cigarette smoke-induced pathology in a murine model of COPD. Next, we studied the effect of chronic bronchitis on clinical manifestations of COPD in a large population-based cohort.

Whereas the mucociliary clearance system is the first line of defense against inhaled noxious stimuli, the adaptive immune response steps in later in mucosal immunity. This adaptive response is quite extensive with the formation of B cell-rich lymphoid follicles which are predominantly formed peri-bronchially. A thought to consider is the possibility that lack of adequate innate immune defense leads to an exaggerated adaptive immune response. In this research, we aimed to investigate the role of the lymphoid follicles by antagonizing B cell activating factor (BAFF).

10.1 Role of Mucus Dysfunction in COPD

10.1.1 Airway Surface Dehydration Aggravates Cigarette Smoke-Induced Hallmarks of COPD

Mucociliary dysfunction is evident in individuals with chronic bronchitis, a feature that is highly prevalent in subjects with COPD. Furthermore, unrelated to the presence of chronic bronchitis, mucus obstruction of small airways is cardinal in the pathogenesis of COPD. In recent years, the concept of mucociliary structure, function and physicochemical properties has changed dramatically. It has become clear that adequate hydration of the airway surface liquid, regulated by ion channels, is crucial to maintain the function of this defense mechanism. In patients with COPD, Bodas et al. showed that CFTR (cystic fibrosis transmembrane conductance regulator; a Cl− channel) protein expression correlates with lung function and inversely correlates with disease severity. Zhao et al. confirmed these results and additionally showed that the expression of ENaC – the Na+ channel - is associated with disease severity. Although these studies imply a functional role for airway surface dehydration in cigarette smoke-induced pathology, this has not been investigated in vivo.

To examine whether airway surface dehydration contributes to the development and progression of COPD, we exposed βENaC-Tg mice and their wild-type littermates to cigarette smoke. As described by Livraghi et al. and Mall et al., we observed airway inflammation, increased numbers of goblet cells, elevated mucin expression and distal airspace enlargement in air-exposed βENaC-Tg mice, adding to the notion that mucus stasis is sufficient to trigger inflammation. The synergistic effect of airway surface dehydration and CS exposure, resulted in significantly higher numbers of macrophages, neutrophils and
lymphocytes, especially CD8+ T cells, in βENaC-Tg mice compared to their wild-type littermates. Gehrig et al. found that neutrophil elastase plays an important role in the development of the airway inflammation, mucin expression and emphysema present in βENaC-Tg mice 304. Of note, accumulations of lymphocytes were detected in βENaC-Tg mice after 8 weeks of CS exposure and have not been detected in wild-type mice after such a relative short exposure period. This suggests that the lack of mucociliary clearance (innate defense) might contribute to the initiation of lymphoid follicles. This might be through an increased susceptibility to infections or through the aggravated airway inflammation which leads to a higher amount of proteases, reactive oxygen intermediates and other products of inflammatory cells that break down lung tissue. This enhanced destruction of lung parenchyma may promote the formation of neo- or self-antigens.

In βENaC-Tg mice, an increased number of goblet cells was reported previously and is confirmed in our study 176-178. Although CS exposure did not induce a further increase of the number of goblet cells, we consistently measured a significant CS-induced upregulation of Muc5ac in βENaC-Tg mice but not in wild-type littermates, coinciding with the observation that Muc5ac rapidly responds to injurious substances 288. Moderate mucin hypersecretion alone will probably not induce mucociliary dysfunction, since Muc5ac overexpressing mice do not exhibit mucus plugging 299. However, severe hypersecretion or mucin hypersecretion in combination with ion channel impairment will result in a hyperconcentrated mucus layer, because mucins are stored intracellular in dehydrated form and expand enormously by binding H2O once secreted 123, 138, 158, 344. Hyperconcentrated mucus increases the osmotic pressure, resulting in mucus stasis and ultimately adhesion, which predisposes for bacterial infections 125, 344.

Alongside mucociliary dysfunction and airway inflammation, βENaC-Tg mice develop severe emphysema, illustrated in our study by a substantial difference in mean linear intercept and in static and dynamic compliance between transgenic and wild-type mice. As in patients with COPD, MMP12 and neutrophil elastase play key roles in the development of emphysema 300, 304. Mean linear intercept did not increase significantly following CS exposure, which might be due to the severity of the constitutive emphysema. However, the destructive index, a measure for the degree of alveolar destruction, did significantly increase after subacute CS exposure in βENaC-Tg mice whereas no effect was detected in wild-type mice, indicating that in the presence of airway surface dehydration, CS exposure induces accelerated development of emphysema.

Exposing βENaC-Tg mice to CS allowed us to study the effect of airway surface dehydration in vivo, however, this model does include some limitations. Recently, it has become clear that interactions between host and microbiome can steer the inflammatory response 117. It is likely that the presence of
airway surface dehydration induces alterations in the composition of the microbiome, which might influence the outcome. Next, recent papers show that acidification of airway surface liquid diminishes bacterial killing. Since CS in itself is already quite acidic, it might contribute to the acidification created by the loss of HCO$_3^-$ secretion as a consequence of CFTR dysfunction in cigarette smokers. In this model, airway surface dehydration is achieved by Na$^+$ hyperabsorption and does not alter Cl$^-$ secretion by CFTR and hence, presumably not the HCO$_3^-$ secretion, excluding the possibility to research this feature in these mice.

In conclusion, CS exposure of βENaC-Tg mice results in significantly increased neutrophilic airway inflammation, mucin expression and alveolar destruction, indicating that airway surface dehydration contributes to the development and progression of COPD.

10.1.2 Epidemiology of Chronic Bronchitis in COPD

Chronic bronchitis, defined as chronic cough and sputum production for at least 3 months per year for 2 consecutive years, is highly prevalent in patients with COPD and to a lesser extend in the general public. Several different definitions have been used, explaining the variability in reported prevalence in part. However, whether the classic definition - as stated above - is used or the less stringent definition of ‘chronic phlegm for at least 3 months per year for 2 consecutive years’, the effect of chronic bronchitis on clinically important outcomes is similar both in our study and in the PLATINO study.

In the Rotterdam Study, COPD subjects with chronic bronchitis were more frequently current smokers, smoked more pack-years, had a worse quality of life and importantly experienced an accelerated lung function decline. These results corroborate outcomes obtained in other studies, although there is debate concerning the effect on lung function decline. Furthermore, in our study, the presence of chronic bronchitis is also associated with the number of moderate and severe exacerbations per year.

Chronic bronchitis is the result of chronic mucociliary dysfunction, which is associated with bacterial infections. An increased vulnerability for pulmonary infections, has also been demonstrated in epidemiological studies. In healthy mucociliary clearance, bacteria interact with carbohydrates of mucins and are subsequently transported towards the pharynx. The adherence of mucus implies that bacteria are not eliminated and thus chronic bacterial infection is favored. In addition, acidification of airway surface liquid, due to lack of HCO$_3^-$ secretion or due to the acidic nature of cigarette smoke itself, impairs bacterial killing. This may explain why subjects with COPD and concomitant chronic bronchitis are more at risk for experiencing COPD exacerbations, since the majority of exacerbations are caused by infections. However, chronic bronchitis is often not a stable feature and the majority of subjects with
chronic bronchitis seem to follow a remitting-relapsing course. This implies that cross-sectional studies or studies with a short follow-up period might not detect associations. The Rotterdam Study allowed us to measure exacerbation rate during a long follow-up period, resulting in the observation that COPD subjects with chronic bronchitis have an increased risk for being a frequent exacerbator.

In the Rotterdam Study, we detected that especially women with COPD and chronic bronchitis have an increased mortality risk. Stratifying for the cause of death revealed that in the presence of chronic bronchitis, subjects with COPD died more frequently due to pulmonary related causes compared to COPD subjects without chronic bronchitis. This might explain the increased mortality risk in women detected here, since it has been observed before that women are more vulnerable for COPD-related death and hospitalizations due to pulmonary infections.

In conclusion, in the Rotterdam Study, a large prospective, population-based cohort study, we have shown that subjects with COPD and concomitant chronic bronchitis have an increased risk for accelerated lung function decline, COPD exacerbations and for being a frequent exacerbator. Furthermore, in the presence of chronic bronchitis, subjects with COPD have an increased risk of mortality and importantly, women with COPD and chronic bronchitis have a higher mortality risk than men with COPD and chronic bronchitis.

10.1.3 Mucociliary dysfunction: Future perspectives

Currently, there is not a satisfying therapy available to treat chronic bronchitis symptoms. Several attempts have been made to increase mucociliary function resulting in, at best, partial improvement of the symptoms. A thorough knowledge of the contributing factors can aid in the search for new therapeutic options. Therefore, it would be interesting to unravel the contribution of airway surface liquid acidification to cigarette smoke-induced chronic bronchitis. In COPD patients with chronic bronchitis, the vulnerability to bacterial infections could potentially be explained by the decreased activity of antimicrobials in the presence of an acidic pH. Reduced pH has been measured in exhaled breath condensate of patients with COPD. Interestingly, ex-smokers with COPD had lower pH values in exhaled breath condensates than current smokers with COPD. Of note, recently it was shown that administration of resolvin D1, a specialized proresolvin lipid mediator, reduced chronic inflammation and emphysema in cigarette smoke-exposed mice. However, mice are less vulnerable to acidification due to lack of H+/K+ adenosine triphosphatase which is present in human airway epithelial cells. With proper correction for acidic pH, these naturally occurring lipid mediators could be a candidate for a new therapeutic option.
Another interesting line of inquiry is the expression and function of Short Palate Lung and Nasal epithelial Clone 1 (SPLUNC1) in patients with COPD. SPLUNC1 is thought to be a soluble volume sensor that regulates the airway surface liquid hydration by inhibiting ENaC. SPLUNC1’s capacity to negatively regulate ENaC is pH-sensitive; pH ≤ 6.5 inactivates SPLUNC1 \(^{124}\). Cleavage of SPLUNC1 results in a peptide, S18, that can inactivate ENaC in a pH-independent manner, and is therefore currently considered a molecule of interest in cystic fibrosis \(^{352}\).

Whether or not chronic bronchitis is a pre-stage of COPD for a subgroup of patients is currently extensively discussed. Earlier this year, two papers were published describing the importance of chronic bronchitis in subjects without COPD. Allinson \textit{et al.} analyzed data from the Medical Research Council National Survey of Health and Development and found that chronic mucus hypersecretion in smokers between 36 and 43 years old predisposes for the development of COPD. The longer the mucus hypersecretion was present over 20 years, the greater the impact on lung function decline \(^{314}\). In addition, Woodruff \textit{et al.} observed that smokers with respiratory symptoms but without airflow limitation are more vulnerable for respiratory exacerbations and experience more physical limitations than asymptomatic smokers, underlining the impact of respiratory symptoms \(^{353}\). Considering that middle-aged symptomatic (ex-)smokers without COPD represent a population which has been excluded from clinical COPD trials, it would be interesting to study the impact of chronic bronchitis in subjects without COPD and determine the impact on several clinically important outcomes such as risk for developing COPD, risk for hospitalization, medication use, risk for cardiovascular disease, mortality... in the Rotterdam Study.
10.2 B CELL RICH LYMPHOID FOLLICLES IN COPD

10.2.1 ROLE OF B CELL-ACTIVATING FACTOR IN COPD

Along with disease severity, an increased number of B cells and lymphoid follicles are observed in lungs of patients with COPD. Although the demonstration of oligoclonality in the follicular B cells suggests a local antigen-specific immune response, the role of these lymphoid follicles in COPD is still controversial, especially since it is not known which antigens are involved. Different options come to mind why these lymphoid follicles are generated. In a first scenario, these lymphoid follicles are formed in reaction to infections. Lymphoid follicles are predominantly observed in patients with severe and very severe COPD and these patients also experience significantly more acute exacerbations than patients with mild-to-moderate COPD. As discussed before, the majority of exacerbations are infectious by nature. In addition, lymphoid follicles in COPD patients can be a response to the altered microbiome or chronic infection in the lung. The lung microbiome of COPD patients is significantly different from that of control patients. A recent murine study showed that host-microbiome crosstalk favored lymphoid neogenesis. Furthermore, chronic pulmonary infection might predispose for acute exacerbations. Another option is that lymphoid follicles are generated as part of an autoimmune response. Several different auto-antibodies have been described. In addition, an increasing number of studies suggest a crucial role for IL-17A, a cytokine implicated in autoimmunity, in CS-induced pathology. A final alternative that cannot be excluded as long as the inducing antigens are not identified and confirmed, is that the options stated here, might all be valid. Baraldo et al. reported both oligoclonal and polyclonal Ig rearrangements. Moreover, since oligoclonality has only been investigated in small numbers of patients, it is far from certain that all COPD patients generate lymphoid follicles against the same type of antigen. In patients with (very) severe COPD, the immune system has been overstimulated for decades and is perhaps primed to easily form lymphoid follicles. Immune cell-stromal cell interactions might be important in this respect.

In two consecutive experiments, we have attempted to elucidate the functional role of lymphoid follicles in COPD by interrupting their formation. In a first experiment, we prevented lymphoid follicle formation by administering anti-CXCL13 to CS-exposed mice. Prophylactic administration, i.e. from the start of the CS exposure, attenuated CS-induced airway inflammation and alveolar destruction. Although the intervention with anti-CXCL13 prevented the formation of organized follicles, B cell aggregates were still present. Next, we antagonized BAFF by administration of soluble BAFFR-Fc in chronic cigarette smoke-exposed mice. This intervention resulted in almost complete loss of B cells in BAL, lung and mediastinal...
lymph nodes, thus preventing the formation of lymphoid follicles and aggregates. As a consequence, immunoglobulin levels in BAL and serum were significantly decreased. Prophylactic administration of BAFFR-Fc, significantly attenuated airway and pulmonary inflammation, to a greater extent than anti-CXCL13 treatment. Furthermore, as did prophylactic anti-CXCL13 treatment, prophylactic administration of BAFFR-Fc partially protected CS-exposed mice against alveolar wall destruction. This indicates that organized lymphoid follicles, rather than the mere presence of B-cells, contribute to the development of emphysema.

The importance of lymphoid follicles in the development and progression of emphysema is recently substantiated by the recent network analysis of lung transcriptomics that identified a B cell signature and lymphoid follicle formation in COPD patients with emphysema but not in those with obstructive bronchiolitis 250. In addition, the 3D reconstructions of Mori et al. show that the majority of lymphoid follicles are in contact with alveolar epithelium and a vast lymphatic vessel network 243. Moreover, B cell-deficient mice which lack lymphoid follicles, are completely protected against CS-induced development of emphysema 276. All together, these observations suggest that the antigens inducing lymphoid follicle formation in COPD are likely sampled from within the alveoli and not only from within the airways. Although this does not pin down the identity of the responsible antigens, it narrows down the search and might contribute to determine whether these lymphoid follicles are protective or harmful in the pathogenesis of COPD. Of note, the lack of protection against the development of emphysema by therapeutic administration of anti-CXCL13 or BAFFR-Fc, i.e. administration that starts after 3 months of CS exposure, supports the idea that immunoglobulins contribute to the development of emphysema. In mice, the presence of lymphoid follicles after only 3 months of CS exposure is uncommon; however, immunoglobulins against alveolar antigens may be generated in lung-draining lymph nodes.

In contrast to prophylactic administration of BAFFR-Fc, therapeutic administration did not have an effect on CS-induced inflammation, although B cells and lymphoid follicles were absent and immunoglobulin levels were decreased to the same level as in the prophylactic setting. This discrepancy suggests that during the first 3 months of CS exposure, B cells and/or immunoglobulins play an active role in CS-induced pathology. It has been repeatedly described that B cells modulate inflammatory responses in their capacity of cytokine-secreting and antigen-presenting cells 273-277. To a lesser extent than dendritic cells, B cells also carry antigen to the lung-draining lymph nodes 356. Additionally, in the first 3 months of CS exposure, BAFF could have affected the immune response. BAFF is known to promote monocyte survival, dendritic cell activation and skew the T cell response towards a Th1 and Th17 response 229-232.
Importantly, in lung tissue of patients with COPD, we confirmed the work of Polverino et al. and Ladjemi et al. by demonstrating an upregulation of BAFF mRNA expression and an elevated BAFF protein level in lung homogenate \(^{263, 264}\). We continued to investigate BAFF in patients with COPD by performing a series of co-staining experiments on lymphoid follicles. We co-localized BAFF with stromal and immune cell types. Importantly, we corroborated recent observations that fibroblastic reticular cells contribute to the production of BAFF \(^{227}\). The high amount of BAFF staining in lymphoid follicles suggest the generation of a micro-environment that favors B cell maturation and survival.

10.2.2 Lymphoid follicles in COPD: Future perspectives

Although substantial progress has been made to elucidate the role of lymphoid follicles in patients with COPD, the inducing antigens are still unknown. Microbiota are suggested to be critical and in that regard it would be interesting to expose germ-free mice chronically to CS. Yadava et al. reported that the microbiome promotes the formation of lymphoid follicles in an LPS/elastase model \(^{109}\). However, CS is a far more complex stimulus and the chronicity of the exposure might also influence the host-microbiome interactions. By exposing germ-free mice chronically to CS, the contribution of the host-microbiome interactions to the pathogenesis of COPD can be studied. In addition, depletion (either selective or complete, of microbiota in mice chronically exposed to CS, can also contribute to the role of microbiota in the persistence of inflammation and lymphoid follicles.

Stromal cell – immune cell interactions have stepped into the spotlight in chronic inflammatory diseases \(^{254}\). Investigating these interactions might help to identify which cell type acts as lymphoid tissue organizer cell and which immune cell acts as lymphoid tissue inducer cell. Furthermore, it is highly possible that stromal cell – immune cell interactions change due to chronic infections of the lung, a feature that is highly prevalent in patients with COPD. The most common bacteria that cause chronic infections in patients with COPD are non-typeable *Haemophilus influenzae* (NTHi), *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* \(^{357, 358}\). The reaction of the host to the infectious bacteria might play a key role in the pathogenesis of COPD \(^{117}\). Therefore, combining co-cultures of stromal cell and immune cells with bacterial lysates or live bacteria can help in elucidating the initiating factors of lymphoid follicle formation.

Finally, lymphoid follicles are important sites for local induction of IgA responses. As mentioned in the introduction, IgA is a crucial component of the mucociliary clearance system. In patients with COPD, increased production of IgA does not translate into increased secretory IgA levels in BAL due to
downregulation of the polymeric immunoglobulin receptor$^{264,268}$. Cigarette smoke exposure of pIgR$^{-/-}$ mice can help to unravel the importance of this feature in patients with COPD.

### 10.3 General Conclusion

In conclusion, a functional mucociliary clearance is of utmost importance in host defense. In βENaC-Tg mice, airway surface dehydration enhances airway inflammation, mucin expression and alveolar destruction. Furthermore, chronic bronchitis, the clinical discourse of dysfunctional mucociliary clearance, increases the risk of COPD subjects for an accelerated decline of lung function, exacerbations and respiratory mortality.

We have also shown that BAFF is increased in patients with COPD and is highly expressed in lymphoid follicles, indicating the development of a micro-environment that favors B cell maturation and survival. Furthermore, antagonizing BAFF with consequent loss of B cells attenuates pulmonary inflammation and alveolar wall destruction in chronic CS-exposed mice, suggesting a more central role for BAFF, B cells and lymphoid follicles in COPD pathogenesis than considered so far.
Figure 23: Schematic overview of the research work presented in this thesis.

Cigarette smoke induces internalization of cystic fibrosis transmembrane conductance regulator (CFTR), leading to loss of Cl⁻ and HCO₃⁻ secretion and stimulation of Na⁺ absorption via the Epithelial Natrium Channel (ENaC). The net result is airway surface dehydration and mucus stasis/adherence which aggravates the CS-induced inflammatory response and mucus production.

Innate immune cells produce B cell-activating factor (BAFF) which stimulates B cell survival, maturation and differentiation. In addition, within lymphoid follicles both stromal and immune cells produce BAFF, resulting in an environment that promotes B cell survival and differentiation. The presence of organized lymphoid follicles aggravates airway and pulmonary inflammation and alveolar wall destruction.
PART II

CHAPTER 11: SUMMARY / SAMENVATTING
SUMMARY

Chronic obstructive pulmonary disease (COPD) is a highly prevalent, disabilitating disease, affecting more than 200 million people worldwide. COPD is characterised by an abnormal inflammatory reaction and destruction of lung tissue in response to noxious particles and gases, most commonly cigarette smoke. The main symptoms are progressive and persistent breathlessness accompanied by chronic cough and sputum.

In healthy individuals, the mucociliary clearance system captures inhaled noxious substances and transports them towards the pharynx. Chronic cigarette smoke exposure compromises the function of this system by increasing mucus production and altering the activity of ion channels, both leading to dehydration of the airway surface liquid. We have studied the effect of airway surface dehydration on cigarette smoke-induced pathology in a murine model of COPD. The presence of airway surface dehydration aggravated airway and pulmonary inflammation, increased mucin expression and aggravated destruction of alveolar tissue.

Chronic bronchitis, the clinical manifestation of dysfunctional mucociliary clearance, is common in subjects with COPD. We investigated whether the presence of chronic bronchitis in COPD subjects altered the risk for acute worsening of COPD and mortality. To study this we used data from a large prospective, population-based cohort: the Rotterdam Study. We observed that COPD subjects with chronic bronchitis have an increased risk for acute exacerbations of COPD and an increased risk for respiratory mortality. Interestingly, especially women with COPD and chronic bronchitis have an increased mortality risk.

In a third investigation, we investigated the role of B cell-rich lymphoid follicles in patients with COPD, by antagonizing B cell-activating factor (BAFF), a necessary factor for B cells to survive, mature and differentiate. First, we studied the expression and localisation of BAFF in patients with COPD. We determined that both stromal cells and immune cells in lymphoid follicles produce BAFF, thus supporting the survival of B cells. Next, we antagonized BAFF in mice that were chronically exposed to cigarette smoke. We observed that antagonizing BAFF resulted in complete loss of B cells and lymphoid follicles and attenuated pulmonary inflammation and destruction of alveolar tissue. We concluded that B cells and BAFF mediate pulmonary inflammation and that organized lymphoid follicles are important in the development of emphysema.
SAMENVATTING

Chronisch obstructief longlijden (COPD) is een zeer frequent voorkomende ziekte die wereldwijd meer dan 200 miljoen mensen treft. COPD wordt gekenmerkt door een abnormale ontstekingsreactie en afbraak van longweefsel na langdurige inhalatie van schadelijke gassen en partikels, meestal sigarettenrook. De voornaamste symptomen zijn progressieve en continue kortademigheid, samen met chronische hoest en slijm productie.

In gezonde luchtwegen zorgt het mucociliair systeem voor het vangen en verwijderen van schadelijke substanties. Chronische blootstelling aan rook compromitteert de functie van dit systeem door de werking van de ionenkanalen aan te tasten en de productie van slijm te verhogen, met dehydratatie van de vochtlaag die de luchtwegen bedekt, als gevolg. Wij hebben het effect van dehydratatie van het luchtwegoppervlak op sigarettenrook-geïnduceerde pathologie onderzocht in een muismodel. Onze studie wijst uit dat dehydratatie van het luchtwegoppervlak bijdraagt tot de ontstekingsreactie in de luchtwegen, expressie van mucines en beschadiging van longweefsel in reactie op sigarettenrook.

Dysfunctionele mucociliaire klaring uit zich klinisch als chronische bronchitis en is veelvoorkomend in personen met COPD. We hebben nagegaan of de aanwezigheid van chronische bronchitis in personen met COPD het risico verhoogt op plotse verergeringen van COPD en mortaliteit. Om dit te bestuderen hebben we gebruik gemaakt van gegevens uit de Rotterdam Studie, een grote cohorte studie bij de algemene bevolking. De studie toont aan dat mensen met COPD en chronische bronchitis een verhoogde kans hebben op acute verergeringen van hun COPD symptomen en een verhoogd risico op sterfte door longaandoeningen. Deze studie wijst ook uit dat vooral vrouwen met COPD en chronische bronchitis een verhoogd risico op exacerbaties en sterfte hebben.

In een derde onderzoek hebben we de rol van lymfoïde follikels in patiënten met COPD onderzocht. B cellen zijn de voornaamste cellen in deze lymfoïde follikels en zijn afhankelijk van B cell-activating factor (BAFF) voor hun overleving, maturatie en differentiatie. Eerst hebben we de expressie en lokalisatie van BAFF in patiënten met COPD bestudeerd. We stelden vast dat zowel stromale als immuun cellen in lymfoïde follikels BAFF produceren en zodoende een gunstig microklimaat genereren dat de overleving van B cellen stimuleert. Vervolgens hebben we BAFF geantagoniseerd in muizen die chronisch aan rook werden blootgesteld. Het antagoniseren van BAFF leidde tot verlies van B cellen en lymfoïde follikels en verminderde de ontstekingsreactie in luchtwegen en de afbraak van longweefsel. We concludeerden dat BAFF en B cellen de ontstekingsreactie in de longen mediëren en dat georganiseerde lymfoïde follikels bijdragen tot de ontwikkeling van emfyseem.
PART III

ADDENDUM
REFERENCES


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Work experience

2012 – present: PhD candidate in Health Sciences – Department Respiratory Medicine – Faculty of Medical and health sciences – Ghent University Hospital - Belgium
2006 – 2012: Independent equine veterinarian

Education

2002-2006: Veterinary medicine – Major in Equine medicine
Faculty of Veterinary medicine – Ghent University – Belgium
1997-2002: Master in Biology, Major in Animal Science
Faculty of Science – Ghent University – Belgium

Additional courses

2016: PhD career focus – Ghent University – Belgium
2015: Translational Biomedical in vivo imaging – Ghent University – Belgium
Speed Reading – Ghent University – Belgium
2014: Multicolor flow cytometry course - BD Biosciences – Erembodegem – Belgium
2013: Advanced academic English: writing skills– Ghent University - Belgium
Advanced academic English: conference skills– Ghent University – Belgium
2012 Laboratory animal science – Ghent University - Belgium
Clinical studies: study design, implementation and reporting– Ghent University - Belgium
Statistical analysis with SPSS (advanced course) – Ghent University - Belgium

Awards/grants

2016 Facultair opvangmandaat November 2016 – September 2017


2006 Award for best Master thesis from the Belgian Equine Practitioner Society (BEPS)

Attended conferences


2015 European Congress of Immunology, Vienna, Austria, September 6th – 9th 2015
IUAP meeting AIReWAY II, Leuven, Belgium, June 2015

ATS international Conference, San Diego, USA, May 16th – 29th 2014
IUAP meeting AIReWAY II, Liège, Belgium, June 13th 2014

2013 IUAP meeting AIReWAY II, Ghent, Belgium, March 8th 2013

2012 Chronic inflammatory disorders of the lung. Freiburg, Germany, September 28th – 29th 2012
Abstracts and presentations

- **IRC Symposium**, Ghent, Belgium, September 14th 2016
  Absence of polymeric immunoglobulin receptor aggravates cigarette smoke-induced inflammation in mice (poster presentation).

- **American thoracic society conference**, San Francisco, USA, May 2016
  - Epidemiology of Chronic Bronchitis in Chronic Obstructive Pulmonary Disease (COPD) (Poster presentation)

- **Belgische Vereniging voor Pneumologie**, GSK awards, Brussel, Belgium, May 6th 2015
  Role of B cell-activating factor in COPD (Oral presentation)

- **American thoracic society conference**, San Diego, USA, May 16th – 21st 2014
  - The role of B cell activating factor in cigarette smoke-induced follicle formation and COPD (Oral presentation)
  - Cigarette smoke exposure of transgenic β-ENaC overexpressing mice as a model for COPD (Poster session)

- **12th Lung Science Conference ‘Lung inflammation and immunity’**, Estoril, Portugal, March 21st – 23rd 2014
  Antagonizing B cell activating factor (BAFF) prevents formation of lymphoid follicles in cigarette smoke-exposed mice (Oral presentation)

- **IUAP meeting AIReWAY II**, Liège, Belgium, June 13th 2014
  The role of B cell activating factor in cigarette smoke-induced follicle formation and COPD (Oral presentation)

- **Belgische Vereniging voor Pneumologie**, GSK awards, Brussel, Belgium, June 11th 2014
  Cigarette smoke-exposure of transgenic β-ENaC overexpressing mice - A model for COPD (Oral presentation)

Peer-reviewed publications

  IF: 11.986, ranking Respiratory System: 1/54

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Leen