From the clinic to the laboratory: azithromycin for prevention of COPD exacerbations

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CHAPTER 5: MANUSCRIPT 2 "THE MECHANISM OF ACTION OF AZITHROMYCIN TO REDUCE THE RISK OF COPD EXACERBATIONS"

ABSTRACT

Background: Chronic obstructive pulmonary disease (COPD) patients often experience exacerbations despite being on optimal therapy. The key feature of COPD exacerbation is inflammation that involves multiple immune cells and mediators. Global Initiative for Chronic Obstructive Lung Disease (GOLD 2017) has recommended macrolides as third-line therapy for patients with recurrent exacerbations already being on optimal inhaled treatment (GOLD D). It was unknown if patients with severe disease and those colonized with *Pseudomonas aeruginosa* would benefit from treatment with macrolides. In addition, benefits beyond one year of treatment had not been studied.

The mechanisms by which macrolides prevent exacerbations are believed not to be the antibacterial effect but to be related to the anti-inflammatory and immune-modulatory effects, preventing the production of proinflammatory mediators from the host. In addition, biofilm development and bacterial adherence of pathogens such as *P. aeruginosa* are affected by macrolides. It is unknown if these patients might benefit from long-term therapy with the macrolide antibiotic azithromycin and if smoking could interfere with the therapeutic response. We hypothesized Long-term treatment with azithromycin decreases exacerbation frequency and health service use in severe COPD patients by modulating inflammation in human airway epithelial cells.

Objectives: First, to evaluate the effectiveness of long-term azithromycin in reducing exacerbations in patients with severe COPD on optimal therapy, considering smoking status, disease characteristics such as presence or absence of bronchiectasis, and colonization with

P. aeruginosa. Second, to assess the mechanism through which azithromycin modulates inflammation in an *in vitro* model of cigarette smoke-exposed airway epithelial cells.

Methods: Firstly, we conducted a retrospective observational study of patients with severe COPD who were prescribed azithromycin (250 mg, at least 3 times weekly for at least 6 months). The control group included patients with severe COPD not exposed to azithromycin. Secondly, in order to study the effect of azithromycin on cigarette smoke-induced inflammation, first BEAS-2B bronchial epithelial cells were incubated with 5% cigarette smoke extract (CSE) for 3h, 6h, and 24h. Expression and release of IL-6 and IL-8 mRNA were analyzed by quantitative real-time PCR (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. Then, airway epithelial cells were pretreated with azithromycin and exposed to 5% CSE. Expression and release of IL-8 and IL-6 were measured by qRT-PCR.

Results: Clinical project: study included 126 cases and 69 controls. Patients had severe airflow obstruction, mostly emphysema, and one-third bronchiectasis. A predominant feature in the case group was respiratory tract colonization with *P. aeruginosa*. The mean number of exacerbations per patient per year in the case group was 3.2± 2.1 before initiating azithromycin, and 2.3 ± 1.6 during the following year on therapy ($p<0.001$). Patients in the control group had 1.7±1.3 and 2.5±1.7 exacerbations during the first and second follow-up year respectively ($p<0.001$). Exacerbation changes from pre to post differed between groups ($p<0.001$). Reduction in emergency visits and hospital admissions was significant in the case group. The reduction in exacerbations appeared to be more modest in active smokers compared to former smokers, although both benefited from treatment with azithromycin. The subgroups of patients with respiratory colonization with *P. aeruginosa,* and those with and without bronchiectasis on CT scan also had a significant reduction in the number of exacerbations after one year of treatment with azithromycin. Exacerbation reductions and patient proportions having ≥2 exacerbations extended to the second year of treatment.

In vitro project: we observed a significant increase of IL-6 and IL-8 mRNA following 3h, 6h and 24h exposure to 5% CSE. Similarly, IL-8 secretion was significantly increased after exposure to 5% CSE for 24h. when cells were pre-treated with azithromycin and exposed to 5% CSE for 3h, a significant dose-dependent decrease in the expression of IL-6 mRNA was observed. Finally, our results revealed an inhibition of IL-6 and IL-8 mRNA levels in 5% CSE-exposed BEAS-2B cells when the cells were pretreated with 9μg/mL azithromycin.

Conclusion: Our clinical study showed that in real life of practice, long-term azithromycin reduces the risk of exacerbations that persist beyond one year in patients with severe COPD on optimal therapy still having a history of recurrent exacerbations. Desirable effects are more likely to outweigh the risks and adverse events in patients colonized with *P. aeruginosa.* Our *in vitro* study showed that Incubation with azithromycin resulted in a significant decrease in the expression of inflammatory mediators in BEAS-2B cells exposed to CSE.

In overall, Treatment with azithromycin decreases exacerbation frequency and health service use in severe COPD patients by modulating inflammation in human airway epithelial cells exposed to CSE.

RÉSUMÉ

Contexte : Les patients atteints de maladie pulmonaire obstructive chronique (MPOC) présentent souvent des exacerbations malgré un traitement optimal. La principale caractéristique de l'exacerbation de la MPOC est l'inflammation impliquant plusieurs cellules immunitaires et médiateurs. L'Initiative mondiale contre les maladies pulmonaires obstructives chroniques (GOLD 2017) a recommandé les macrolides en tant que traitement de troisième intention chez les patients présentant des exacerbations récurrentes déjà sous traitement inhalé optimal (GOLD D). Il était inconnu si les patients atteints d'une maladie grave et ceux colonisés par Pseudomonas aeruginosa bénéficieraient d'un traitement par macrolides. De plus, les bénéfices au-delà d'un an de traitement n'ont pas été étudiés.
Les mécanismes par lesquels les macrolides préviennent les exacerbations ne semblent pas être l'effet antibactérien, mais sont liés aux effets anti-inflammatoires et immunomodulateurs, empêchant la production de médiateurs pro-inflammatoires par l'hôte. De plus, le développement de biofilms et l'adhérence bactérienne d'agents pathogènes tels que *P. aeruginosa* sont affectés par les macrolides. On ignore si ces patients pourraient bénéficier d'un traitement à long terme par l'azithromycine, un antibiotique macrolide, et si le fait de fumer pourrait interférer avec la réponse thérapeutique. Nous avons émis l'hypothèse qu'un traitement à long terme par l'azithromycine diminue la fréquence d'exacerbation et l'utilisation des services de santé chez les patients atteints de BPCO sévère en modulant l'inflammation dans les cellules épithéliales des voies aériennes.
Objectifs : D'abord, évaluer l'efficacité de l'azithromycine à long terme pour réduire les exacerbations chez les patients atteints de BPCO sévère sous traitement optimal, en tenant compte du tabagisme, des caractéristiques de la maladie telles que la présence ou l'absence de bronchectasie et la colonisation avec *P. aeruginosa.* Deuxièmement, évaluer le mécanisme

par lequel l'azithromycine module l'inflammation dans un modèle in vitro de cellules épithéliales des voies aériennes exposées à la fumée de cigarette.

Méthodes : Dans un premier temps, nous avons mené une étude observationnelle rétrospective sur des patients atteints de MPOC grave à qui on avait prescrit de l'azithromycine (250 mg, au moins 3 fois par semaine pendant au moins 6 mois). Le groupe témoin comprenait des patients atteints de BPCO sévère non exposés à l'azithromycine. Deuxièmement, afin d'étudier l'effet de l'azithromycine sur l'inflammation induite par la fumée de cigarette, les premières cellules épithéliales bronchiques BEAS-2B ont été incubées avec un extrait de fumée de cigarette à 5% pendant 3h, 6h et 24h. L'expression et la libération de l'ARNm d'IL-6 et d'IL-8 ont été analysées par PCR en temps réel quantitative (qRT-PCR) et par dosage immunoenzymatique (ELISA). Ensuite, les cellules épithéliales des voies aériennes ont été prétraitées avec de l'azithromycine et exposées à 5% de CSE. L'expression et la libération d'IL-8 et d'IL-6 ont été mesurées par qRT-PCR.

Résultats :

Projet clinique : l'étude comprenait 126 cas et 69 contrôles. Les patients présentaient une obstruction respiratoire sévère, principalement un emphysème et un tiers de bronchectasie. Une caractéristique prédominante dans le groupe de cas était la colonisation des voies respiratoires avec *P. aeruginosa*. Le nombre moyen d'exacerbations par patient et par an dans le groupe de cas était de 3,2 ± 2,1 avant l'initiation de l'azithromycine et de 2,3 ± 1,6 au cours de l'année suivant le traitement ($p < 0,001$). Les patientes du groupe témoin présentaient des exacerbations de 1,7 ± 1,3 et 2,5 ± 1,7 au cours de la première et de la deuxième année de suivi, respectivement ($p < 0,001$). Les changements d'exacerbation de pré à post différaient entre les groupes ($p < 0,001$). La diminution des visites d'urgence et des admissions à l'hôpital était significative dans le groupe de cas. La réduction des exacerbations semblait être plus modeste chez les fumeurs actifs que chez les anciens fumeurs, bien que tous deux

aient bénéficié d'un traitement à l'azithromycine. Les sous-groupes de patients présentant une colonisation respiratoire avec *P. aeruginosa* et ceux avec et sans bronchectasie au scanner ont également connu une réduction significative du nombre d'exacerbations après un an de traitement par l'azithromycine. La réduction des exacerbations et les proportions de patients ayant acer2 exacerbations étendues à la deuxième année de traitement.

Projet *in vitro* : nous avons observé une augmentation significative de l'ARNm de l'IL-6 et de l'IL-8 après une exposition de 3h, 6h et 24h à 5% de CSE. De même, la sécrétion d'IL8 était significativement augmentée après une exposition à 5% de CSE pendant 24 heures. Lorsque les cellules ont été prétraitées avec de l'azithromycine et exposées à 5% de CSE pendant 3H, une tendance à une diminution dose-dépendante de l'expression de l'ARNm de l'IL-6 a été observée. Enfin, nos résultats ont révélé une inhibition des taux d'ARNm d'IL-6 et d'IL-8 dans des cellules BEAS-2B exposées à 5% de CSE lorsque les cellules ont été prétraitées avec 9 μg/ml d'azithromycine.

Conclusion : Notre étude clinique a montré que dans la pratique, l'azithromycine à long terme réduit le risque d'exacerbations qui persistent au-delà d'un an chez les patients atteints de BPCO sévère avec un traitement optimal ayant encore des exacerbations récurrentes. Les effets souhaitables sont plus susceptibles de l'emporter sur les risques et les effets indésirables chez les patients colonisés par *P. aeruginosa*. Notre étude in vitro a montré que l'incubation avec l'azithromycine entraînait une diminution significative de l'expression des médiateurs inflammatoires dans les cellules BEAS-2B exposées à l'ESC.

Dans l'ensemble, le traitement par l'azithromycine diminue la fréquence d'exacerbation et l'utilisation des services de santé chez les patients atteints de BPCO sévère en modulant l'inflammation dans les cellules épithéliales des voies respiratoires humaines exposées à l'ESC.

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suggestions, which were determinant for the accomplishment of the work presented in this thesis.

A special thanks to Raquel Farias, the person who organized and supervised the translational research project, for serving as an invaluable mentor. When I started this study, I had no previous experience in a research lab and she had always encouraging words and was always ready to help me. I appreciate her patience, efforts and productive comments in guiding me in every step of the work described in *in vitro* study. She continually tried to solve complicated and hard aspects of research and guided me in the best way.

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Finally, I must express my very profound special thanks to my parents for providing me with endless love, support, and encouragement throughout my years of study as well as all of my life.

PREFACE AND CONTRIBUTION OF AUTHORS

One of the major goals of the projects constituting this Master thesis was to evaluate the effectiveness of long-term azithromycin to reduce exacerbations in severe COPD patient on optimal therapy. This thesis complies with the Graduate and Postdoctoral Studies' guidelines and general requirements of a manuscript- based (article-based) Master's theses at McGill University. This thesis consists of two manuscripts (one already published and another to be submitted in the first trimester of 2019) that address important research topics related to COPD patients.

This thesis contains **six chapters**:

Chapter 1 provides a comprehensive literature review on chronic obstructive pulmonary disease (COPD), acute exacerbations of COPD, treatment, and prevention of acute exacerbations, including prevention of acute exacerbations by the long-term therapy with macrolides and its mechanisms;

Chapter 2 introduces the thesis rationale, hypothesis, and objectives of the 2 projects, the clinical project and the laboratory project;

Chapters 3 to 5 include the two manuscripts, which constitute my thesis. Chapter 3 is the clinical project (manuscript 1) that evaluates the effects of long-term azithromycin to reduce exacerbations in severe COPD patients; chapter 4 is the bridging chapter which highlights the importance of developing a translational research project; chapter 5 is the laboratory research project (manuscript 2) that assess the mechanism through which azithromycin modulates inflammation in an *in vitro* model of cigarette smoke-exposed airway epithelial cells;

Chapter 6 summarises and discuss the overall findings and provides the final conclusions;

Chapter 7 provides the complete reference list; and

Chapter 8 contains supplementary material, methods, results with tables and/or figures.

My thesis supervisor, Jean Bourbeau, instrumentally contributed to all stages of the research from the conception and design of the research projects, implementation of the work to the analysis, active discussion of the results and thoughtful manuscript revisions. My co-supervisor, Carolyn Baglole, and Raquel Farias, a research associate in our team, contributed efficiently to all stages of *in vitro* project. They also provided guidance through informative feedback on the *in vitro* study design, experiments and in the interpretation of data, and edited the manuscripts and the thesis. Pei Zhi Li assisted in data acquisition and imparted support on the statistical analyses.

All chapters were written and completed by Nafiseh Naderi. Jean Bourbeau and Raquel Farias reviewed and edited the text in this thesis.

Chapters 3 is formatted and written according to the respective peer-reviewed journal's specifications. The manuscript in chapter 3 was submitted in the revised form to the Respiratory Medicine journal on December 05, 2017 and published on April 05, 2018.

For the clinical project (Manuscript 1), all authors made substantive intellectual contributions to the development of this study. Nafiseh Naderi was the first author and with Deborah Assayag and Jean Bourbeau, she contributed to the conception, planning, and design of the study protocol.

Nafiseh Naderi, Deborah Assayag, Seyed-Mohammad-Yousof Mostafavi-Pour-Manshadi, and Zeina Kaddaha have done the chart review and data extraction. Nafiseh Naderi, Deborah Assayag, Seyed-Mohammad-Yousof Mostafavi-Pour-Manshadi have reviewed the study protocol, participated in the analysis, and participated in the redaction and reviewed the

manuscript. Pei Zhi Li has done the analysis and reviewed the manuscript. Alexandre Joubert, Isabelle Ouellet, Isabelle Drouin have assisted in the chart review and reviewed the manuscript. Jean Bourbeau has written the protocol, planned and participated in the analysis, written and reviewed the manuscript. All authors approved the final version of the manuscript to be submitted, resubmitted and published to the peer-reviewed journal. Figures and tables are embedded in the manuscripts

For the *in vitro* project (Manuscript 2), which is still ongoing, Nafiseh Naderi is the lead author, while Raquel Farias, Jean Bourbeau, Carolyn Baglole contributed to the conception, planning, and design of the study. Immortalized human bronchial epithelial Beas-2B cells were provided by Dr. Simon Rousseau. Mira Abou Rjeili collaborated in ELISA experiments. All experiments were performed by Nafiseh Naderi in Carolyn Baglole's laboratory and in collaboration with the Meakins Christie laboratories.

CHAPTER 1: INTRODUCTION

1. Background

1.1. COPD

1.1.1 Definition and clinical manifestation

According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2017, Chronic obstructive pulmonary disease (COPD) is a preventable and treatable disease characterized by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases (1). The chronic airflow limitation is caused by a simultaneous presence of small airways disease (e.g., obstructive bronchiolitis) and parenchymal destruction (emphysema), the relative contributions of which vary from person to person (1). This structural dysfunction is associated with the enhanced chronic inflammatory response of the lung to noxious particles and gases. COPD is characterized by decreased lung function, shortness of breath, reduced capacity, and reduced quality of life (2).

Symptom burden, defined as symptom frequency, severity, and distress, is associated with reduced physical, psychological and social functioning as well as decreased health-related quality of life (3). Chronic and progressive dyspnea is the most characteristic symptom of COPD. Patients use different terms to describe their dyspnea including a sense of increased effort to breathe, chest heaviness, air hunger, or gasping. Cough with sputum production is present in up to 30% of patients. These symptoms may vary from day-to-day (4).

Chronic cough is often the first symptom of COPD, which is described by the patients as an expected consequence of smoking and/or environmental exposures. Chronic cough in COPD

may be productive or unproductive. A small amount of tenacious sputum is commonly seen in COPD patients with coughing. Regular production of sputum for three or more months in two consecutive years (in the absence of any other conditions that may explain it) is the clinical definition of chronic bronchitis. The presence of purulent sputum indicates an increase in inflammatory mediators and its development might be contributed to the onset of a bacterial exacerbation, though the association is relatively weak (1, 5). Wheezing and chest tightness follow exertion are some other symptoms that might be seen in these patients and varied between the days. Fatigue, weight loss, and anorexia are common problems in patients with severe and very severe COPD. Although COPD is defined on the basis of airflow limitation, patient's functional status including chronic respiratory symptoms or an acute, transient episode of exacerbated respiratory symptoms is a key factor in practice which is contributed to seeking medical help (1). In COPD patients with high symptom levels, increasing symptom burden is associated with increased health service resource use (HCRU) and had a detrimental impact on work productivity (6). Furthermore, the presence of COPD symptoms is associated with an increased risk of exacerbations and a worse disease prognosis.

1.1.2 Epidemiology of COPD

COPD is the fourth leading cause of death in the world and it is estimated to become the third leading cause of death by the year 2020. In the coming decades, due to consecutive exposure to many risk factors as well as population aging, COPD burden is anticipated to growth (1, 7).

According to the Global Burden of Disease (GBD) studies, COPD led to about 5% of global disability-adjusted life years (DALYs) (76.7 million), and 5% of total deaths (2.9 million) (8). In 2015, 3·2 million people died from COPD worldwide, an increase of 11·6% compared

with 1990 (9). Interestingly, while COPD was once more common in men than women, it is now being reported more in women than in men under age 75 (10). Chronic exposure to toxic substances and gases, especially tobacco smoke leads to abnormal inflammatory pulmonary and systemic response causing in susceptible individuals the onset of COPD (11). In fact, COPD emerges due to multiple factors, including genetic disorders (the only one known is alpha-1 antitrypsin (AAT) deficiency) and environmental factors (most commonly smoking, biomass fuel exposure and possibly air pollution) (1, 12).

As would be expected, smoking affects COPD prevalence, with 13·3% of current smokers, 6·8% of former smokers, and 2·8% of never-smokers reporting COPD (13, 14). However, data from both the USA and Canada suggest that 25% of those with COPD are never-smokers (14, 15). COPD is responsible for high death rates, early mortality, and significant cost to the healthcare system. Likewise, COPD accounts for a considerable amount of morbidity in the USA, including 10·3 million physician office visits,1·5 million emergency department visits, and 699 000 hospital discharges in 2010. In the US, costs from hospital admission and absenteeism in 2010 estimated at $36 billion. However, the actual cost could be much higher than these estimates (14).

1.1.3 COPD and cigarette smoke

Cigarette smoking is the most well studied and easily identifiable COPD risk factor. Cigarette smokers have a higher prevalence of respiratory symptoms and lung function abnormalities, a greater annual rate of decline in FEV1, and a greater COPD mortality rate than non-smokers (1, 16). Studies have shown that chronic airway irritation induced by cigarette smoke and other noxious particles and gases cause an increased number of goblet cells and enlarged submucosal glands, resulting in mucus hypersecretion (17).

In the lungs, exposure to cigarette smoke induces inflammation in several cell types, including epithelial cells, fibroblasts, and macrophages (18, 19). Cigarette smoke recruits inflammatory cells to the site of exposure, maintains their persistence, and increases inflammatory mediators, leads to damage to the airways and lungs (20, 21).

Cigarette smoke, which has been shown to activate several mediators and proteases, stimulates mucus hypersecretion through the activation of epidermal growth factor receptor (*EGFR*) and mucin MUC5AC in the epithelium of proximal airways (1, 17). Furthermore, it has been indicated that inhaled stimuli such as cigarette smoke are able to interfere with wound healing by inhibiting human bronchial epithelial cell repair process (17).
Cigarette smoking appears to attenuate innate immune responses, which results in pathogen proliferation and persistence in the airways of COPD patients. Cigarette smoke contains an extremely high concentration of oxidants. The reactive oxidant substances (ROS) generated by smoking induce inflammation in the central airways, peripheral airways, and lung parenchyma (22). In primary human bronchial epithelial cells (PHBECs) differentiated at the air-liquid interface, acute exposure to cigarette smoke extract (CSE) impairs barrier function and tight junction organization *in vitr*o (23, 24). These smoking-associated functional abnormalities in combination with mucus overproduction and decreased mucociliary clearance promote pathogen colonization and development of smoking-related lung disorders, such as COPD and lung cancer. Cigarette smoke specifically alters the cellular composition of the airway epithelium by affecting basal cell differentiation in a post-transcriptional manner through a reduction of acetylated tubulin levels, an increased expression of the basal cell marker keratin 14 (KRT14), and increased secretion of Clara cell-specific 10-kDa protein (CC10) (24). These findings indicate that CSE exposure significantly reduced the number of ciliated cells, while it increased the number of Clara and goblet cells (24).

Some studies have shown that the production of proinflammatory cytokines decrease in bronchial epithelial cells exposed to cigarette smoke in the presence of viruses and bacteria (25, 26) while other studies have demonstrated that cigarette smoke leads to the production of interleukin(IL)-8 and IL-6 (27, 28). This controversy is probably due to activation of different pathways which regulate the expression of proinflammatory cytokines.

1.1.4 Pathogenic mechanism and airway inflammation of COPD

Hyperplasia of mucus glands, chronic inflammation and an elevated number of goblet cells are airway abnormalities that have been observed in COPD, particularly chronic bronchitis. As well, alveolar wall destruction in emphysema causes loss of tethering and leads to reduced elastic recoil, narrowing, and disability of the airways to remain open during expiration, resulting in airflow limitation (29). Although lung inflammation is observed in all cigarette smokers, COPD develops in susceptible smokers due to enhanced or abnormal response to inhaling toxic substances leading to hypersecretion of mucus/sputum (chronic bronchitis), destruction of airway sacs (emphysema), and small airway inflammation and fibrosis (bronchiolitis) due to dysfunction of mechanism of normal repair and defence (30). The airway epithelium, which is the first line host defense against pathogens or irritants (e.g., cigarette smoke), produces various mediators such as proinflammatory cytokines (e.g., IL-8), mucins, and antimicrobial substances (e.g., β defensin-2). Likewise, the protective role of the epithelium includes recognition of potentially dangerous particulates and microbes. Although the production of these inflammatory cytokines and antimicrobial substances is tightly regulated, persistent and repeated infections in COPD patients suggest an abnormal epithelial cell function (31). It has been demonstrated that these bronchial epithelial cell abnormalities also persist in former smokers with COPD. Therefore, the abnormal structure of bronchial

epithelium and defective immune barrier functions are associated with the progression of airway inflammation, which is thought to be a key factor in the pathogenesis of COPD (32). Generally speaking, the inflammatory and structural changes in COPD patients are associated with the severity of airflow limitation. These changes will be persistent even after smoking cessation. An imbalance between proteases and anti-proteases and an inconsistency between oxidants and antioxidants (oxidative stress) in the lungs are also other components in the pathogenesis of COPD (30). Moreover, the severity of inflammation in COPD patients is related to the degree of airflow obstruction and disease activity which is associated with an annual decline in FEV1. During this inflammation process, many cytokines and mediators are released from inflammatory cells. Studies have shown that in COPD, there is an increase in levels of many inflammatory factors, such as leukotriene B4, T cell chemoattractants produced by macrophages, neutrophils, and epithelial cells, chemotactic factors, including the CXC chemokines interleukin 8 and growth-related oncogene produced by macrophages and epithelial cells. These cells play a key role as cell attraction and absorb many cells from the circulation and increase pro-inflammatory responses (30).

1.2. Acute exacerbations of COPD

1.2.1 Definition and clinical manifestations

Acute exacerbations, which are a common complication of COPD, are defined as an acute worsening of respiratory symptoms that often results in the need of additional therapy and account for the greatest proportion of the total COPD burden on the healthcare system (1, 33). COPD exacerbations are associated with increased airway inflammation, mucus production, and gas trapping. These changes contribute to worsening dyspnea which is the main characteristic of an exacerbation. Other prominent symptoms include increased sputum

purulence and/or volume together, often with an increased cough. Exacerbation defined as mild if increases in regular inhaled medication are required, moderate if courses of steroids and/or antibiotics are prescribed, and severe if the patient requires hospital admission (1, 34). During a COPD exacerbation, symptoms usually last between 7 to 10 days, but some events may last longer. Evidence suggests that COPD exacerbations contribute to disease progression (35). Exacerbations can also cluster in time and increase the susceptibility of COPD patients to another event. It is well established that the most important predictor of patient's future exacerbation is the presence of exacerbations in the previous year (36).

1.2.2 Epidemiology of COPD exacerbations

More than 50% of the total cost of COPD is accounted for by services related to exacerbations. For instance, in the UK, they are the most common cause of medical hospital admission, accounting for 15·9% of hospital admissions, at a cost to the National Health System of over £253 million a year (37). It has been reported that 70–80% of COPD exacerbations are triggered by viral or bacterial respiratory infections and the remaining 20–30% are related to exposure to environmental pollution or have an unknown etiology (38). Exacerbations of COPD impose a substantial burden on the healthcare systems worldwide (35). Patients experiencing COPD exacerbations will have accelerated disease progression, worse health status, and morbidity than patients with less frequent exacerbations.
COPD frequent exacerbations are associated with a faster decline in FEV1 and lung volumes, poorer quality of life, reduced physical activity and increase mortality, morbidity, and hospitalizations (39, 40).

1.2.3 Pathogenic mechanism of acute exacerbations

Evidence suggests that inflammation is a key factor of COPD exacerbations that involves multiple immune cells and mediators. Bacteria, viruses and environmental pollutants are the major cause of inflammation during exacerbations which lead to an increase in airway edema, bronchoconstriction and sputum production (Figure 1.1). During exacerbations there are physiological consequences such as increased hyperinflation and gas trapping, with the reduced expiratory flow, thus accounting for increased dyspnea and worsening of COPD symptoms (33). These triggers stimulate the cells in the lung parenchyma and airways to produce a variety of inflammatory mediators including cytokines, chemokines and reactive oxygen species (ROS). The epithelial cells, macrophages, and neutrophils have an important role in mediating inflammation in COPD. Crucial mediators produced by these cells include tumor necrosis factor alpha (TNF-α), IL-1, IL-6, and IL-8. These cytokines are markedly proinflammatory in COPD (41).

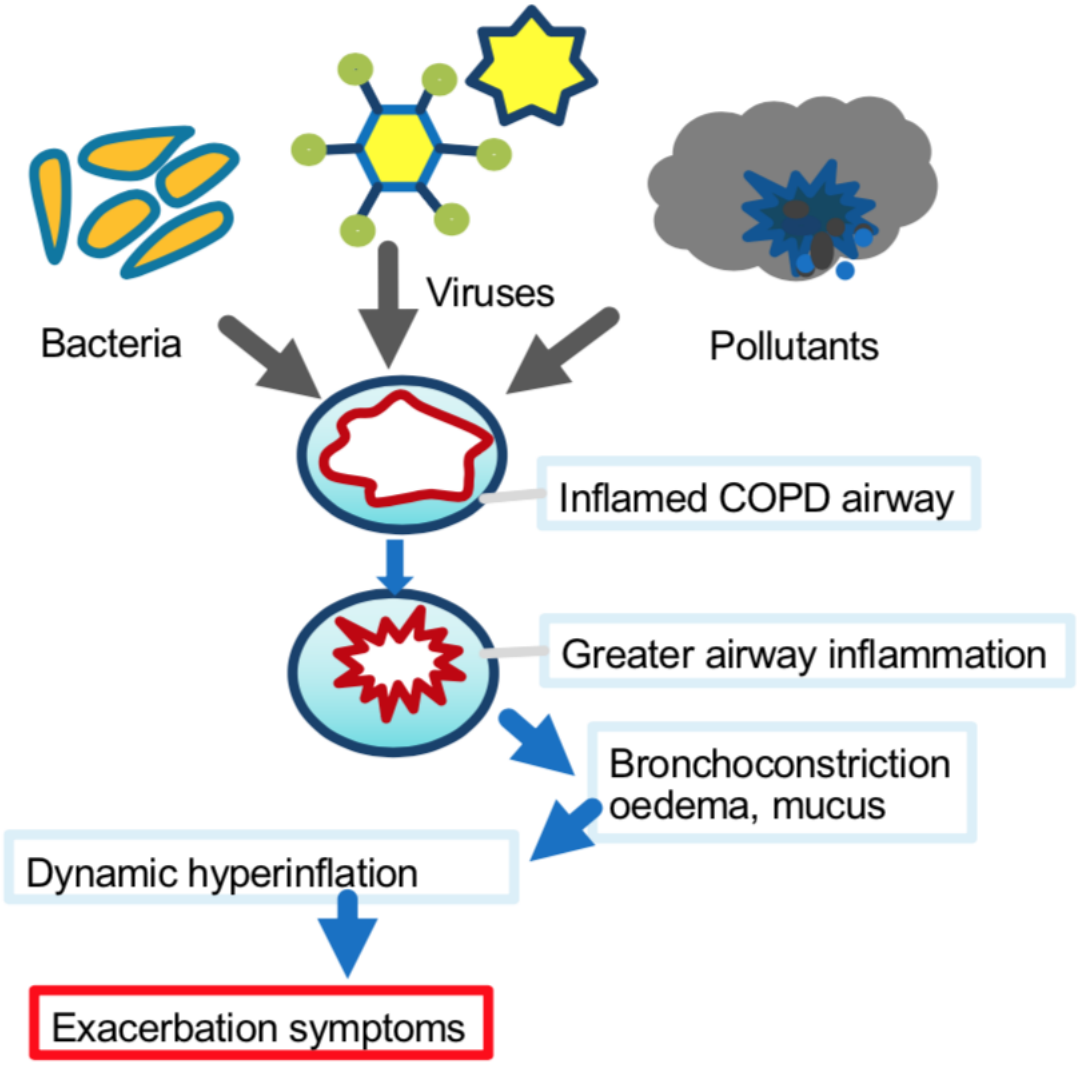

Figure 1.1: Exacerbation of COPD. Viral and bacterial infections, as well as pollutants, increase inflammation further in a chronically inflamed airway, lead to an increase in bronchoconstriction, edema and mucus production, resulting in promoting dynamic hyperinflation and symptoms of increased dyspnea. Adapted from Wedzicha JA, et al (33).

1.2.3.1 COPD exacerbations and *Pseudomonas aeruginosa (P. aeruginosa)*

Studies have shown that approximately 50% of COPD exacerbations are caused by bacterial infections which are associated with increased airway and systemic inflammation compared to nonbacterial exacerbations (38). Patients with severe COPD are often chronically

colonized with *P. aeruginosa*, where the risk of resistant *P. aeruginosa* (*PA-R*) is probably high. During adaptation to chronic infection, numerous virulence factors in *P. aeruginosa* are down-regulated, including invasiveness, protease production and secretion of toxins (42, 43). *H. influenzae and P. aeruginosa*, which are the most prevalent chronic bacterial pathogens in COPD, enhance mucus secretion, disrupt normal ciliary activity and cause airway epithelial injury, thereby further impairing mucociliary clearance. Levels of sputum cytokines or chemokines, such as TNF-α, IL-8, and LTB4 are elevated in patients with COPD whose sputum is colonized with bacterial pathogens as compared with patients without such pathogens (38). It has been reported that 5 to 10% of COPD exacerbations are caused by *P. aeruginosa* and this has been associated in patients with severe COPD (38). Individuals having FEV1 below 50% of the reference value have a six-fold higher risk of suffering exacerbations due to *H. influenzae* and *P. aeruginosa* than patients with mild or moderate COPD and this has been observed particularly in patients who have risk factors such as previous hospital admissions and use of oral corticosteroids or antibiotics. In these patients, the presence of *P. aeruginosa* is associated with higher mortality and worse clinical outcomes (44, 45).

1.2.4 Treatment of Acute Exacerbations

The goals of treatment for COPD exacerbations are twofold, to minimize their negative impact and, if possible, to prevent the development of subsequent events (46). According to the severity of an exacerbation and/or the underlying disease, an exacerbation can be managed in either an outpatient basis, with pharmacologic therapies including bronchodilators, corticosteroids, and antibiotics, or inpatient setting (47).

More than 80% of exacerbations are managed in an outpatient basis. Supplemental oxygen should be considered for all patients with COPD exacerbation present in the emergency and if

the situation is life-threatening with increased work of breath and impaired gas exchange they should be admitted to the respiratory or intensive care unit of the hospital, otherwise the patient might be managed in emergency department or medical ward (1).

In severe but not life-threatening exacerbations, management includes assessing the severity of the symptoms, obtaining serial spirometry, arterial and venous blood gas, measuring pulse oximetry, performing chest radiography as well as pharmacological interventions such as administration of bronchodilator and oral corticosteroid and consideration of oral antibiotics when signs of infections are present (1). Data from studies indicate that systemic glucocorticoids in COPD exacerbations shorten recovery time and improve lung function (FEV1) (48).

Antibiotics have been recommended for patients with COPD exacerbations who have two or three cardinal symptoms: increase in dyspnea, sputum volume, and sputum purulence or require mechanical ventilation (49). The recommended length of antibiotic therapy is 5-7 days. The choice of antibiotic should be considered based on the local bacterial resistant pattern. Usually, initial empirical treatment is an aminopenicillin with clavulanic acid, macrolide, or tetracycline**.** If patients have frequent exacerbations, severe airflow limitation, and/or exacerbations requiring mechanical ventilation, sputum culture should be considered as gram-negative bacteria (e.g., *Pseudomonas species*) or resistant pathogens that are not sensitive to the above-mentioned antibiotics (1).

1.2.5 Prevention of Acute COPD Exacerbations

Following an acute COPD exacerbation, appropriate measures for the prevention of further exacerbations should be implemented and this is part of the goals of treatment for COPD exacerbations. Optimizing treatment for stable COPD will help to reduce the risk of exacerbations. Pharmacological treatments such as long-acting bronchodilators, alone or

combined with inhaled corticosteroids, have demonstrated to be effective in reducing the rate of exacerbations in patients with COPD (1, 50). In order to prevent future exacerbations, non-pharmacological interventions such as vaccination, rehabilitation, smoking cessation and pharmacological interventions including inhaled bronchodilators, corticosteroids, and oral anti-inflammatory medications are recommended (1, 51). Despite optimal pharmacological interventions such as triple inhaled therapy, many COPD patients will still experience exacerbations (52).

1.2.5.1 Antibiotics for prevention of COPD exacerbations

Control of infective exacerbations and thereby the inflammation with oral or aerosol antibiotics have beneficial effects in many respiratory disorders such as pneumonia. Specific to COPD, GOLD strategy recommends long-term use of macrolides as a third line therapy in order to prevent further exacerbations in patients with moderate to severe COPD; however, it is mentioned that patient has to be former smoker in addition to having at least one exacerbation of moderate or greater severity in the previous year despite optimal maintenance inhaler therapy (1). This may have some biological plausibility although it has not been validated in further studies. Evidence of the efficacy of macrolides has been demonstrated in a large randomized clinical trial (53). In this latter study, it has been demonstrated that macrolide use is associated with an increased incidence of bacterial resistance. The recommendation of using macrolide in former smokers is based on a secondary analysis of the same large trial that suggested the effectiveness of azithromycin in the prevention of acute exacerbation in former smokers and milder GOLD stages (53). In the GOLD recommendations, they have retained from the secondary analysis that patient had to be a former smoker although for moderate to severe disease severity.

In summary, treatment with macrolide antibiotics, especially in those patients who have frequent exacerbations, has been demonstrated to prevent COPD exacerbations and improve patient quality of life and symptoms. However, uncertainty remains about the specific patient population that is most likely to benefit from long-term macrolide treatment (current and/or ex-smoker, patients with or without bronchiectasis and those colonized or not with *P. aeruginosa*), the optimal dose and duration of macrolide treatment, and the potential impact of long-term macrolide treatment on bacterial resistance (54).

1.2.5.1.1 Macrolide Mechanisms

Macrolide antibiotics are effective broad-spectrum bacteriostatic agents, exerting their effect by binding to the 50S ribosomes of both prokaryotes and eukaryotes and inhibiting transpeptidation or translocation of nascent peptides. Macrolides have excellent tissue penetration, prolonged tissue persistence, and favorable side-effect profiles. For these reasons and their broad efficacy against Gram-positive, some Gram-negative and atypical bacteria, macrolides are well established in the therapy of respiratory infections (55). Beyond their antimicrobial activity, macrolides have been shown to have significant anti-inflammatory and immunomodulatory effects related to the macrocyclic lactone ring. Immunomodulation, which is defined as modifying or regulating one or more functions of the immune system, describes the nonlinear downregulation of a hyperimmunity or hyperinflammation without impairing the normal immune or inflammatory response to defend against bacteria (56).
It has been shown that macrolides have effects on airway secretion by decreasing mucus hypersecretion both *in vitro* and *in vivo* (56, 57). Airway mucin is synthesized by epithelial goblet cells and mucous cells of the submucosal glands. MUC5AC and MUC5B are the major gel-forming mucins in the human airway. It has been suggested that azithromycin or clarithromycin significantly inhibit TNF-α-induced MUC5AC secretion from human

mucoepidermoid carcinoma cells (NCI-H292) and human nasal epithelial cells. MUC5AC mRNA expression is also significantly inhibited by azithromycin (57). Also, Ca2+ -activated airway epithelial chloride channel is inhibited by macrolides, result in reducing water and, possibly, mucin secretion (56, 58). Macrolides appear to decrease the production of proinflammatory cytokines that are detrimental to the host, including TNF-α, granulocyte-macrophage-colony-stimulating factor [GM-CSF], IL-1, IL-6, IL-8, IL-10 and IFN-γ (59, 60). Adhesion molecules, which are necessary for neutrophils and other inflammatory cells to migrate into the airway in response to inflammatory signals, have been shown to be affected by macrolides, leading to a reduction in resolution of airway neutrophilic inflammation (56). Leukotriene B4 (LTB4), a metabolite of arachidonic acid, is an important chemotactic factor for neutrophils and is elevated in patients with chronic airway disease. Evidence suggests that erythromycin and roxithromycin reduce LTB4 production in broncoalveolar lavage fluid (BALF) or epithelial lining fluid (ELF) in subjects with diffuse pan bronchiolitis associated with decreased neutrophil numbers and chemotactic activity (56). The production of ROS, which are known mediators of cell and tissue injury, could also be inhibited from neutrophils by macrolides and this effect is due in part to cell membrane stabilization (56, 61). Furthermore, macrolides may induce apoptosis of activated neutrophils. Clarithromycin and azithromycin are able to promote the phagocytosis of apoptotic epithelial cells and neutrophils by alveolar macrophages and contribute to improving the resolution of inflammation (62). Data suggest that macrolides may help stabilize the epithelial cell membrane by protecting against activated phospholipases, which cleave arachidonic acid-induce proinflammatory mediators in the inflammatory airway. Azithromycin increases the trans-epithelial electrical resistance (barrier) of human airway epithelial cells cultured on filter supports (63). This effect was associated with increasing tight junction proteins claudin-1, claudin-4, occludin, and junctional adhesion molecule A. In cultured human primary

tracheal cells, bronchial BEAS-2B cells and pneumocyte II-like A549 cells, erythromycin showed induction of bactericidal activity of the airway surface liquid (64) which might be secondary to the reduction in airway inflammation.

Moreover, it has been suggested that macrolides have an effect on gram-negative bacteria such as *P. aeruginosa* (65), including inhibition of bacterial adherence by reducing the number of pili and the motility of *P. aeruginosa*, and inhibition of virulence factors, biofilms, and quorum sensing genes. These effects are associated with suppressing alginate production and antibody reaction to alginate, result in a reduction in immune complex formation (56, 66) (Figure 1.2).

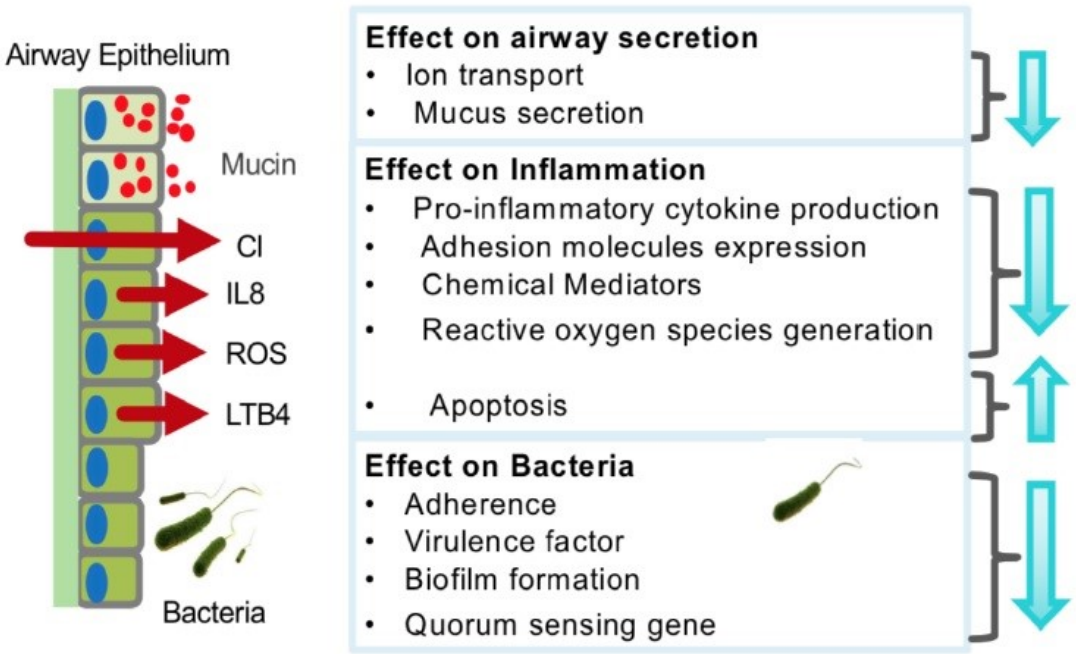

Figure 1.2: Effects of macrolides on the inflamed airway

Maintenance treatment with azithromycin decreases exacerbation rates compared with placebo and should, therefore, be considered for use in COPD patients who have frequent

exacerbations and are refractory to standard care (53). However, the risk of macrolide resistance is increasing with consumption of azithromycin in a large group of patients (67). It has been demonstrated that, although azithromycin has no bactericidal effect on *P. aeruginosa*, the drug interferes with *P. aeruginosa* colonization by inhibiting biofilm formation. These properties have made azithromycin therapy an attractive adjunct in the treatment of chronic inflammatory pulmonary diseases (68).

CHAPTER 2: RATIONALE, HYPOTHESIS, AND OBJECTIVES

2.1. Rationale

COPD patients often experience exacerbations despite being on optimal therapy. The key feature of COPD exacerbations is inflammation that involves multiple immune cells and mediators. Viral and bacterial infections are the major identifiable causes of inflammation during COPD exacerbations. *P. aeruginosa* has been associated with acute exacerbations in patients with severe COPD, important history of smoking and previous hospitalizations (44). Recently, an interest in using prophylactic antibiotics in COPD to prevent exacerbations has emerged, with an emphasis on the use of macrolide antibiotics.

GOLD 2017 has recommended macrolides as third-line therapy for patients GOLD D already on optimal inhaled therapy (i.e. exacerbation history of ≥2 or ≥1 leading to the hospital admission and symptoms as mMRC≥2 and CAT≥10) (1). However, the setting in which COPD patients have been studied in a large randomized clinical trial (69) are those of a general population of patients not necessarily on optimal inhaled therapy. Furthermore, secondary analysis has suggested that macrolides are more effective in mild COPD and former smokers (53). GOLD D patients are usually not mild COPD and patients in that category may still be current smokers.

Many questions remain unanswered in practice, making clinical decisions a challenge. It is unknown if patients with severe disease and at risk of being colonized with *P. aeruginosa* would benefit from treatment with macrolides. Current smokers represent a group of patients who are at risk of recurrent and severe exacerbations. This needs to be revisited as we presently recommend against using macrolides in that high-risk group of COPD patients. In addition, benefits beyond one year of treatment have not been well studied, all the trials have been done less than a period of treatment of one year.

Furthermore, evidence suggests that the mechanisms by which macrolides prevent exacerbations cannot be fully explained by their antibacterial effect and that their anti-inflammatory and immune-modulatory effects could be playing significant roles, preventing the production of proinflammatory cytokines from the host. In addition, biofilm development and bacterial adherence of pathogens such as *P. aeruginosa* are affected by macrolides (65). These effects make macrolides a therapeutic choice for patients who are colonized with *P. aeruginosa*. It is unknown if these patients might benefit from long-term therapy with the macrolide antibiotic azithromycin and if smoking could interfere with the therapeutic response.

2.2 Central Hypothesis

Long-term treatment with azithromycin decreases exacerbation frequency and health service use in severe COPD patients already treated with optimal inhaled medications, and this effect is obtained by modulating inflammation in airway epithelial cells.

2.3 Objectives

2.3.1 Clinical Project

2.3.1.1 General objective

To determine whether, in real-life clinical practice, long-term azithromycin is associated with decreased exacerbations in patients with severe COPD known for recurrent exacerbations while on optimal inhaled therapy.

2.3.1.2 Secondary objectives

To assess in real-life clinical practice, in patients with severe COPD known for recurrent exacerbations while on optimal inhaled therapy:

i) the effect of azithromycin on health service use, specifically emergency medical visits and hospitalizations;

ii) the sustained effect of azithromycin on exacerbations beyond 1 year and;

iii) the safety of azithromycin in real-life clinical practice.

2.3.2 Laboratory project

2.3.2.1 General objective

To assess the mechanism through which azithromycin modulates inflammation in an *in vitro* model of human airway epithelial cell injury by cigarette smoke.

2.3.2.2 Specific objectives

i) To characterize the inflammatory response of epithelial cells exposed to cigarette smoke;

ii) To assess the effects of azithromycin on airway epithelial cell expression and release of the inflammatory mediators IL-6 and IL-8 in response to cigarette smoke.

CHAPTER 3: MANUSCRIPT 1 "LONG-TERM AZITHROMYCIN THERAPY TO REDUCE ACUTE EXACERBATIONS IN PATIENTS WITH SEVERE CHRONIC OBSTRUCTIVE PULMONARY DISEASE"

Title: Long-term azithromycin therapy to reduce acute exacerbations in patients with severe chronic obstructive pulmonary disease

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Statement of each author's contributions to the study:

NN, DA, SMYMPM, and ZK have done the chart review and data extraction. NN, DA, and SMYMPM have reviewed the study protocol, participated in the analysis, and participated in the redaction and reviewed the manuscript. PZL has done the analysis and reviewed the manuscript. AJ, IO, and ID have assisted in the chart review and reviewed the manuscript. JB has written the protocol, planned and participated in the analysis, written and reviewed the manuscript.

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3.1 Abstract

Rationale: According to clinical trials, azithromycin taken daily for 1year, decreased exacerbations of chronic obstructive pulmonary disease (COPD).

Objectives: Effectiveness evaluation of long-term azithromycin to reduce exacerbations in severe COPD patient on optimal therapy in real-life practice.

Methods: We conducted a retrospective observational study of severe COPD patients who were prescribed azithromycin (PA) (250 mg, at least 3 times weekly for at least 6 months). The comparison group included severe COPD patients not prescribed azithromycin (NPA). Data were extracted from the clinical chart review.

Main Results: Study included 126 PA and 69 NPA patients. They had severe airflow obstruction, mostly emphysema, and one-third bronchiectasis. A predominant feature in the PA group was respiratory tract colonization with *Pseudomonas aeruginosa*. The mean number of exacerbations per patient per year in the PA group was 3.2± 2.1 before initiating azithromycin, and 2.3 ± 1.6 during the following year on therapy ($p<0.001$). Patients in the NPA group had 1.7±1.3 and 2.5±1.7 exacerbations during the first and second follow-up year respectively ($p<0.001$). Exacerbation changes from pre to post differed between groups ($p<0.001$). The decrease in emergency visits and hospital admissions was significant in the PA group. Exacerbation reductions and patient proportions having ≥2 exacerbations extended to the second year of treatment.

Conclusion: These data showed that long-term azithromycin reduces exacerbation numbers in severe COPD patients, and benefits persist beyond one year. Desirable effects are more likely to outweigh the risks and adverse events in patients colonized with *Pseudomonas aeruginosa*.

3.2 Introduction

Chronic obstructive pulmonary disease (COPD) is currently the fourth leading cause of death and will become the third leading cause of death by 2030 according to the World Health Organization (7, 70). In spite of a remarkable decrease in mortality from cardiovascular disease in the past three decades, mortality due to COPD has almost doubled (70, 71). Acute exacerbations are a common complication of COPD a major cause of increased utilization of health care resources such as emergency department visits and hospital admissions, and the most common cause of death (72-74). Evidence suggests that recurrent exacerbations are associated with a faster decline in lung function and a negative impact on health status (75-77). About a third of patients with moderate to severe COPD experience two or more exacerbations per year (78, 79). There seems to be a specific phenotype of patients who are at increased risk, where the most important predictor of future exacerbations is the number of exacerbations in the previous year (78). Prevention of exacerbations should be one of the principal management goals in these patients (70, 80).

Prevention of acute exacerbations of COPD can be improved, especially for those patients having a high burden of exacerbations despite being on optimal therapy (80). Current therapies such as long-acting bronchodilators and/or inhaled corticosteroids, smoking cessation, pulmonary rehabilitation, and influenza immunization are recommended to prevent exacerbations (33, 79). Even though these interventions have shown a reduction in exacerbations, there is a need for further pharmacological therapies in patients who continue to have recurrent exacerbations despite optimal care. Recently, an interest in using prophylactic antibiotics in COPD to prevent exacerbations has emerged, with an emphasis on the use of macrolide antibiotics. Macrolides have been used in several chronic respiratory conditions for their anti-inflammatory and immunomodulatory effects. Low-dose macrolide therapy has been reported to decrease the number of exacerbations in a large number of

chronic inflammatory pulmonary diseases (81-84). Macrolides interfere with biofilm development (65) and bacterial adherence of pathogens (85, 86) such as *Pseudomonas aeruginosa*. Administration of macrolides may be helpful in patients who have been colonized by *Pseudomonas aeruginosa* (86). Macrolides also have prokinetic activity on the gastrointestinal tract, leading to a reduction in gastroesophageal reflux and microaspiration, common in chronic pulmonary conditions (65, 86-88). Macrolides have been shown to reduce the number of exacerbations in patients with cystic fibrosis (CF), especially in those colonized with *Pseudomonas aeruginosa* (89-92).

In patients with COPD who have had at least one exacerbation, two recent large trials have shown that regular macrolides reduce the time to subsequent exacerbation and the frequency of exacerbations at one year (93, 94). Use of macrolides has been recommended in the Global Initiative for Chronic Obstructive Lung Disease (GOLD 2017) as third-line therapy for patients with recurrent exacerbations (GOLD D) (80). Treatment with azithromycin is also associated with an increased incidence of bacterial resistance (Evidence A) and hearing test impairments (Evidence B) (80). Macrolides will unlikely be used in every patient with COPD known for exacerbations considering those undesirable effects and the small increase in cardiovascular adverse events (95). Many questions remain unanswered in practice making clinical decisions a challenge. It is still unknown if patients with severe disease and those colonized with *Pseudomonas aeruginosa* would benefit from treatment with macrolides. In addition, benefits beyond one year of treatment have not been studied.

3.3 Objectives

The principal objective of this study was to determine whether long-term azithromycin

is associated with a decrease in exacerbations in patients with severe COPD known for recurrent exacerbations while on optimal inhaled therapy. Secondary objectives were to evaluate: i) the effect of azithromycin on health service use, specifically emergency medical visits and hospitalizations; ii) the sustained effect on exacerbations beyond 1 year and; iii) the safety of azithromycin in real-life clinical practice.

3.4 Material and methods

3.4.1 Study design

This retrospective observational study was performed using data from chart review of patients with COPD followed by pulmonary physicians working at a single specialized respiratory hospital, the Montreal Chest Institute (MCI), McGill University Health Centre, Montreal, Quebec, Canada. Patients with severe COPD are followed in a multidisciplinary clinical program that includes a pulmonologist and nurses acting as case managers. Patients are strongly encouraged to contact the team when they have an increase in respiratory symptoms and to seek care at the center's walk-in clinic or emergency department. Every visit and phone interventions are systematically recorded using standardized forms that are included in the patients' medical records. Information gathered includes symptoms, use of an action plan, initiation of antibiotics or corticosteroids, emergency visits, and hospital admissions.

Ethics approval for this project was obtained from the local institutional review board.

3.4.2 Patient population

All patients who had an outpatient visit at the Montreal Chest Institute (MCI) from July 2010 to December 2016 with a diagnosis of COPD had their chart reviewed. Patients of the non exposed and those of the exposed groups were selected according to their exposure to

azithromycin before any information was retrieved on the outcomes under study. The prescribed azithromycin group included patients with severe or very severe COPD and documented exacerbations, who were prescribed regular azithromycin for the prevention of exacerbations. The severity of COPD was defined as per the GOLD spirometry criteria, i.e., FEV1/FVC ratio ≤ 0.70 and FEV1 of < 50% predicted (GOLD 3 and 4) (80). They had to have been prescribed a dose of 250 mg, at least 3 times per week, for a minimum of 6 months. Short courses of macrolide antibiotics to treat acute exacerbations were disregarded. Patients could not receive azithromycin if they had prolonged QT on baseline electrocardiogram (ECG), concurrent lung cancer, tuberculosis and bronchiectasis as the primary diagnosis. The non-prescribed azithromycin group included patients with severe COPD (GOLD 3 or 4) and documented recurrent exacerbations within one year who did not receive azithromycin. Identifying non-prescribed patients as comparison group was possible as some pulmonologists at the time were reluctant to use macrolide regularly for prevention of exacerbations.

3.4.3 Data collection and extraction of information on exacerbations

We used a standardized data collection form to record study information. Two physicians reviewed each inpatient and outpatient medical charts. Baseline clinical information was extracted, including demographic data, baseline respiratory medications, comorbid conditions, pulmonary function tests, dyspnea (using the Modified Medical Research Council scale from 1 to 5), and lower respiratory tract microbial colonization (sputum culture results). The presence of any emphysema or bronchiectasis on computed tomography (CT) scan of the chest was noted, based on the official radiologist's report.

Data were extracted one year prior to initiation of azithromycin for the exposed patients, and until December 2016, or until the patient stopped taking azithromycin. In the non-exposed

group, information was extracted for 2 consecutive years to allow comparison with the exposed group. With respect to patients in the non-exposed group, we took advantage of a period of time when many respirologists were still not prescribing azithromycin for COPD patients with frequent exacerbations. The follow-up period of the patients in the non exposed group had to be limited to 24 months; beyond 24 months there are patients who could have been initiated on azithromycin. For this reason, the period of comparison between patients of the non exposed and the exposed group was limited to 2 years. In the exposed group, we were able to follow patients beyond 24 months and to report on the long-term benefit of azithromycin. The follow-up after taking azithromycin was 32±22 (mean±SD) months.

The following information was extracted for each exacerbation: severity of exacerbation, duration of antibiotic and/or corticosteroid course, emergency visit, hospitalization and duration of hospital admission (if applicable). Exacerbations were defined as event-based, meaning an increase in respiratory symptoms from baseline requiring medication change (antibiotic and/or systemic corticosteroids) and contact with the health care provider. These contacts may have been a phone call to a COPD nurse case manager, a physician visit, an unscheduled visit to the clinic or emergency department visit, or a hospital admission. Exacerbations were considered moderate if patients required a prescription of systemic corticosteroids, a course of antibiotics, or both. They were deemed as severe if patients required hospitalization.

Adverse effects known to be associated with azithromycin were recorded, including QT prolongation on electrocardiogram, and gastrointestinal dysfunction. Hearing tests were not done routinely, and this information was not available from chart review. Repeat sputum cultures following treatment with azithromycin were noted when available. Death was identified from the chart and reviewed with the treating physician and the COPD case managers.

3.4.4 Statistical analysis

Descriptive data were reported using means and standard deviations for continuous variables or counts and proportions for categorical variables. Statistically significant differences for exacerbations and healthcare utilization between the year pre-treatment and the year post-treatment were then compared using the GEE model with Poisson distribution for count data or binary distribution as appropriate. Time*group interaction term was added into the model to estimate whether the average change in the outcome from pre to post-treatment differed between two groups. We also performed a logistic regression model to estimate the P values for the three responders exacerbations, emergency room (ER) visits, and hospitalizations between azithromycin and control groups, adjusted for baseline age, sex, current smokers, and FEV1%predicted. GEE model with the repeated statement was also performed to compare a number of exacerbations, ER visits, and hospitalizations among pre-treatment year and post-treatment years in the azithromycin group who completed 2-year follow-up. Analysis was performed using SAS version 9.4.

3.5 Results

Clinical characteristics of patients on treatment (azithromycin group) and those not on azithromycin (comparison group) are summarized in Table 1. There were 126 patients in the treatment group and 69 in the comparison group. Patients prescribed azithromycin had more severe COPD with lower FEV1, higher dyspnea score (MRC 4-5/5) and more frequent exacerbations at baseline. Patients from both groups were already on triple inhaled therapy but more patients were on long-term oxygen therapy in the azithromycin group. CT scan of the chest showed that emphysema was the underlying primary lung condition in most patients, while bronchiectasis was present in one-third of the patients from either group. The

patients in the azithromycin group had more frequent colonization of the respiratory tract with *Pseudomonas aeruginosa* compared to those in the comparison group.

Table 1. Clinical characteristics of patients on treatment (azithromycin group) and those not on azithromycin (control group)

Variable	Azithromycin Group N=126	Control Group N=69	P-value*
Age, years	67.8 ± 9.3	70.8 ± 9.4	0.035
Male sex, n (%)	73 (59.8)	33 (47.8)	0.109
Smoking status, n (%)			
Ex-smokers	98 (79.0)	49 (71.0)	0.21
Current smokers	26 (21.0)	20 (29.0)	0.21
Cigarette smoking pack-years	48.3 ± 17.2	52.1 ± 19.6	0.265
MRC dyspnea Scale, n (%)			
2	0 (0.0)	2 (3.8)	0.155
3	24 (29.6)	19 (35.8)	0.451
4 or 5	57 (70.4)	32 (60.4)	0.231
Chronic bronchitis, n (%)	15 (41.7)	12 (48.0)	0.624
Exacerbation previous year	3.2 ± 2.1	1.7 ± 1.3	<0.001
Respiratory medication, n (%)			
LABA/LAMA/ICS	124 (98.4)	69 (100.0)	0.293
Theophylline	13 (10.4)	9 (13.0)	0.578
Leukotriene receptor antagonist	10 (8.0)	2 (2.9)	0.219
Systemic corticosteroids	10 (8.0)	2 (2.9)	0.219

Long-term oxygen therapy	33 (26.6)	9 (13.0)	0.029
FEV1 (L)	0.9 ± 0.4	1.0 ± 0.4	0.042
FEV1, % predicted	34.8 ± 14.1	39.9 ± 13.7	0.006
FVC (L)	2.1 ± 0.8	2.2 ± 0.8	0.463
FEV1/FVC (%)	42.9 ± 12.5	46.1 ± 11.9	0.062
Comorbid conditions, n (%)			
Asthma	15 (12.0)	4 (5.8)	0.211
Sleep disordered	23 (18.4)	11 (15.9)	0.666
Osteoporosis	19 (15.2)	13 (18.8)	0.513
Coronary artery disease	20 (16.1)	10 (14.5)	0.764
Atrial fibrillation	15 (12.0)	11 (15.9)	0.44
Hypertension	46 (36.8)	30 (43.5)	0.362
Diabetes	16 (12.8)	11 (15.9)	0.545
Pulmonary hypertension	5 (4.0)	8 (11.6)	0.043
Chest CT scan, n (%)	n=106	n=53	
Bronchiectasis	29 (27.4)	15 (28.3)	0.9
Emphysema	99 (93.4)	44 (83.0)	0.04
Colonization, n (%)	n=111	n=59	
Pseudomonas aeruginosa	69 (62.2)	23 (39.0)	0.004
Stenotrophomonas maltophilia	14 (12.6)	5 (8.5)	0.415
Others †	36 (32.4)	39 (66.1)	<0.001
Normal flora	25 (22.5)	11 (18.6)	0.556
Number of exacerbations in the previous 1-year n (%)	3.2 ± 2.1	1.7 ± 1.3	<0.001
1 exacerbation	16 (12.7)	19 (27.5)	0.009

2+ exacerbations	104 (82.5)	36 (52.2)	<0.001

Values were mean ±SD or n (%).

MRC: Medical Research Council; LABA: Long Acting Beta Agonist; LAMA: Long-Acting Muscarinic Antagonist; ICS: Inhaled Corticosteroid; FEV1: Forced Expiratory Volume in 1 second; FVC: Forced Vital Capacity; CT: Computerized Tomography

*P-value was obtained by performing T-test (normal distribution) or Wilcoxon two-sample test (non-normal distribution) or Chi-square analysis for category variables.

† Others include: *Escherichia coli*, *Enterobacter*, *Acinetobacter*, *Staphylococcus aureus*, *Streptococcus pneumoniae, Moraxella catarrhalis, Serratia marcescens, Candid*

Table 2 shows within-group changes in acute exacerbations and health service use in patients on treatment (azithromycin group) and those not on azithromycin (comparison group), and between-group differences in those outcomes. The mean number of exacerbations decreased significantly in the year after initiation of azithromycin (3.2 vs 2.3 exacerbation per year, $p<0.001$), whereas in the comparison group, the mean number of exacerbations increased between year one and two (1.7 vs 2.7 exacerbations per year, $p<0.001$).

Table 2. Changes in exacerbations and health service use within treatment and comparison groups and between groups

	Azithromycin Group (N = 126)			Non-Azithromycin Group (N = 69)		
	0–12 months PreRx*	12–24 months Post Rx†	P-Value‡	0 -12 months	12-24 months	P-Value‡
Number of exacerbations mean±SD	3.2 ± 2.1	2.3 ± 1.6	<0.001	1.7 ± 1.3	2.5 ± 1.7	<0.001
Patients with exacerbations, n (%)						
1 exacerbation	16 (12.7)	37 (29.4)	0.001	19 (27.5)	13 (18.8)	0.226
2+ exacerbations	104(82.5)	78 (61.9)	<0.001	36 (52.2)	50 (72.5)	0.014
Health care utilization						
ER visit/patient/year mean±SD	1.5 ± 1.7	1.0 ± 1.2	0.007	1.1 ± 1.1	1.3 ± 1.5	0.926
Hospitalization/patient/year mean±SD	1.4 ± 1.7	0.9 ± 1.1	0.003	0.8 ± 1.0	1.0 ± 1.5	0.763
Hospital stay for the patients who have hospitalizations, mean±SD	9.1 ± 10.0	6.5 ± 4.7	0.039	8.7 ± 8.9	10.4 ± 9.8	0.274

*Pre Rx-refers to the year before the patient has been prescribed Azithromycin

†Post Rx the year while the patient has been taken Azithromycin.

‡P-value was obtained by performing a GEE model within the group.

§P-value was obtained by performing a GEE model between the azithromycin and control group. For the two groups comparison (between-group comparison), we used GEE model with a repeated statement to test time*group interaction term and estimate whether the average change in the outcome from pre to post differed in the two groups.

Fig. 1 shows a higher proportion of responders, i.e., having a reduction in exacerbations, ER visits or hospital admissions, between azithromycin and comparison group.

Table 3 shows changes in exacerbations in the azithromycin and the comparison groups according to patient and disease characteristics. Although the reduction in exacerbations appeared to be more modest in active smokers compared to former smokers, both benefited from treatment with azithromycin. The subgroups of patients with respiratory colonization with *Pseudomonas aeruginosa,* and those with and without bronchiectasis on CT scan also had a significant reduction in the number of exacerbations after one year of treatment with azithromycin.

Figure 1. The proportion of responders between azithromycin and non-prescribed azithromycin groups. The definition for responder is patients with the number of exacerbations during 12-24 months PostRx (post-treatment) being less than 0-12 months PreRx (pre-treatment). Compared to the non-azithromycin group, responders were all significantly higher for the azithromycin group using logistics model adjusted for baseline age, sex, current smokers, and FEV1%predicted. ER: emergency room.

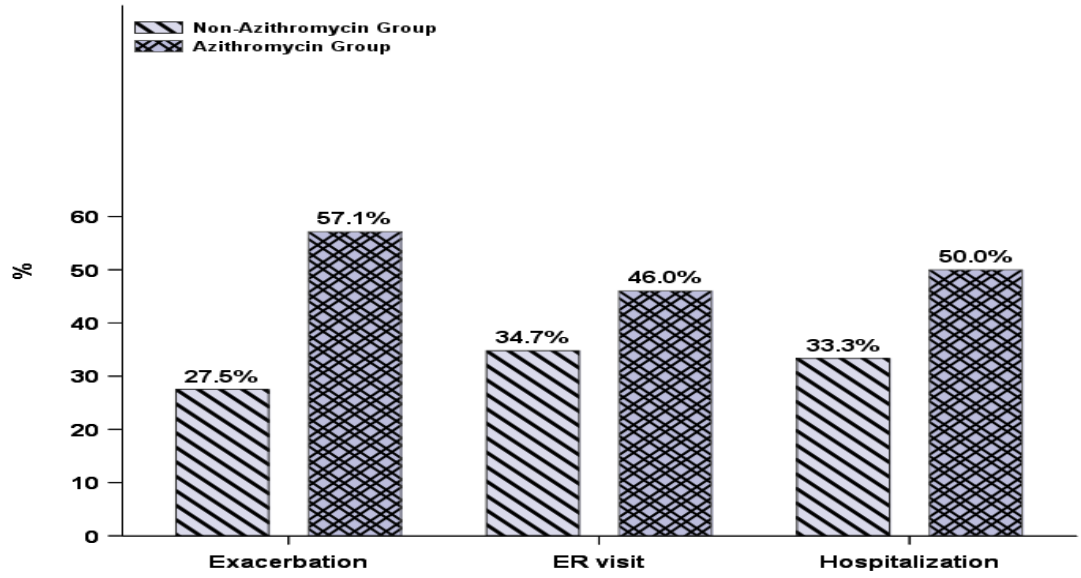


Figure 1. The proportion of responders between azithromycin and non-prescribed azithromycin groups. The definition for responder is patients with the number of exacerbations during 12-24 months PostRx (post-treatment) being less than 0-12 months PreRx (pre-treatment). Compared to the non-azithromycin group, responders were all significantly higher for the azithromycin group using logistics model adjusted for baseline age, sex, current smokers, and FEV1%predicted. ER: emergency room.

Table 3. Changes in exacerbations in the azithromycin and the control groups according to patient and disease characteristics.

Patient and disease characteristics	Number of exacerbations						
	Azithromycin Group			Non-Azithromycin Group			Two-group comparison P-value*
	N	mean±SD	P-Value*	N	mean±SD	P-Value*	

Smoking status							
Ex-smokers							
0–12 months PreRx†	98	3.2 ± 2.2	<0.001	49	1.6 ± 1.4	0.006	<0.001
12-24 months PostRx‡	98	2.2 ± 1.7		49	2.5 ± 1.6		
Current smokers							
0–12 months PreRx	26	3.3 ± 1.7	0.022	20	1.8 ± 1.1	0.082	0.010
12-24 months PostRx	26	2.6 ± 1.4		20	2.6 ± 1.9		
Sputum culture							
Pseudomonas aeruginosa							
0–12 months PreRx	60	2.7 ± 1.7	0.236	21	1.5 ± 1.2	0.006	0.002
12-24 months PostRx	60	2.3 ± 1.7		21	2.9 ± 2.3		
Stenotrophomonas							
0–12 months PreRx	5	6.2 ± 3.2	0.018	3	2.0 ± 2.0	0.346	0.039
12-24 months PostRx	5	1.4 ± 1.7		3	4.0 ± 2.6		

Pseudomonas or *Stenotrophomonas*							
0–12 months PreRx	74	3.0 ± 2.1	0.035	26	1.6 ± 1.4	0.011	0.001
12-24 months PostRx	74	2.3 ± 1.7		26	3.0 ± 2.3		
Any others § (not *Pseudomonas* or S*tenotrophomonas*)							
0–12 months PreRx	20	3.3 ± 2.4	0.054	22	1.7 ± 1.1	0.255	0.032
12-24 months PostRx	20	2.3 ± 1.1		22	2.1 ± 1.3		
CT scan *Bronchiectasis*							
0–12 months PreRx	29	3.3 ± 2.2	0.036	15	2.1 ± 1.5	0.085	0.007
12-24 months PostRx	29	2.4 ± 1.6		15	3.2 ± 2.2		
No bronchiectasis							
0–12 months PreRx	100	3.3 ± 2.1	<0.001	50	1.8 ± 1.3	0.008	<0.001
12-24 months PostRx	100	2.3 ± 1.6		50	2.7 ± 1.7		

*P-value was obtained by performing GEE model.

For the two groups comparison, we used GEE model with a repeated statement to test time*group interaction term and estimate

whether the average change in the outcome from pre to post differed in the two groups.

†PreRx refers to the year before the patient has been prescribed azithromycin.

‡Post Rx refers to the year while the patient has been taken azithromycin.

§Others include: *Escherichia coli*, *Enterobacter*, *Acinetobacter*, *Staphylococcus aureus*, *Streptococcus pneumoniae, Moraxella catarrhalis, Serratia marcescens, Candida*

Figures 2 and 3 show the number of exacerbations and proportion of patients having ≥2 exacerbations, and ER and hospitalizations among patients on azithromycin treatment who completed 2-year follow-up. There was a significant reduction of exacerbations, ER visits and hospitalizations beyond one year of follow-up.

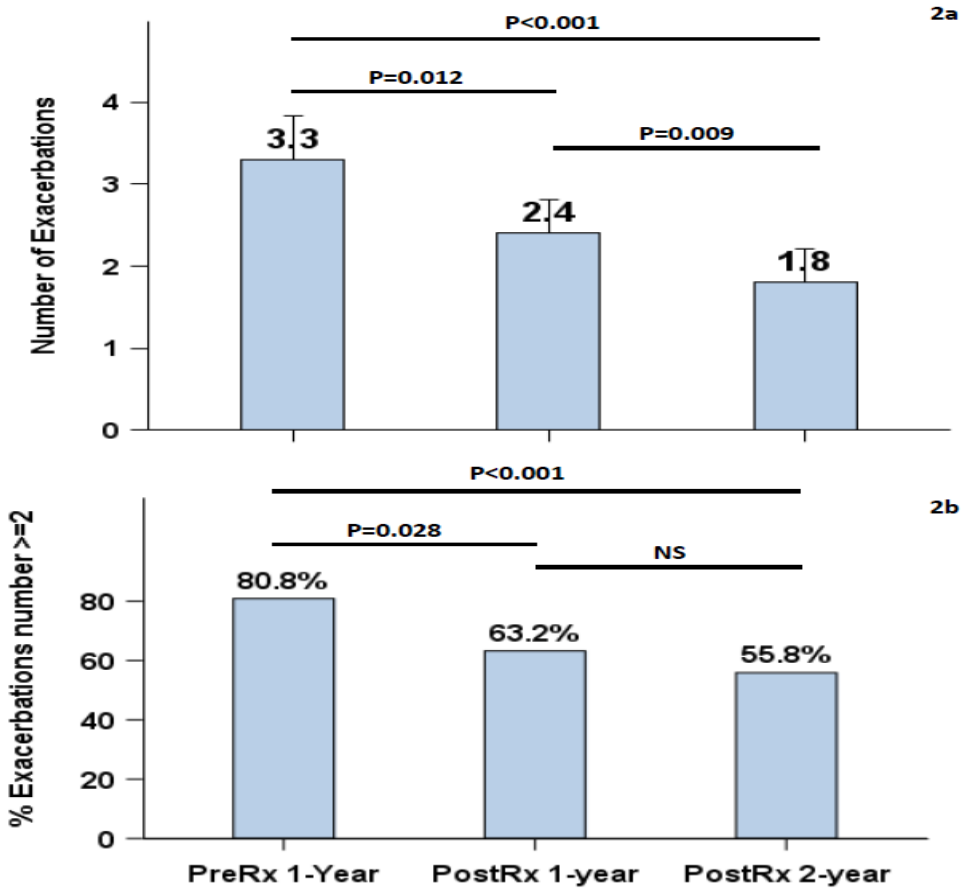


Figure 2: The number of >=2 exacerbations (2a) and the proportion of >=2 exacerbations (2b) among patients of the azithromycin group who completed 2-year follow-up (n=68).

Figure 2a, GEE model with Poisson distribution and repeated statement was performed to compare the number of >=2 exacerbations among the three-time points; compared to PreRx (pre-treatment) 1 year, there are significant differences for PostRx (post-treatment) 1 year and PostRx 2-year, with a P value 0.012 and <0.001, respectively. Also, there are significant differences between PostRx 1-year and PostRx 2-year, with a p=0.009. Figure 2b, GEE model with binary distribution and the repeated statement was performed to compare the proportion of >=2 exacerbation among the three-time points; compared to PreRx 1 year, there are significant differences for PostRx 1 year and PostRx 2-year, with a P value 0.028 and <0.001, respectively. NS: Non-significant.

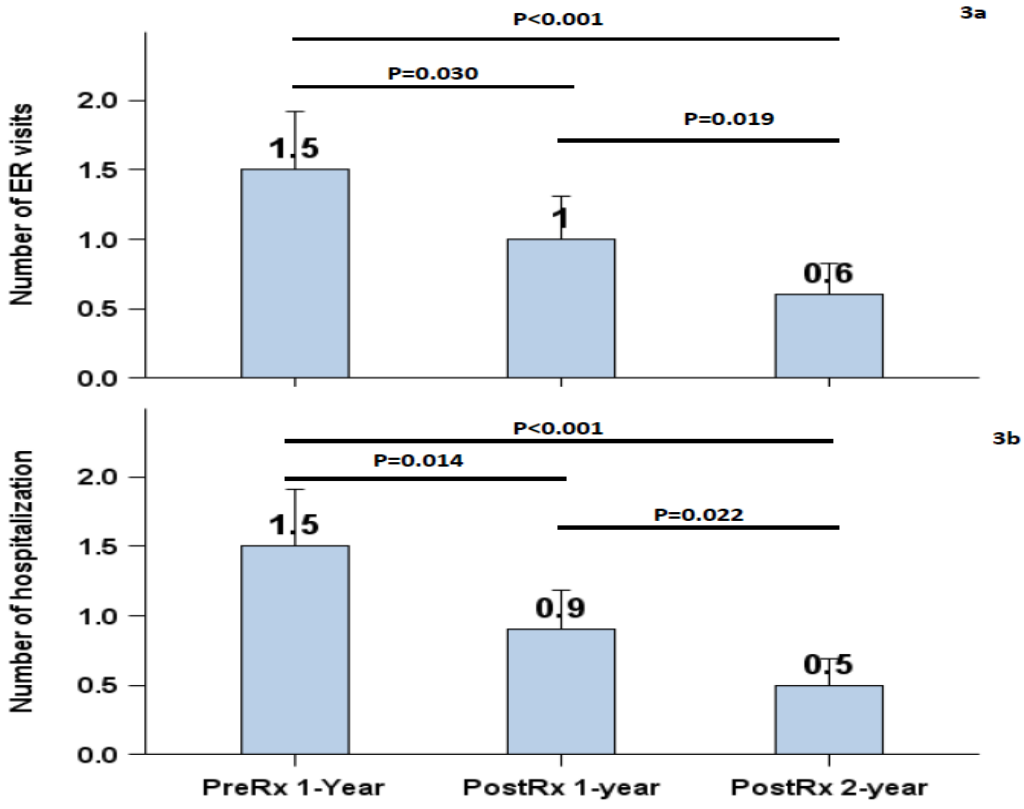


Figure 3. The number of ER visits (3a) and hospital admissions (3b) among patients of the azithromycin group who completed 2-year follow-up (n=68). GEE model with Poisson distribution and the repeated statement was performed to compare ER visits/hospitalizations

among the three-time points. Figure3a, compared to PreRx (pre-treatment) 1 year, there are significant differences for PostRx (post-treatment) 1 year and PostRx 2-year, with a P value 0.030 and <0.001, respectively, for ER visit. Also, there are significant differences between PostRx 1-year and PostRx 2-year, with a p=0.019 for an ER visit. Figure 3b, compared to PreRx (pre-treatment) 1 year, there are significant differences for PostRx (post-treatment) 1 year and PostRx 2-year and P value 0.014 and <0.001, respectively and 0.022 for comparison between post Rx 1 year and post Rx 2 year for hospitalization. ER; emergency room

There were few documented adverse effects attributable to azithromycin, and the medication rarely had to be discontinued. Seven patients with COPD (5.6%) discontinued azithromycin, of whom only 2 discontinued due to QT prolongation (1.6%), and 3 because of gastrointestinal symptoms such as diarrhea and/or tenesmus (2.4%). Only 2 patients (1.6%) discontinued azithromycin because their physician did not consider the treatment beneficial. Post-treatment sputum culture was collected primarily in patients with *Pseudomonas aeruginosa* respiratory colonization; 60% of the patients remained colonized after 2 years or more being on azithromycin. The rate of death respiratory cause during the 2 or more years of follow-up was 1.5% (2 patients) and 1.4% (1 patient) in the azithromycin and control groups, respectively.

3.6 Discussion

This study of real-life practice has shown that patients with severe and very severe COPD and frequent exacerbations on optimal inhaled pharmacotherapy benefit from the addition of regular azithromycin to reduce the number of acute exacerbations. This is in contrast to a control group of patients who did not receive long-term azithromycin who experienced increased yearly exacerbations during the same time period. The probability of being a

"responder", i.e., having a reduction in exacerbations and health service use in patients in the azithromycin group, was significantly higher compared to those in the control group. The benefit of azithromycin treatment was demonstrated in smokers and former smokers, in patients colonized with *Pseudomonas aeruginosa*, and in those with and without bronchiectasis. We have also shown that the benefits of reducing exacerbations, emergency visits, and hospital admissions were prolonged beyond one year of treatment. Finally, very few patients had to discontinue azithromycin treatment because of adverse effects. Seemungal and colleagues (94) performed a randomized double-blind control trial evaluating erythromycin for a year versus placebo in 109 patients with COPD. Subjects receiving erythromycin had significantly fewer exacerbations (81 per year) compared to the subjects receiving placebo (125 per year) (94). However, patients having moderate to severe COPD (mean FEV1 50% predicted) were not receiving what would be considered optimal medical therapy these days, the majority being on inhaled corticosteroids, 60% on a long-acting beta2-agonist, and less than 40% on the long-acting muscarinic antagonist. More recently, Albert and colleagues (93) performed a multisite randomized control trial evaluating on the year of azithromycin (250 mg daily) versus placebo in patients with COPD. This study showed the number of exacerbations requiring both antibiotics and steroids was 0.76 times fewer in the azithromycin group than the placebo group (95% CI, 0.63–0.91; P = 0.002). Han and colleagues (53) reported the results of a secondary analysis of this cohort, taking into consideration potentially relevant confounders such as the use of various concomitant therapies, clinical characteristics, disease severity, and the type of COPD exacerbations. They showed that azithromycin had greater efficacy in older patients, lower GOLD stage and those not actively smoking. This trial was not designed to include patients with severe or very severe COPD and recurrent exacerbations despite optimal inhaled therapy. The patients in our study had more severe disease, the higher recurrent rate of exacerbations, and more were

colonized with *Pseudomonas aeruginosa*. Many clinicians may be reluctant to use long-term macrolides in mild to moderate COPD considering that benefits do not clearly outweigh the risks and adverse events, in particular, an increased incidence of microbial resistance.

Few studies reflecting real-life clinical practice have been published where macrolide was initiated in patients with COPD and recurrent exacerbations. One Japanese study (96) performed a retrospective analysis of 123 patients with COPD in seven academic hospitals, 45 were treated with macrolides (clarithromycin or erythromycin), compared to a control group of 78 patients (96). There was no difference in the mean frequency of yearly exacerbations between the two groups (0.51 exacerbation per year in the treatment group versus 0.63 in the control group, p=0.54). However, there were fewer patients with >1.5 exacerbations per year receiving macrolides compared to control patients (4.4% versus 15.4%. p=0.01). Again, in this study, most patients were mild to moderate (GOLD 1 or 2, 76% in the macrolide group and 59% in the controls). This group with fewer acute exacerbations is more likely to benefit from inhaled medication and unlikely to require additional treatment with azithromycin.

In our study, a predominant feature of the azithromycin group was the colonization of the respiratory tract with *Pseudomonas aeruginosa,* representing two-thirds of the patients. They had a significant reduction in a number of exacerbations on azithromycin treatment. This is in keeping with the BLESS study,(97) in which patients with non-cystic fibrosis bronchiectasis colonized with *Pseudomonas aeruginosa* had a significant reduction in total exacerbations. In these patients, it has been demonstrated that long-term macrolides change the composition of the respiratory microbiota. In patients without *Pseudomonas aeruginosa* airway infection, macrolides did not significantly reduce exacerbations and promoted displacement of *Haemophilus influenza* by more macrolide-tolerant pathogens including *Pseudomonas aeruginosa*. These findings argue for a cautious approach to chronic macrolide use in patients

without *Pseudomonas aeruginosa* airway colonization (98). It has also been demonstrated that inhibition of *Pseudomonas aeruginosa* quorum sensing genes within the airways of patients with bronchiectasis receiving long-term low-dose macrolides, without a reduction in bacterial load, represents a potential mechanism of therapeutic impact beyond a classical antimicrobial or anti-inflammatory pathway (97).

3.6.1 Strengths and Limitations

One of the strengths of the present study was that therapy with azithromycin was initiated in a well-defined patient population with severe or very severe COPD and recurrent exacerbations despite optimal medical therapy (combined long-acting bronchodilators and inhaled corticosteroids). Another strength of this study was the length of treatment with azithromycin therapy beyond 1 year, as most studies previously published stop intervention at one year. Finally, we have shown that in patients colonized with *Pseudomonas aeruginosa*, the potential benefits of azithromycin outweighed the risks and adverse events, most particularly the increased incidence of microbial resistance.

This study has potential limitations. Due to the retrospective nature of the study design, we had access to both exposure and outcomes at the time of doing the chart review. Although reviewers of the medical chart were instructed to select patients based on their exposure, they were not blind to the patient outcomes. Patients in the comparison group had less severe disease and were therefore different than the treatment group. However, following a comparison group of non-exposed patients with severe COPD for the same period of time and tracking their exacerbations allowed us to study the natural clinical evolution of these patients compared to the treatment group. Furthermore, the differences of more frequent exacerbations in the exposed compared to the non-exposed group with fewer exacerbations would likely bias our results toward the null hypothesis. The fact that we found a significant between-group difference in exacerbations and health care utilization suggests that

azithromycin is truly beneficial. Our study was not designed or powered to accurately assess adverse events and the risk of long-term use of azithromycin in a COPD population. Adverse effects were rarely a cause of discontinuing the macrolide treatment. A careful review of cardiac history and a baseline electrocardiogram was done before initiation of therapy and patient with long QT were not considered for treatment with azithromycin, which likely helps reduce potential harm. Ototoxicity was not clinically reported, and it was not systematically assessed in the study.

3.7 Conclusions

In conclusion, our study showed that regular use of azithromycin in patients with severe and very severe COPD and recurrent acute exacerbations despite optimal therapy (triple inhaled therapy) has the potential to reduce the risk of exacerbations, emergency visits, and hospital admissions and that these benefits are sustained beyond one year. Benefits are more likely to outweigh the risks and adverse events in patients with COPD who are colonized with *Pseudomonas aeruginosa*. However, it is necessary to carefully select patients since long-term use of macrolides can have deleterious effects. Further studies are needed to determine if intermittent therapy is just as effective as daily azithromycin in preventing COPD exacerbations. Our study, in a real-life practice, showed that regular use of azithromycin in patients with severe and very severe COPD and recurrent acute exacerbations despite optimal therapy (triple inhaled therapy) has the potential to reduce the risk of exacerbations, emergency visits, and hospital admissions, and these benefits are sustained beyond one year. Benefits have been shown in subgroups of patients, smokers, and ex-smokers, those with and without bronchiectasis and patients colonized with *Pseudomonas aeruginosa*.

The present study originality was that therapy with azithromycin was initiated in a well-defined patient population with severe or very severe COPD and recurrent exacerbations

despite optimal medical therapy (combined long-acting bronchodilators and inhaled corticosteroids). Furthermore, the length of treatment with azithromycin therapy showed a benefit beyond 1 year; most studies previously published were at one year. Finally, we have shown that patients colonized with *Pseudomonas aeruginosa* would be a population to target considering that the potential benefits of azithromycin outweigh the risks and adverse events, most particularly the increased incidence of microbial resistance.

CHAPTER 4: BRIDGING CHAPTER "THE IMPORTANCE OF DEVELOPING TRANSLATIONAL RESEARCH PROJECT TO ASSESS THE MECHANISM BY WHICH AZITHROMYCIN DECREASES EXACERBATIONS"

Our clinical observational study (manuscript 1) showed that long-term azithromycin reduces the number of exacerbations in severe COPD patients already on optimal inhaled therapy. Beneficial effects of azithromycin were observed not only in ex-smokers but also in current smokers, not only in those having COPD with bronchiectasis but also COPD without bronchiectasis and in patients colonized with *P. aeruginosa.* Finally, the study demonstrated that the benefit was maintained beyond one year. This new knowledge is reinforcing the GOLD recommendations (1) and will help the clinicians targeting patients with severe COPD who are on optimal therapy and will most likely benefit from taking long-term azithromycin. Furthermore, these results led us to develop a translational research project to study the mechanisms by which macrolides reduce exacerbations in COPD patients.

Treatment with long-term macrolide therapy has been shown to be effective in the reduction of exacerbations not only in patients with COPD but also in some other chronic inflammatory lung disease such as non-CF bronchiectasis. Two large RCTs among patients with non-CF bronchiectasis, BLESS (Bronchiectasis and Low-dose Erythromycin Study) and BAT (Bronchiectasis and Long-term Azithromycin Treatment), have demonstrated that long-term use of erythromycin and azithromycin compared with placebo resulted in a decrease in the rate of pulmonary exacerbations and an increased rate of macrolide resistance (83, 99).

Although the precise mechanism of action of macrolides has not been clearly shown, *in vitro* data has demonstrated that macrolides achieve clinical benefit through anti-inflammatory and

immunomodulatory properties, preventing the production of proinflammatory mediators from the host (65, 97).

Macrolides are bacteriostatic through interference with bacterial protein biosynthesis. Although the beneficial effect of macrolides is against a broad range of species particularly Gram-positive bacteria, patients whose airway is colonized by *P. aeruginosa* still benefit from taking macrolides. This effect is independent of a reduction in *P. aeruginosa* load, suggesting a mechanism other than a classical antimicrobial effect (83, 97).

Several inflammatory mediators are involved in the immune response during COPD exacerbations among which IL-6, IL-8, and TNFα have been well characterized (41).

The immunomodulatory effect of macrolides is related to the lactone ring that is seen with the 14 (erythromycin, clarithromycin, and roxithromycin) and the 15 (azithromycin) member macrolides. Studies have demonstrated that macrolides are able to reduce production of pro-inflammatory cytokines, lead to the resolution of inflammation by preventing accumulation and proliferation of neutrophil in the mucosal epithelium and suppressing lymphocytic activity (65). In human and, animal models macrolides suppress the production of cytokines such as IL-5, IL-6, IL-8, IL-1β, IL-10, TNFα, and GM-CSF, decrease neutrophil adhesion to epithelial cells and the mucous secretion from airways (65).

Furthermore, azithromycin is known to attenuate cigarette smoke extract-induced oxidative stress in human alveolar epithelial cells (100). According to our results that COPD patients who are smoking may still benefit from long-term azithromycin, we decided to develop a translational research approach incorporating both clinical and basic science. The next chapter (manuscript 2), will be limited to laboratory experiments studying the effects of macrolide on cigarette smoke interaction with airway epithelial cells.

CHAPTER 5: MANUSCRIPT 2 "THE MECHANISM OF ACTION OF AZITHROMYCIN TO REDUCE COPD EXACERBATIONS"

5.1 Abstract

Rationale: Recently, we published a retrospective observational study (101) showing that long-term azithromycin reduces the number of exacerbations in severe COPD patients already on optimal inhaled therapy. Beneficial effects of azithromycin were observed not only in ex-smokers but also in current smokers, not only in patients having COPD with bronchiectasis but also COPD without bronchiectasis and in those colonized with *P. aeruginosa.* Finally, the study demonstrated that the benefit was maintained beyond one year. These results led us to develop a translational project to study the mechanisms by which macrolides reduce COPD exacerbations. We hypothesized that treatment with azithromycin decreases exacerbation frequency by modulating inflammation in human airway epithelial cells exposed to cigarette smoke.

Specific objectives:

i) To characterize the inflammatory response of epithelial cells exposed to cigarette smoke;
ii) To evaluate the effects of azithromycin on airway epithelial cell expression of the inflammatory mediators IL-6 and IL-8 in response to cigarette smoke.

Methods: In order to study the effect of azithromycin on cigarette smoke-induced inflammation, BEAS-2B bronchial epithelial cells were incubated with 5% cigarette smoke extract (CSE) for 3h, 6h, and 24h. Expression and release of IL-6 and IL-8 mRNA were analyzed by quantitative real-time PCR (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. Then, airway epithelial cells were pretreated with azithromycin and exposed to 5% CSE. Expression of IL-8 and IL-6 were measured by qRT-PCR.

Results: We observed a significant increase of IL-6 and IL-8 mRNA following 3h, 6h and 24h exposure to 5% CSE. Similarly, IL8 secretion was significantly increased after exposure to 5% CSE for 24h. When cells were pre-treated with azithromycin and exposed to 5% CSE for 3H, we observed a significant dose-dependent decrease in the expression of IL-6 mRNA. Finally, when cells were pre-treated with 9 μg/mL of azithromycin and exposed to 5% CSE for 3h, we observed a significant decrease in the expression of IL-6 and IL-8 mRNA.
Conclusion: Incubation with azithromycin resulted in a significant decrease in the expression of the inflammatory mediators IL-6 and IL-8 in BEAS-2B cells exposed to CSE.
Future directions**:** The intracellular signaling pathways known to modulate IL-6 and IL-8 expression will be assessed by immunoblotting. Also, we will establish the impact of azithromycin on airway epithelial cell inflammation in response to an infection with *P. aeruginosa.*

5.2 Introduction

Azithromycin is a broad-spectrum macrolide antibiotic, preventing bacterial growth by interfering with their protein synthesis. It binds to the 50S subunit of the bacterial ribosome, thus inhibiting peptidyl transferase activity and interfering with amino acid translocation during the process of translation (102). However, azithromycin is different from other macrolide antibacterial drugs, accumulating at a higher rate in cells and tissues and has a plasma half-life of >40 hours (103). It has been proposed that macrolides exert their immunomodulatory properties by regulating leukocyte function and production of inflammatory mediators such as TNF-α, GM-CSF, IL-6, IL-8 and IFN-γ, controlling mucus hypersecretion and resolution of inflammation and modulating host defense mechanisms (56, 104). *In vitro* data suggests that azithromycin inhibits release of neutrophil chemoattractant IL-8 induced by lipopolysaccharide (LPS) in human bronchial epithelial cells (105). In potential agreement with these activities, azithromycin significantly decreased the rate of exacerbation in chronic obstructive pulmonary disease (COPD) patients (101).

IL-6, which is an interleukin, acts as both anti-inflammatory and proinflammatory cytokine, secreted by T cells and macrophages in response to infections and tissue injuries. IL-6 production contributes to host defense through the stimulation of acute phase responses, hematopoiesis, and immune reactions (106). In the lung, different cell types including alveolar macrophages, epithelial cells, endothelial cells, lymphocytes, and dendritic cells have been shown to secrete IL-6 (107). Data suggest that IL-6 production and secretion increase when cells are exposed to certain conditions, such as exposure to environmental allergens, infectious stimuli and cigarette smoke (28). There are some transcriptional and posttranscriptional mechanisms that strictly control the expression of IL-6. However, dysregulated continual synthesis of this interleukin plays a pathological effect on chronic inflammation and autoimmunity (106).

IL-8 is a multifunctional cytokine, produced by macrophages and other cell types such as epithelial cells, airway smooth muscle cells, and endothelial cells, that has significant neutrophil chemoattractant properties. It is produced in response to oxidative stress, a process thought to be mediated by upregulation of redox-sensitive transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) and activator protein-1(AP-1) (27).

Cigarette smoking has been demonstrated to be one of the most important etiological factors causing the development of chronic obstructive pulmonary disease (1). Evidence suggests that CSE causes oxidative stress in alveolar epithelial cells as well as primary human small airway epithelial cells. Also, CSE triggers NF-κB activation and the production of pro-inflammatory cytokines such as IL-6 and IL-8 release in primary human small airway epithelial cells (108). It has been demonstrated that azithromycin has the antioxidant capacity and attenuates CSE-induced oxidative stress injury in human alveolar epithelial cells (100). However, the precise mechanism by which azithromycin modulates inflammatory mediators IL-6 and IL-8 in airway epithelial cells in response to cigarette smoke is not yet understood.

5.3 Hypothesis/Objectives

We hypothesize that treatment with azithromycin decreases exacerbation frequency by modulating inflammation in human airway epithelial cells exposed to cigarette smoke.

Specific objectives: To assess the mechanism through which azithromycin modulates inflammation in an *in vitro* model of cigarette smoke-exposed airway epithelial cells by:

i) Characterizing inflammatory response of epithelial cells exposed to cigarette smoke;
ii) Evaluating the effects of azithromycin on airway epithelial cell expression of the inflammatory mediators IL-6 and IL-8 In response to cigarette smoke.

5.4 Materials

Immortalized human bronchial epithelial Beas-2B cells were provided by Dr. Simon Rousseau (Meakins Christie Laboratories, McGill University Health Centre, Montreal, QC, Canada). Following optimization experiments, Beas-2B cells were purchased from ATCC (Manassas, VA, USA).

DMEM (Dulbecco's Modified Eagle Medium: 4.5 g/L D-glucose, (+) L-glutamine & phenol red, without sodium pyruvate), penicillin/streptomycin 100X, 0.25% trypsin-0.04% EDTA, DPBS (Dulbecco's Phosphate Buffered Saline), qualified foetal bovine serum (FBS) were obtained from Wisent bioproducts (Quebec, Canada).

Human IL-6 and IL-8 ELISA Duo set kits were purchased from R&D (Ottawa, ON, Canada)

RNeasy Mini Kit for RNA extraction was purchased from Qiagen (Toronto, ON, Canada)

5.5 Methods

5.5.1 Cell culture

BEAS-2B cells were cultured in DMEM supplemented with 10% FBS and 100 U/mL of penicillin /streptomycin (complete DMEM). Cells were maintained in 10 cm petri dishes at 37°C, 5% CO2, 100% humidity. The medium was changed every 48-72 hours until cells reached 90% confluence. Cells were then washed with DPBS, incubated with trypsin 0.25%EDTA at 37°C for 5 minutes, resuspended in complete DMEM and harvested by centrifugation at 900 X *g* for 5 minutes. The supernatant was then aspirated, and the cells were resuspended in complete DMEM, counted using a standard hemocytometer and seeded in 6 well plates at a density of 75000 cells/well and grown to 70-80% confluency. At this time the cells were then incubated in serum-free medium (0%DMEM) for an additional 16-18 hours.

5.5.2 Preparation of Cigarette Smoke Extract (CSE)

Research grade cigarettes (3R4F) with a filter were acquired from the Kentucky Tobacco Research Council (Lexington, KT). Each cigarette contains 0.73 mg of nicotine, 9.4 mg of tar, and 12.0 mg of CO as described by the manufacturer. CSE was produced as previously described (109). Briefly, CSE was prepared by bubbling smoke from a cigarette through 10 mL of serum-free DMEM, sterile-filtering with a 0.45-μm filter (Filtropur S 0.45, membrane: PES, filtration surface: 5.3 cm2) and was used within 30 minutes of preparation. An optical density of 0.65 (320 nm) was regarded as 100% CSE (109) and diluted in serum-free DMEM to the appropriate concentration. In order to determine whether CSE induces the expression of IL-6 and IL-8 at different timepoints, BEAS-2B bronchial epithelial cells were incubated with 5% CSE for 3, 6 and 24h. The concentration of 5% CSE has been well characterized to induce inflammation *in vitro* (110).

5.5.3 Azithromycin preparation and cell treatment

We next evaluated the effects of azithromycin on airway epithelial cell expression of the inflammatory mediators IL-6 and IL-8 in response to CSE. For this purpose, BEAS-2B cells were pretreated with azithromycin at concentrations of 1, 3, 9, 18 and 27μg/mL for 1h before exposing them to 5% CSE. To prepare azithromycin for the experiments, 5mg azithromycin powder (Sigma-Aldrich, St. Louise, Missouri, United States) was dissolved in ethanol (PH:7.2) to achieve a concentration of 16μg/mL. One hour prior to incubation with or without CSE, azithromycin was diluted in PBS to reach 1:1 solution of ethanol; PBS and added to the cells at the indicated concentrations.

5.5.4 Assessment of cell death with trypan blue staining

To determine whether exposure to 5% CSE for 3h, 6h or 24h induces cell death, the percentage of unviable BEAS-2B cells was calculated using trypan blue staining (Wisent bioproducts, Quebec, Canada) according to the manufacturer's instructions. First 50µl of cell suspension was placed in a polystyrene tube and 50µL of 0.4% trypan blue was then added and mixed well with the cell suspension. The mixture was incubated at room temperature for less than 3 minutes and counted by using hemocytometer on the stage of a light microscope. Based on the principle that living cells possess intact cell membranes that exclude certain dyes, such as trypan blue, the cells uptaking trypan blue were considered non-viable. The percentage of viable cells was calculated by dividing the number of viable cells by the total number of the cells multiplying by 100 or % viable cells.

5.5.5 RNA extraction

Following treatment and harvesting, messenger ribonucleic acid (mRNA) was extracted from Beas-2B cells with Quiagen RNeasy Mini Kits according to the manufacturer's instructions (111). Briefly, cells were incubated with 350µL RLT lysis buffer mixed containing 10µl

B-mercaptoethanol (B-ME) (Sigma Aldrich, St. Louise, Missouri, United States) at room temperature for 5 minutes. The cell lysates were harvested and 350µL of 70% ethanol was added to the lysate and mixed well by pipetting. The solution was transferred to an RNeasy Mini spin column placed in a 2mL collection tube, centrifuged for 15seconds at ≥8000 X *g* and then the flow through was discarded, while the column retained mRNA. Following addition of 700µL Buffer RW1 to the spin columns and spin down for 15 seconds, 2*500µL Buffer RPE was added to the same columns and spun down for 2 minutes and that step was repeated. Finally, the RNeasy spin column was placed in a new 1.5 ml collection tube. mRNA was eluted by adding 30µL RNase-free water and centrifuging for 1 minute.

Quantification of mRNA was conducted using a Nanodrop 1000 spectrophotometer (infinite M200 pro, TECAN, CA).

5.5.6 Reverse transcription and quantitative real-time PCR

To remove any potential DNA contamination, DNase digestion was used according to manufacturer's instruction (111). 350 µl Buffer RW1 was added to RNeasy column and centrifuged 15 sec at ≥8000 Xg. Then 10µL of DNase 1 stock solution was added to 70µL Buffer RDD, mixed very well by inverting the tube. 80µL DNase 1 incubation mix was then added directly to the RNeasy column membrane, incubated at room temperature for 15 min. The complementary DNA strand was synthesized by adding 15µL reverse transcription master mix containing iScript TM Reverse Transcription Supermix (BIO-RAD, Canada) and nuclease-free water to 5µL RNA template containing 100µg of RNA template. The mixture was incubated in a BioRad Thermal Cycler as follows: 5 minutes at 25 °C, 20 minutes at 46 °C to achieve full polymerase activity and 1 minute at 95 °C to inactivate the enzyme. Then, the mRNA levels of IL-8 and IL-6 were analyzed using this cDNA template and gene-specific primers (Table1).

For quantitative real-time PCR 96-well reaction plate (Diamed, Mississauga, ON, Canada) were used with each condition containing 50 to 100 ng of cDNA in a total volume of 2.5 µL sterile water, 0.3 µM of each forward and reverse primer, 5µl iQTM SYBR Green Supermix (BioRad, South San Francisco, CA, USA) as well as 1.9 µl of sterile water. The plate was sealed and cycled as follows using a Thermal Cycler machine (BIO-RAD C1000 Touch TM, CA): Thermal cycling was initiated at 95°C for 3minutes and followed by 39 cycles denaturation at 95°C for 10 seconds and annealing at 60ºC for 45 seconds. Each condition was normalized to the housekeeping gene GAPDH. Relative fluorescence and therefore gene

amplification were interpreted as fold induction from cycle threshold values using the Pfaffl mathematical model. Primer efficiencies were determined using a standard curve generated from a 3-fold serial dilution of cDNA.

Table 1. Primer sequences used for qRT-PCR analysis

Gene	Forward Primer Sequence	Reverse Primer Sequence
GAPDH	AGC AAT GCC TCC TGC ACC ACC	CCG GAG GGG CCA TCC ACA GTC
IL-6	GTG TGA AAG CAG CAA AGA GG	TGC AGG AAC TGG ATC AGG
IL-8	GTG CAG TTT TGC CAA GGA GT	CTC TGC ACC CAG TTT TCC TT

5.5.7 Enzyme-linked immunosorbent assay (ELISA)

Following the incubation with 5% CSE for 3, 6, or 24 h, cell culture supernatants were harvested and centrifuged at 8000 × *g* for 10 min. The concentrations of IL-6 and IL-8 in the cell culture supernatant were determined by ELISA according to the manufacturer's instructions. Briefly, the Capture Antibody was diluted to a concentration of 360µg/mL in PBS and a 96-well microplate was coated with 100 µL per well of the diluted capture Antibody. The plate was sealed and incubated overnight at room temperature. Each well was aspirated and washed with 400µL wash buffer containing 10 mL of 5% Tween 20 Solution with 990 mL of BPS for a total of 3washes. The plate was then blocked by adding 300 µL of Reagent Diluent containing 1% bovine serum albumin (BSA) in PBS to each well, incubated at room temperature for a minimum of 1 hour and the aspiration/wash step was repeated. This

step was followed by adding 100 µL of samples or standards in Reagent Diluent to each well, covering with an adhesive strip and incubating 2 hours at room temperature. The standards had known concentrations of recombinant human IL-6 or IL-8. The aspiration/wash was repeated again. 100 µL of the Detection Antibody, diluted in Reagent Diluent to a concentration of 54µg/mL, was then added to each well, covered again with a new adhesive strip, and washed after 2-hour incubation at room temperature. Next step included adding 100 µL of the Streptavidin-HRP diluted to a concentration of 250 µL to each well, washing with Wash Buffer after 20 minutes of leaving the plates at room temperature. Then, after adding 100µL of Substrate Solution containing 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine) were added to each well for 20 minutes, the plate was protected against the light. Finally, 50 µL of Stop Solution (2 N H_2SO_4) was added to each well and the absorbance was read at 450 nm and 570 nm within fifteen minutes by an iMark microplate reader (Bio-Rad Laboratories, Mississauga, Ontario). Data were analyzed by taking averages of optical density (OD) duplicates and constructing a standard curve by plotting absorbance against concentration. The best fit curve was drawn for 7 points on the graph by using the third order multinomial regression equation to calculate sample concentration from OD values.

5.5.8 Statistical Analysis

Using GraphPad Prism 6 (v. 6.02; La Jolla, CA), statistical analysis was performed for normally distributed data using a one-way analysis of variance (ANOVA) and non-parametric test followed by Dunnett's multiple comparisons test to compare all pairs to control or Bonferroni post-test comparing untreated vs CSE and CSE vs different concentrations of azithromycin. In all cases, a p-value < 0.05 is considered statistically

significant. Results are presented as mean ± standard error of the mean (SEM) of the fold-changes compared to control cells.

5.6 Results

5.6.1 IL-6 and IL-8 mRNA expression increases significantly following exposure of BEAS 2B cells to 5% CSE

We observed a significant increase in the expression of IL-6 and IL-8 mRNA in response to 5% CSE for 3, 6 and 24h (Figure 1 A and B). To validate our results in another cell line, A549 alveolar epithelial cells were exposed to 5% CSE for 3 and 6h. This exposure also resulted in a significant increase in IL-6 an IL-8 mRNA expression (supplementary figure 1). Collectively, exposure to 5% CSE increases the total expression of IL-6 and IL-8 mRNA in BEAS-2B and A549 cell lines.

A B

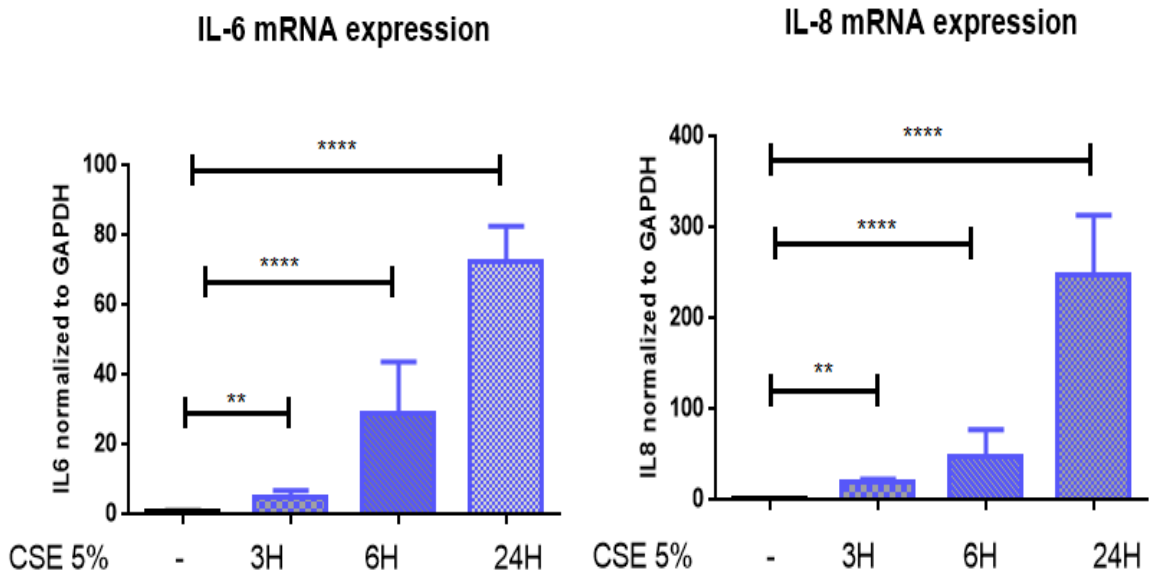


Figure 1. IL-6 and IL-8 mRNA expression is increased in BEAS-2B cells exposed to 5%

CSE for 3h, 6h, and 24h. BEAS-2B cells were exposed to 5% CSE for 3, 6 and 24h. IL-6 and IL-8 mRNA expression was measured by qRT-PCR. Exposure to 5% CSE resulted in a significant increase in IL-6 (A) and IL-8 (B) mRNA. Gene expression was normalized to GAPDH**.** Results are expressed as the mean ± SEM of 5 independent experiments. *p< 0.05, **p= 0.001, ****p=0.0001

5.6.2 Exposure of BEAS-2B cells to 5% CSE results in a significant increase in IL-8 secretion

Although we did not observe an increase in extracellular IL-6 protein level compared to untreated cells (figure 2A), extracellular IL-8 levels were significantly increased in BEAS-2B cells following 24h exposure to 5% CSE (figure 2B).

A B

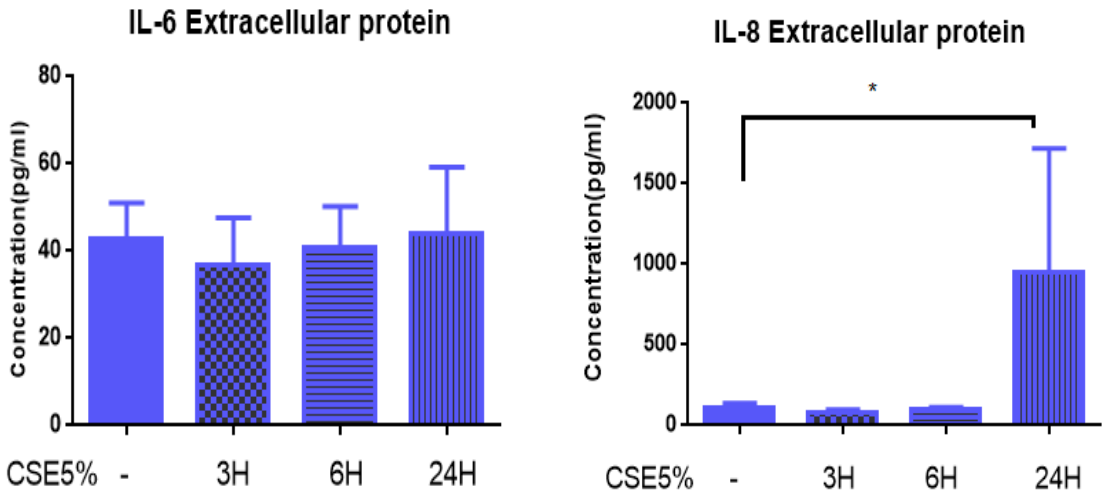


Figure 2. Exposure to 5%CSE for 24h increases IL-8 protein secretion in BEAS-2B cells. BEAS-2B cells were exposed to 5% CSE for 3, 6 and 24h and IL-6 and IL-8 protein secretion was measured by ELISA. While extracellular IL-6 protein level is not significantly increased after exposure to 5% CSE for 3, 6 and 24h (A)**,** exposure to 5% CSE for 24h

significantly increased IL-8 secretion (B). Results are expressed as the mean ± SEM of 4 independent experiments. *p< 0.05

5.6.3 Incubation of BEAS-2B cells with 5%CSE for 3h, 6h or 24h does not increase cell death

Incubation of BEAS-2B cells with 5% CSE for 3h, 6h or 24h did not significantly increase cell death compared to untreated cells (Figure 3).

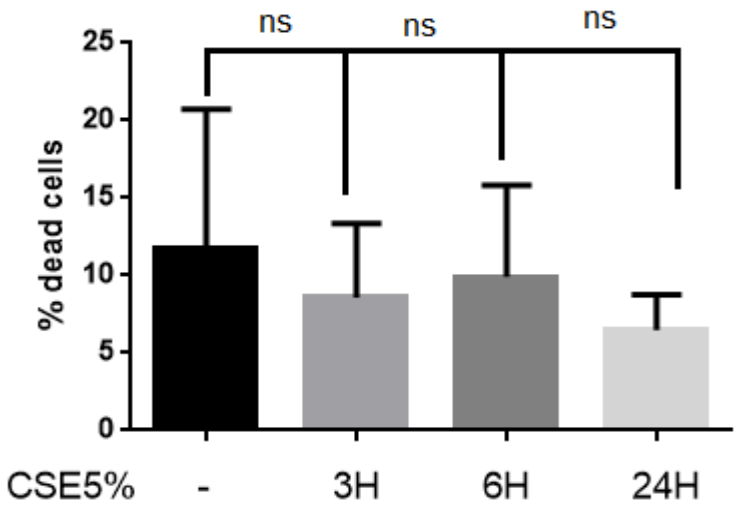


Figure 3. Trypan blue positive cell percentage. BEAS-2B cells were incubated with 5%CSE for 3, 6 or 24h. Cell death was assessed with trypan blue staining. Exposure to 5% CSE does not significantly increase cell death in BEAS-2B cells. Results are expressed as the mean ± SEM of 4 independent experiments.

5.6.4 Treatment with azithromycin resulted in a dose-dependent reduction of IL-6 mRNA level in BEAS-2B cells exposed to CSE

We observed a significant dose-dependent reduction in the expression of IL-6 mRNA in BEAS-2B cells treated with azithromycin at concentrations of 1, 3, 9, 18 and 27µg/mL, and exposed to 5% CSE (Figure 4).

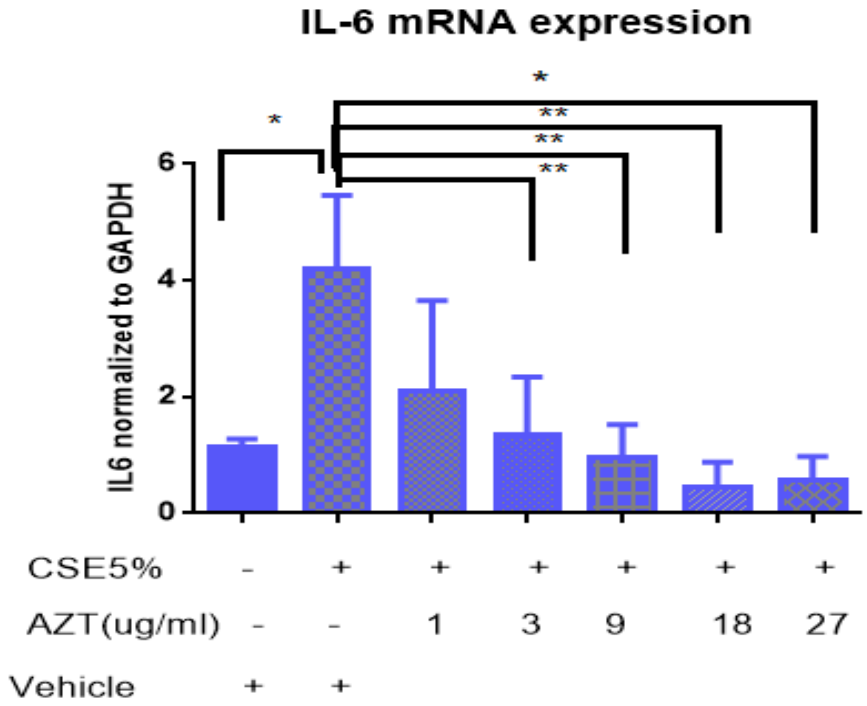
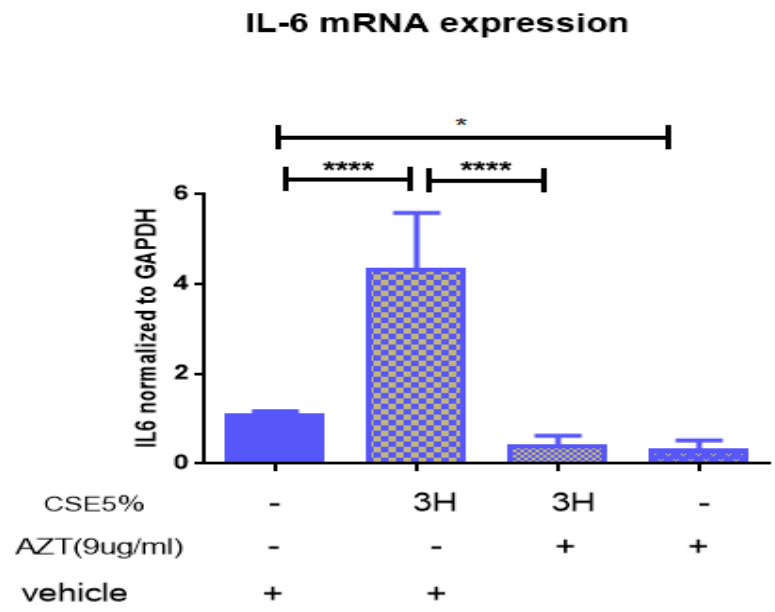


Figure 4. Pre-treatment with azithromycin leads to a significant decrease in IL-6 mRNA following exposure to 5% CSE. BEAS-2B cells were pre-incubated with azithromycin at concentrations of 1, 3, 9, 18 and 27µg/mL for 1h. Cells were then exposed to 5%CSE for 3h and IL-6 expression was measured by qRT-PCR. Treatment with azithromycin resulted in a significant dose-dependent decrease in the expression of IL-6 mRNA. Results were analyzed by One-Way ANOVA followed by Bonferroni post-test comparing untreated vs CSE and CSE vs different concentrations of azithromycin. Results are expressed as the mean ± SEM of 3 independent experiments.

5.6.5 Incubation with azithromycin decreases the expression of IL-6 and IL-8 mRNA in BEAS-2B cells exposed to 5% CSE

Based on our previous results, we selected the concentration of 9 µg/mL of azithromycin to assess its effects on IL-6 and IL-8 mRNA expression in BEAS-2B cells exposed to cigarette smoke. Our results reveal an inhibition of IL-6 and IL-8 mRNA levels in 5% CSE-exposed BEAS-2B cells when the cells are pretreated with 9 µg/mL azithromycin (figures 5 A and B, respectively). Additionally, exposure to 9 µg/mL of azithromycin alone reduced the baseline expression of IL-6 and IL-8 mRNA in BEAS-2B cells compared to the cells exposed to vehicle only (Figure 5 A and B).

A



B

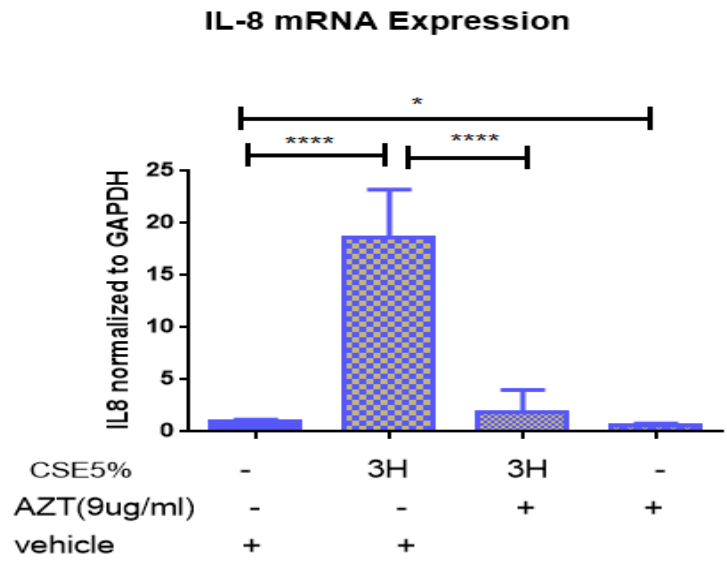


Figure 5. Azithromycin significantly decreases IL-6 and IL-8 mRNA expression in BEAS-2B cells. BEAS-2B cells were pre-incubated with 9 µg/mL of azithromycin for 1h. Cells were then exposed to 5% CSE for 3h and expression of IL-6 and IL-8 mRNA was measured by qRT-PCR. Treatment with azithromycin resulted in a significant decrease in the expression of IL-6 (A) and IL-8 (B) mRNA. Exposure to 9 µg/mL of azithromycin alone significantly reduce the baseline expression of IL-6 (A) and IL-8 (B) mRNA in BEAS-2B cells compared to cells exposed to vehicle only. Results were analyzed by One-Way ANOVA followed by Bonferroni. Results are expressed as the mean ± SEM of 8 and 4 independent experiments for IL-6 and IL-8 mRNA respectively. *p< 0.05, ****p=0.0001

5.7 Discussion

As previously reported, exposure to CSE induced the expression of IL-6 and IL-8 in bronchial epithelial cells (28, 112). Interestingly, an important aspect of our study was the

inhibition of IL-6 and IL-8 mRNA level in cigarette smoke exposed-BEAS-2B cells, when they were pretreated with azithromycin, which has not been reported in any other studies. Furthermore, our results have demonstrated that pre-treatment with 9μg/ mL azithromycin alone decreased the expression of IL-6 and IL-8 significantly compared to untreated cells. The latter could be reflecting that azithromycin affects pathways regulating the constitutive expression of IL-6 and IL-8 mRNA in bronchial epithelial cells.

To gain new insights into the mode of action of azithromycin, we evaluated its effect on gene expression in bronchial epithelial cells exposed to cigarette smoke, using qRT-PCR. When the cells were pretreated with multiple concentrations of azithromycin, we observed a dose-dependent decrease in the expression of IL-6 mRNA in bronchial epithelial cells incubated with 5%CSE. This result indicates that the higher the concentration of azithromycin, the greater the reduction in pro-inflammatory cytokines such as IL-6 in the presence of CSE. We selected a concentration of 9 μg/mL of azithromycin due to its similarity to the concentration of azithromycin in sputum of patients treated with this antibiotic, which is 9.5 μg/mL when the drug reached the steady state after taking 250 mg of azithromycin daily for four weeks (113). Likewise, this concentration closely represents the levels of azithromycin found in plasma in patients who were treated with short-term azithromycin (114).

The reported effects of macrolides on inflammatory responses have recently been reviewed (56). They include suppression of pro-inflammatory cytokines, such as IL-1, IL-6, and IL-8, inhibition of chloride and water secretion across airways, decreased mucus synthesis and secretion, promotion of inflammatory cell apoptosis, a decrease in production of nuclear transcription factors, such as NF-kB and AP-1, and interruption of bacterial virulence (56). The key novel aspect of our study is that we have demonstrated for the first

time the role of azithromycin in cigarette smoke-induced inflammation in human airway epithelial cells.

Cigarette smoke which is the most well-studied risk factor of COPD, has been shown to activate several mediators and cytokines, including TNFα, IL-8 and IL-6 (27, 28). Cigarette smoke increases intracellular ROS levels in lung epithelial cells that leads to activate several ROS-sensitive signaling pathways, including the mitogen-activated protein kinases (MAPKs) and various downstream transcriptional factors, such as NF-*κ*B; these effects then ultimately promote inflammatory gene expression (117, 118). Consistent with these findings, our results were able to show that the expression of IL-6 and IL-8 mRNA increased significantly, although we did not observe a significant increase in the secretion of IL-6 protein from BEAS 2B cells. The lack of IL-6 secretion might be due to the effect of cigarette smoke to alter the cellular composition of the airway epithelium in a post-transcriptional manner or post-translational modifications, such as a post-translational modification of p65 e.g. phosphorylation, acetylation and ubiquitination, which modulates DNA binding and transcriptional activity (119).

However, cigarette smoke is able to attenuate immune responses in the presence of viruses and bacteria, resulting in pathogen proliferation and persistence in the airways (22). The study of Kulkarni R, et al showed that CSE exposure attenuates the production of proinflammatory cytokines such as IL-6, IL-8, and IL-10 in both primary and immortalized epithelial cells exposed to *Staphylococcus aureus* and *Haemophilus influenzae* infection (25). Macrolides are effective broad-spectrum antibiotics, which have been shown to have significant anti-inflammatory and immunomodulatory effects related to the macrocyclic lactone ring. Studies have demonstrated that macrolides exert their immunomodulatory properties by regulating the production of inflammatory mediators such as TNF-α, GM-CSF, IL-6, IL-8 and IFN-γ and modulating host defense mechanisms (56, 104, 120).

Other macrolides such as erythromycin and clarithromycin have shown to inhibit expression and release of IL-8 at the therapeutic and noncytotoxic concentrations in human bronchial epithelial cells (121). Study of Murphy DM, et al, has reported that azithromycin caused a significant decrease in the LPS-stimulated IL-8 and GM-CSF release in primary bronchial epithelial cells derived from stable lung allografts (105). Our results are compatible with these findings, as we have shown that azithromycin is able to decrease the expression of the proinflammatory cytokines IL-6 and IL-8.

5.7.1 Strengths and Limitations

The main strength of this *in vitro* model was the design of the experiment that allows us to assess directly the effect of azithromycin in CSE-induced inflammation in airway epithelial cells. Another strength of this study was choosing a concentration of azithromycin which closely represents the level of azithromycin in sputum and plasma of patients treated with this antibiotic.

However, this *in vitro* study was limited to one type of the cells which does not represent the physiological conditions of the airway epithelium in vivo.e.g. complex of the cells, oxygen and air liquid. The study was limited to short term azithromycin therapy while in our clinical study the effect of long-term therapy was evaluated.

This study introduces a translational research approach from clinical findings showing the direct effect of azithromycin on inflammatory mediators in inflamed bronchial epithelial cells which leads to logical and guided use of azithromycin in preventing exacerbation in COPD patients. Further studies are required in order to improve the result of this study regarding the inflammatory effects of azithromycin in COPD patients.

CHAPTER 6: SUMMARY OF THE THESIS FINDINGS, DISCUSSION, AND CONCLUSIONS

The major goal of this Master thesis was to evaluate the effectiveness of long-term azithromycin in reducing exacerbations in patients with severe COPD and assess the mechanism through which azithromycin modulates inflammation.

First, the clinical retrospective observational study showed that long-term azithromycin therapy reduces acute exacerbations in patients with severe COPD known for recurrent exacerbations while on optimal inhaled therapy. Through this study of real-life practice, we were able to show that long-term azithromycin therapy reduces not only acute exacerbations but also health service use, such as emergency visits and hospital admissions in severe COPD patients beyond one year. Beneficial effects of azithromycin were observed in subgroups of smokers/ex-smokers, as well as in patients with or without bronchiectasis. Furthermore, in this study, desirable effects were more likely to outweigh the risks and adverse events in patients colonized with *P. aeruginosa.*

One of the most important strengths of this study was treatment with azithromycin in a well-defined patient population with severe or very severe COPD and recurrent exacerbations despite optimal medical therapy. While most studies previously published were at one year or less, our study was designed to study the effects of treatment with azithromycin beyond 1 year. Finally, we have shown that in patients colonized with *P. aeruginosa*, the potential benefits of azithromycin outweigh the risks and adverse events, most particularly the increased incidence of microbial resistance. These results reinforce the GOLD recommendations and will help the clinician, guiding the therapeutic decision of prescribing azithromycin in patients with COPD who are on optimal therapy and will most likely benefit from taking long-term azithromycin. Further studies are recommended to confirm the effects

and the appropriate dose of long-term azithromycin therapy especially on severe COPD patients who are colonized with *P. aeruginosa.*

Although azithromycin has been demonstrated to have the antioxidant capacity through attenuating CSE-induced oxidative stress, the precise mechanism by which azithromycin modulates inflammatory mediators in airway epithelial cells in response to cigarette smoke was not clearly understood. Based on our clinical findings that COPD patients who are current smokers still benefit from taking azithromycin, we decided to develop a translational research approach to study the effects of azithromycin on cigarette smoke interaction with airway epithelial cells.

Second, our *in vitro* study showed a significant increase of proinflammatory cytokines, IL-6, and IL-8 mRNA, following exposure of BEAS-2B cells to 5% CSE. Similarly, IL8 secretion was significantly increased after exposure to 5% CSE for 24h. When cells were pre-treated with azithromycin and exposed to 5% CSE for 3H, a significant dose-dependent decrease in the expression of IL6 mRNA was observed. Finally, when the cells were pretreated with azithromycin and exposed to 5% CSE for 3h, the result revealed a significant decrease in the expression of IL6 and IL8 mRNA.

Interestingly, an important aspect of our study, which has not been reported in any other studies, was the inhibition of pro-inflammatory cytokines, IL-6, and IL-8 mRNA level, in cigarette smoke exposed-bronchial epithelial cells, when they were pretreated with azithromycin. Furthermore, our results revealed that pre-treatment with azithromycin alone decreased the expression of IL-6 and IL-8 significantly compared to untreated cells which could be related to the effect of azithromycin on pathways, regulating the expression of IL-6 and IL-8 mRNA in bronchial epithelial cells. The key novel aspect of this study is that we have demonstrated for the first time the role of azithromycin in cigarette smoke-induced inflammation in human airway epithelial cells.

We were able to conduct a translational research approach from a clinical problem and the findings of a clinical study in real life to evaluate the mechanism of action of azithromycin directly on inflammation in human airway epithelial cells in response to cigarette smoke. However, lack of physiological condition and complexity of the cells are limitations of *in vitro* study.

In conclusion, this thesis has introduced a translational research approach incorporating both clinical and basic science to investigate the role of azithromycin in reducing COPD exacerbations. Implementing this research approach allowed us to build further evidence from our observational clinical study suggesting that azithromycin can be effective to prevent exacerbation even in COPD patients who are still currently smoking. This is important because our clinical results were in contradiction with a recent post hoc analysis from a large trial suggesting that azithromycin was only effective in ex-smokers; based on this, GOLD doesn't recommend treating COPD patient currently smoking but only those who are ex-smokers. The *in vitro* study confirmed the biological plausibility and supported underlying mechanisms, showing azithromycin decreases cigarette smoke-induced inflammatory responses, which is consistent with our clinical observations.

Future Directions for *in vitro* and clinical studies

Future studies are required to assess the impact of azithromycin on airway epithelial cell inflammation in response to an infection with *P. aeruginosa.* We recommend conducting other *in vitro* studies evaluating the effects of azithromycin on the activation of intracellular pathways and on the translocation factors that may lead to the discovery of new medication improving inflammatory process in exacerbation of COPD patients. As well, conducting a randomized clinical trial to increase the reliability of our result concerning the effect of azithromycin on COPD exacerbations is recommended.

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CHAPTER 8: APPENDIXES

8.1. For the clinical project: Data collection form

studyID	DOB	Gender	Date Azithro started	Smoking: Never N; Former F; Current C	Pack yrs	MRC dyspnea (x/V)	Chronic bronchitis

		Medications					
Influenza vaccine date	Pneumovax Date	LAAC (mcg)	LABA (mcg)	ICS (mcg)	Uniphyl (mg)	Singulair (mg)	Steroids PO (mg)

			Comorbid conditions					
Oxygen L/min	Asthma	Sleep apnea	Osteoporosis	Coronary disease	Atrial fib	HTN	Diabetes	Pulm HTN

Sputum Culture		CT Chest findings		
Date	Organism	Date	Emphysema:E Fibrosis:F Bronchiectasis:B	Date

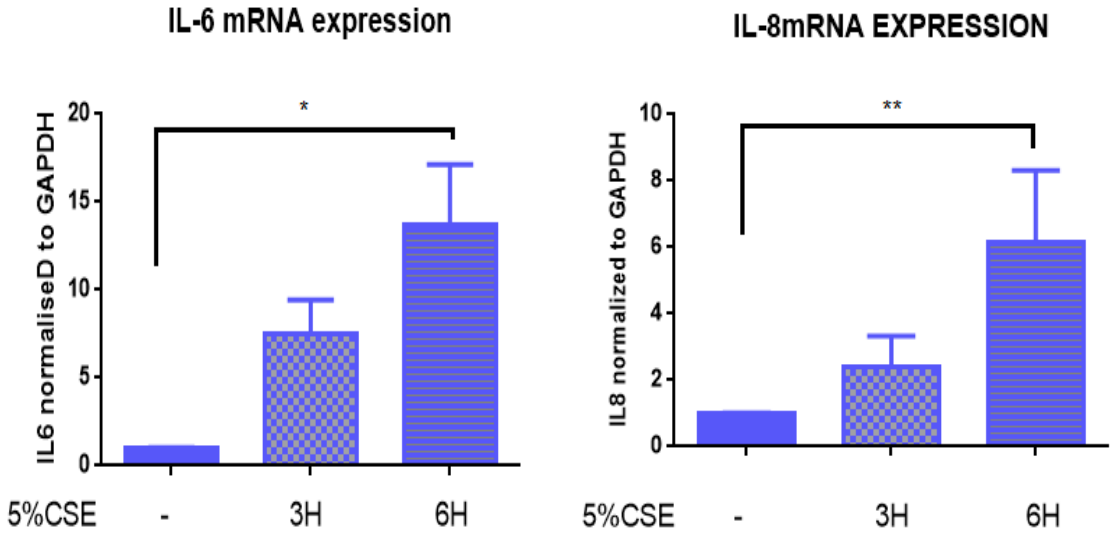
			Exacerbations				
Visit Type Phone:Ph Clinic: CV Emerg:ER	Hospitalization	Duration Hosp (d)	Antibiotics (Y/N)	Duration (d)	Steroids (Y/N)	Duration (d)	Symptoms Dyspnea:D cough:C Sputum:S

			Pulmonary Function tests / spirometry				
Severity Mild:L Moderate:M Severe: S	Date	FEV1 (L)	FEV1% pred	FVC (L)	FVC% pred	Ratio	DLCO% pred

8.2.1. Exposure of A549 cells to 5%CSE for 3h and 6h increases IL-6 and IL-8 mRNA expression

To assess the effect of CSE on mRNA expression in a different lung cell line, we exposed A549 human alveolar epithelial cells to 5%CSE for 3h and 6h and the expression of IL-6 and IL-8 mRNA was analyzed by qPCR. Similar to our previous results, we observed an increase in IL-6 and IL-8 mRNA at 3h and 6h after an exposure to 5% CSE (Figure 5.6.2 A and B).

A B



Supplementary figure 1. Exposure to 5%CSE increases IL-6 and IL-8 mRNA expression in A549 cells. A549 cells were exposed to 5% CSE for 3 and 6h. IL-6 and IL-8 mRNA expression was measured by qRT-PCR. We observed a significant increase in IL-6 (A) and IL-8 (B) mRNA when the cells were exposed to 5% CSE for 6h. Gene expression was normalized to GAPDH**.** Results are expressed as the mean ± SEM of 4 independent experiments. *p< 0.05**,** **p= 0.001