Evaluation of Host Genetic Susceptibility to Predict Nontuberculous Mycobacteria Pulmonary Disease in Patients with Cystic Fibrosis

by

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MD, Universidad del Valle, Colombia, 2013

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate and Postdoctoral Studies

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

May 2022

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Abstract

Patients with cystic fibrosis (CF) have an elevated lifetime risk of infection and disease caused by nontuberculous mycobacteria (NTM). Infection with NTM can be associated with faster decline in lung function for people living with CF. Diagnosis and treatment of pulmonary NTM disease (NTM-PD) remains challenging as there are no accurate estimates of the burden, there is no way to predict progression to disease and the therapeutic guidelines lack high quality evidence for recommendations.

In this thesis, we began by estimating the overall burden of NTM infection and disease in the CF population through a systematic review of prevalence and incidence. We included all available data from registries and observational studies and found a pooled estimate of NTM infection point prevalence of 8%. We identified geographical region and sample size as determinants of heterogeneity in our analysis. Also, we found that estimates were more accurate for NTM infection caused by the *Mycobacterium avium* and *Mycobacterium abscessus* complexes individually. However, we could not identify other sources of heterogeneity due to the lack of primary reporting of microbial identification methods and screening approaches.

Next, we explored the impact of host gene expression on the progression to pulmonary NTM disease (NTM-PD) in a cohort of patients with NTM infection (n = 42). We conducted an RNAseq experiment using whole blood close to the time of first NTM growth and conducted differential gene expression using DESeq2. Our results show that patients who progressed to NTM-PD had higher expression of genes that are associated with innate immunity and inflammation. These findings contrast with results of non-cystic fibrosis studies in humans that show decreased

lymphocyte and immune responses in NTM-PD. However, the pro-inflammatory state of the CF lung and the higher bacterial burden observed in CF, could explain this contradictory result. Overall, in this biomarker discovery study, we identified several functional pathways that may play a role in progression to NTM-PD n the CF population, providing a basis for future biomarker discovery studies.

Lay Summary

Cystic fibrosis is the most common life-limiting genetic disease in Caucasians. It affects multiple organs, but the respiratory system is the main cause of complications and death. The lungs of CF patients are more susceptible to infections by fungi, viruses and bacteria. Nontuberculous mycobacteria are organisms found in water and soil sources that can infect the lungs of predisposed patients, including individuals with CF. These microorganisms are associated with a faster loss of breathing capacity in CF, but can also reside in the lungs without causing significant disease. In this thesis, we examined the burden of infection and disease caused by NTM in the CF population by conducting a systematic review of the literature. Then, in our third chapter, we explored the role of the immune and inflammatory response of the affected individuals in the progression of the infection to clinically relevant disease.

Preface

The research topics included in this document were conceived through an iterative process between Miguel Prieto and Bradley Quon (supervisor). This thesis is an original and unpublished work by the student, Miguel Prieto.

For chapter 2, the systematic review protocol and all research documentation were prepared by me with feedback and supervision by Bradley Quon. The screening of abstracts and full texts, the extraction of data and the appraisal of quality were done independently in collaboration with Mossab Allam, who acted as second reviewer. All data wrangling, analyses, images, tables and report were done by the student with valuable feedback from Bradley Quon and Alex Franciosi.

Chapter 3 is a secondary data analysis that uses information and samples collected in a larger project, the cystic fibrosis biomarker project. The principal investigator of this project is Bradley Quon and the project was approved by the University of British Columbia – Providence Health Care research ethics board number H12-00835. The additional analyses and use of samples performed for chapter 2 are covered by the ethics number H12-00910. I prepared the research protocol for the study, extracted clinical data from primary sources, analyzed all data and prepared reports and summaries. The RNA extraction experiments were performed by Jiah Jang and all relevant sequencing experiments were outsourced to Genome Quebec.

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List of Abbreviations

16S rRNA – Gene for the ribosomal component of the 30s ribosomal subunit of bacteria

ATS – American Thoracic Society

BACTEC MGIT – BACTEC™ Mycobacterial Growth Indicator Tubes

BAL – Broncho-Alveolar Lavage

BCSA – *Burkholderia cepacia* selective agar

CBC – Complete Blood cell Counts

CF – Cystic Fibrosis

CFTR – Cystic Fibrosis Transmembrane Regulator

CI – Confidence interval

CT – Computed tomography

DNA – Deoxy-Ribonucleic acid

EMBASE – Excerpta Medica dataBASE

FDR – False Discovery Rate

FEV1 – Forced expiratory volume in 1 second (lung function measurement)

HP-LC – High Performance Liquid Chromatography

IL – Interleukin

IQR – Inter-Quartile Range

JAK – Janus Kinase

L-J – Lowenstein-Jensen culture medium

LOGIT – Log of the proportion divided by one minus the proportion

MABs – *Mycobacterium abscessus* complex

MAC – *Mycobacterium avium* complex

MALDI-TOF (MS) – Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (coupled to Mass Spectrometry)

MEDLINE – Medical Literature Analysis and Retrieval System Online

Non-CF – Non-cystic fibrosis (other diseases)

NR – Not reported

NTM – Nontuberculous mycobacteria

NTM-PD – Pulmonary nontuberculous mycobacterial disease

PCA – Principal Component Analysis

PCR – Polymerase chain reaction for nucleic acids amplification

PEX – Pulmonary exacerbations

PI – Prediction interval

ppFEV – Percentage of predicted forced expiratory volume in 1 second, based on age, height and ethnicity

PRISMA – Preferred Reporting Items for Systematic Reviews and Meta-Analyses

RGM – Rapid growing mycobacteria

RNA – Ribonucleic acid

SD – Standard Deviation

STAT – Signal transducer and activator of transcription

US – United Stated of America

Acknowledgements

I want to thank my supervisor Dr. Bradley Quon for his support in the conception, conduction and analysis of my thesis. Particularly, I want to highlight his commitment to career focused training and his capacity to actively listen and support my studies through these unprecedented times.

Furthermore, I want to extend my gratitude to Jiah Jang for helping me in all wet lab procedures and Kang Dong for his kindness and support in all analytical and bioinformatics aspects of this project. In addition, the discussions, feedback and expertise of Sameer Desai, Alex Franciosi, Grace Lam and Naomi Potter contributed to enrich all aspects of my projects.

Finally, I want to acknowledge and thank all the support received by my wonderful wife, Diana Giron, throughout these years. She was instrumental not only in helping me keep a healthy work-life balance and enjoy the graduate student experience, but also provided encouragement and academic insights to my thesis and presentations.

Chapter 1: Introduction

1.1 Epidemiology of cystic fibrosis

1.1.1 Historical perspective of Cystic Fibrosis

Cystic fibrosis (CF) is a genetic autosomal recessive disease caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. The initial description of the disease in 1938 described only pediatric manifestations as the prognosis was poor (1). In the 1950s, the fortuitous association of abnormal "saltiness" in sweat with CF led to the first large improvement in clinical care: the diagnostic sweat chloride test. Then, in 1989, decades of research led to the discovery of mutations in the CFTR gene as the cause of CF (2,3). Since then, studies about the underlying mechanisms of disease have led to significant improvement in life expectancy, with most CF patients reaching adulthood currently (4). Furthermore, the current paradigm has shifted from symptomatic and preventive treatment to correction of the inherent causative protein dysfunction using highly specific CFTR modulator drugs (5).

1.1.2 Incidence and prevalence

An estimated 80,000 patients live with Cystic Fibrosis worldwide. The disease affects mostly Caucasian populations (incidence of 1 in 3,000-4,000 newborns), with lower incidences in other ethnic groups, particularly in Africa and Asia. (6–9). In 2019 in Canada, 4,300 individuals were living with CF with 116 new diagnoses in the year (10). A vast majority of epidemiological reports come from high-income countries in Europe, North America and Australia. Most of these countries have newborn screening for CF and record longitudinal demographic and clinical data in registry databases (7,11). In contrast, Lower-Middle-Income Countries lack the technical and financial

capacity to provide newborn screening and multidisciplinary care to CF patients (12,13). Furthermore, competing healthcare needs and lack of awareness about CF also lead to diagnostic delays and poorer outcomes in Lower-Middle-Income Countries. Thus, the burden of disease in Latin America, Asia and Africa is largely unknown (13,14).

1.1.3 Life expectancy

The life expectancy for Cystic fibrosis patients was less than five years before the 1960s (9,15), while now it is above 50 years (6,16–18). Multiple studies show an increase in life expectancy in European and North American populations over the last couple of decades (17,19). The improvement extends to patients with severe lung disease, defined by forced expiratory volume in 1 second (FEV1) below 30% of predicted, and surpasses the life-expectancy increase seen in the general population. Optimized detection of cases, multidisciplinary care starting at an early age, implementation of *Pseudomonas* spp. eradication regimes, aggressive therapy for pulmonary exacerbations, lung transplantation and improved gastrointestinal therapies are commonly cited as the sources of improved survival in CF (16,19–21). However, with an increase in adult survivors, late complications of CF like diabetes and allergic bronchopulmonary aspergillosis are seen more commonly (22). Finally, the predominant causes of death in CF are respiratory failure and transplant-related complications, in contrast to pediatric gastrointestinal complications (like meconium ileus and pancreatic insufficiency) in the 1960s (2,18,23,24).

1.2 Pathophysiology of cystic fibrosis lung disease

1.2.1 Etiology

The *CFTR* gene encodes the CFTR protein, which is expressed in epithelial cells of the respiratory, gastrointestinal and reproductive tracts (25–27). The CFTR protein is a multidomain cell membrane protein that allows ATP-dependent transport of chloride at the apical cellular membrane (28,29). Causative mutations of CF reduce or abolish the function of the CFTR protein. Historically and to provide a common framework for therapeutic interventions, mutations are classified according to the molecular defect they produce. Group I mutations cause premature termination of transcription, those in group II produce protein misfolding, group III mutations affect the ATP-binding domains, group IV mutations reduce conductance of chloride (Cl^-) and bicarbonate (HCO_3^-) ions, group V defects decrease the number of functional CFTR proteins and group VI mutations increase degradation of functional proteins (25,26,29–31). Currently, more than 380 causative mutations of CF have been identified. The predominant mutation in CF patients with European ancestry is the F508 deletion that causes a misfolded protein (13,26). **Table 1-1** summarizes the most common mutations in 2019 Canadian CF population (10).

Table 1-1. Distribution of genotypes in the Canadian CF population for 2019 (n=4344)

Mutation	Percentage
Homozygous F508del	47.1%
Heterozygous F508del	40.7%
Other	11.3 %
Unknown	1.3%

1.2.2 Pathophysiological mechanisms

The pathophysiological events leading to the CF respiratory compromise are not completely understood. The most widely accepted hypothesis proposes that CFTR dysfunction produces abnormal mucus with subsequent obstruction of the small airways. In CF, the absence of CFTR mediated chloride efflux creates an exacerbated compensatory influx of sodium and water promoted by the epithelial sodium channel. The result is a reduced volume of extracellular fluid and a poorly hydrated mucus layer (32,33). Also, the airway surface liquid that covers the respiratory epithelium is disrupted by CFTR dysfunction. The reduced airway surface liquid volume impairs mucociliary function and contributes to the accumulation of debris and obstruction of small airways (25,34,35).

Abnormalities in pH homeostasis, caused by diminished secretion of bicarbonate, can contribute to persistent inflammation in the lung. The airway surface liquid in the respiratory epithelium contains antimicrobial peptides with a strict range of pH for activity. CFTR-related dysfunction can repress microbicidal function in animal models of CF (33,36,37). Furthermore, Gustaffsson et al. demonstrated that inhibition of bicarbonate secretion reproduces the CF-mucus phenotype in mice models and the addition of soluble bicarbonate $^{-}$ can reverse it (33,35,38).

Abnormal function of CFTR predisposes to chronic infection and inflammation in the lungs. The combination of impaired mucociliary clearance, pH disturbance in the airway surface liquid and thick mucus promotes bacterial colonization in the airway. A disbalance between pro-inflammatory and regulatory signals is believed to promote chronic inflammation (32,39); although the sequence of events is not completely understood (39–41). For instance, IL-17, IL-1β

and IL-8 are abnormally elevated in the CF lung, while anti-inflammatory cytokines like IL-10 and nitric oxide are decreased (32,35,39,42,43). Furthermore, the CF lung parenchyma is rich in leukocyte chemo-attractants, like IL-8, and heavily infiltrated by neutrophils. These CF neutrophils have reduced microbicidal activity, but potentiate the pro-inflammatory state through the sustained release of proteases and reactive oxygen species. Proteases, particularly neutrophil elastase, are associated with tissue damage, lung function decline and neutrophil recruitment (35,39,43). In addition to endogenous pro-inflammatory signals, the CF lung is colonized by bacteria that also promote inflammation. The colonizers are usually aerobic bacteria but can include fastidious organisms and fungi. Thus, the CF lung microenvironment is rich in pathogen-associated molecular patterns and damage-associated molecular patterns which further enhance chronic inflammation (43).

1.3 Cystic fibrosis lung disease

1.3.1 Overview

CF is a systemic disease, but the most important manifestations are seen in the respiratory tract. Respiratory symptoms are uncommon in early childhood and when present, can often be confused with asthmatic episodes or viral infections (44). Over the years, the lungs of CF patients have a progressive obstructive disease as a consequence of chronic inflammation and superimposed infections; common symptoms include cough, chronic sinusitis, nasal polyps, shortness of breath, sputum production and recurrent pneumonia (8,44,45). As a consequence of the progressive damage to the lung, patients have a slow but constant decline of respiratory capacity, measured by pulmonary function tests, that accelerates during early adulthood (18 to 24 years) (46–48). The respiratory symptoms are variable and determined by the amount of residual activity of the CFTR

channel (genotype), other non-CFTR mutations (modifier genes), environmental factors, treatments received and colonizing bacteria (49).

In high-income countries, patients receive multidisciplinary care including regular appointments, and psychological and nutritional support (14). A typical treatment regime for a patient includes physical therapy, airway clearance techniques, pancreatic supplementation and oral/inhaled medications. Overall, a patient with CF requires an approximate investment of up to 68,696 US dollars per year (50).

Pulmonary exacerbations (PEx) of CF are episodes of increased respiratory symptoms that can cause a permanent loss of lung function. No consensus definition is available to diagnose a PEx. In general, they are characterized by changes in baseline respiratory symptoms, a decline in lung function measurements and the appearance of novel radiological changes (51–53). Hypothesized triggers for PEx include disbalances in lung microbiota composition and viral infections. Furthermore, the number of PEx per year is a predictor of the 5-year survival and rate of lung function decline over the following 3 years (53–55). The major consequence of a PEx is the irreversible loss of lung function, 16 - 35% of patients with a PEx fail to reach 90% of their previous lung function measurement despite optimal therapy (54,56–59).

1.3.2 Lung microbiology and microbiome

Patients with CF are colonized by fungi and bacteria at an early age. The most common pathogens isolated from CF respiratory samples are *Haemophilus influenzae, Staphylococcus aureus, Pseudomonas aeruginosa, Stenotrophomonas malthophilia, Achromobacter* spp.*, Burkholderia*

spp and nontuberculous mycobacteria (NTM). However, the lung microbiome is a dynamic environment that also includes fungi and other bacterial organisms.

Microbial colonization has an age-related pattern with microbial diversity decreasing over time. Incidence of *H. influenzae* and *S. aureus* is highest in infancy while *P. aeruginosa* starts to appear in the adolescent years (60,61). Unbiased analysis of bacterial communities by sequencing (16S rRNA) has shown that microbial communities in the CF lung are dynamic and complex (62) including varied anaerobes organisms. Furthermore, microbial diversity seems to decrease over time until typical CF pathogens (*S. aureus* and *P. aeruginosa*) become the predominant populations in adulthood (63–66).

The CF lung microbiome also contains fungal microorganisms and viruses. Fungi like *Aspergillus fumigatus* and *Candida albicans* are frequently reported in clinical specimens. However, molecular approaches have also identified *Candida* spp., other *Aspergillus* spp., *Saccharomyces cerevisiae*, *Cladosporium cladosporioides* complex, *Sporobolomyces roseus* and *Malassezia* spp (64,67). Viruses can be lung residents (bacterial phages) and are possible triggers of PEx, but their role in CF lung disease is unclear (68).

1.4 Nontuberculous mycobacteria in cystic fibrosis

1.4.1 Overview and epidemiology

Nontuberculous mycobacteria (NTM) are free-living organisms found in soil and water that can cause disease in humans (69). Among them, species from the *Mycobacterium avium* complex

(MAC) and *Mycobacterium abscessus* complex (MABs) are the most common pathogens in the CF population.

Recent reports show an increased rate of NTM detection in the non-CF population, particularly in seniors and those with underlying lung conditions. According to a 2014 systematic review, the incidence rates for pulmonary NTM disease (NTM-PD) are rising in industrialized countries, although precise estimates are unclear (70). Data from Ontario, Canada showed an increase in five-year prevalence from 29.3 per 100,000 in 1998-2002 to 41.3 per 100,000 in 2006-2010 (71). In the United States, Adjemian et al. estimated a prevalence of NTM-PD of 112 per 100,000 in patients over 65 with non-CF bronchiectasis or chronic obstructive pulmonary disease. They also highlighted an increase in annual prevalence from 8.2 to 20 per 100,000 persons between 1997 and 2007 (72).

Patients living with CF have increased lifetime susceptibility to infections with NTM. The prevalence of NTM infection in CF has reportedly grown over the last two decades, although improvements in identification methods and surveillance may account for this phenomenon (73). NTM infection prevalence estimates are highly variable and range between 4.2 and 40.9% in the CF population (74–79). The most recent reports from the United States (US) and European registries show estimates of NTM infection prevalence of 13.9% and 4.3% respectively. However, differences in screening rates, the geographical distribution of species and identification methods make them hard to compare (18,24). Nevertheless, NTM are more frequently found in CF patients compared to the general population (80).

1.4.2 Clinical aspects of NTM in CF

Not all CF patients infected by NTM develop NTM-PD. Infected individuals can either clear the bacteria spontaneously, advance to overt pulmonary disease or persist in infection without impact on their lung function (81–83). Only patients with confirmed NTM-PD diagnosis warrant antimicrobial treatment. According to guidelines, NTM-PD is defined by at least two positive respiratory cultures for the same NTM; novel and typical radiological features of NTM-PD; changes in respiratory symptoms and/or lung function tests; and poor response to treatment against conventional CF pathogens (82). Also, as NTM are inherently resistant to most antibiotics, recommended regimens combine parenteral with oral antibiotics and are maintained until 12 months of sustained negative cultures are achieved (69,82). Yet, these recommendations are supported by the low quality of evidence and non-CF studies show low rates of successful treatment: sputum conversion rate for MABs pulmonary disease of around 50 % and up to 75% recurrence/reinfection rates after treatment for MAC pulmonary disease (77,82,84–86). Hence, anti-NTM treatment is an additional burden for patients and exposes them to unwanted drug-interactions and toxicity with a low rate of success.

NTM infection and NTM-PD increase the rate of lung function decline in CF patients and are relative contraindications for a lung transplant. In prospective studies, patients infected with MABs had a faster decline in percentage predicted FEV1 (ppFEV1) compared to uninfected patients, -2.52 vs. -1.64% per year respectively (76). Furthermore, Martiniano et al. found that patients who eventually developed NTM-PD had a heightened decline in FEV1 in the year prior to the first growth of NTM (81). Finally, there is no conclusive evidence of worse outcomes post-transplantation in patients with pre-operative NTM infection. However, current guidelines

recommend treatment of NTM-PD before transplant to mitigate the risk of disseminated disease after the procedure (82).

1.5 Thesis rationale and aims

The burden of NTM infection and NTM-PD in the CF population is difficult to estimate due to differences in screening practices and culturing methods. Registries represent large volumes of data, but only about particular regions. In contrast, non-registry studies represent more diverse locations, although their sample sizes are typically smaller. As an approach to estimate the burden of NTM infection and NTM-PD in the CF population, we conducted a systematic review and meta-analysis of all available published (curated databases) and unpublished literature.

The current algorithm to diagnose NTM-PD is cumbersome and may cause unnecessary delays and complications. Currently, reported risk factors for NTM infection are inconsistent among studies; these proposed risk factors include exposure to corticosteroids or azithromycin, co-infection with *P. aeruginosa* or *A. fumigatus*, increasing age and exposure to water sources (77,79,82,87). In this context, a biomarker for the prediction of NTM-PD in CF could facilitate stratification and optimize resources. Based on non-CF studies evaluating host genetic polymorphisms and gene expression, T cell responses and inflammatory markers (IL10, Interferon-γ) could be linked to susceptibility towards NTM infection and NTM-PD. In our third chapter, we explore if changes in whole blood gene expression can help us predict NTM outcomes (NTM-PD or not).

Chapter 2: Systematic review of prevalence and incidence of NTM infection and NTM pulmonary disease in cystic fibrosis population

2.1 Introduction

The burden of nontuberculous mycobacteria (NTM) appears to be rising in non-CF populations (70,72,88–90). For example, in Canada, NTM-PD prevalence increased from 11.4 to 22.2 cases per 100,000 individuals between 1998 and 2010 (71,91). A similar trend has been described in cystic fibrosis (CF) populations (60,82,92,93). However, the burden of NTM infection and NTM-PD can vary according to age, environmental exposure, geographical region and identification methods used. Furthermore, improved awareness of NTM, due to its potential impact on CF lung disease progression, has promoted enhanced screening practices that could explain the increased prevalence (76,81,82).

Despite the availability of population-based clinical registries for CF, the burden of NTM remains poorly defined. Registries represent data only from certain geographical regions with hig- income countries. Furthermore, divergent screening and detection practices (internationally and nationally) make it difficult to compare or generalize estimates from different locations. Thus, we conducted a systematic review of all available data (including registries) to estimate the incidence and prevalence of NTM-PD and NTM infection among patients living with CF. Using this data, we explored potential sources of heterogeneity and subgroup differences. No prior studies have evaluated epidemiological measures of NTM in CF using systematic review methods. Our data

summarizes the burden before widespread therapy with CFTR modulators in most countries (94,95).

2.2 Methods

2.2.1 Review question

Our review question was formulated based on population, condition, outcome (epidemiological measure) and study design, as recommended by guidelines of prevalence systematic reviews (96,97). Briefly, we screened for studies including CF patients (population), with a prospective or cross-sectional design (study design), reporting NTM infection/disease (condition), and including at least one among incidence rate, incidence proportion, point prevalence or period prevalence (outcome); the detailed criteria are described in **Table 2-1**. We excluded studies that were not in English. The review protocol was registered in July 2020 to the International Prospective Register of Systematic Reviews, PROSPERO (CRD42020200418). In October 2020, before starting screening, it was updated with an improved description of grey literature methods and screening procedures.

2.2.2 Literature search

The databases EMBASE (OVID Inc.) and MEDLINE (OVID Inc.) were searched as specified in **Appendix A-1**. An initial search was conducted in September 2020 and updated in September 2021. For grey literature, we evaluated the Grey Matters checklist (98) (**Appendix A-2**) and performed a manual review of the proceedings from relevant CF conferences (North American Cystic Fibrosis Conference, European Cystic Fibrosis Conference, American Thoracic Society International Conference and the Infectious Diseases Society of America conference) since 2010.

Also, we performed forward and backward searches of the references listed in **Appendix A-2** using Web of Science and Google scholar. Finally, the US, Canadian, European, Australian and Brazilian registry reports published since 2010 were included.

Table 2-1. Eligibility criteria for systematic review

Population (P): Cystic fibrosis patients	• Includes CF patients of any age • Excludes studies with a specific subgroup of CF patients (transplant recipients, Allergic broncho-pulmonary aspergillosis, macrolide exposure, chronic *Pseudomonas* spp. infection)
Condition (C): NTM infection or NTM pulmonary disease	Reporting of NTM infection • Defined by isolation of nontuberculous mycobacteria on at least one occasion • Microbiological detection methods (culture, direct staining, PCR, MALDI-TOF, not reported) Reporting of pulmonary NTM disease • Based on accepted criteria for diagnosis (ATS 1997, ATS 2007, CFF/ECFS 2016)
Outcome (O): Prevalence or incidence	Reporting of NTM: • Point prevalence (at a given point in time) • Period prevalence (over a time period) • Incidence rate (person-time measures) • Incidence proportion (percentage of new cases/ at risk patients)
Study design (S): Prospective or cross-sectional	• The study design must be cohort, clinical trial or cross-sectional (including registry reports). • Excludes reviews, letters to the editor, commentaries and case reports.
Others	• English language reports • No restriction on the date of publication • No restriction by geographic region

CFF: Cystic Fibrosis Foundation. **ATS**: American Thoracic Society. **ECFS**: European Cystic Fibrosis Society.

2.2.3 Screening process and data extraction

All records were retrieved and exported in Research Information Systems format to the SRA DeDuplicator software (99). Manual removal of duplicates was performed based on Author, Title and Year of publication in RefWorks. A final deduplication step was automatically performed by Covidence (100).

Abstract and full-text were independently screened by two reviewers (Miguel Prieto - MP and Mossab Allam -MA) using Covidence. Discrepancies were solved by consensus or by a third reviewer (Bradley Quon - BQ) if necessary. The abstract screening was based on language, study type, the inclusion of CF population and report of outcomes of interest. Full-text screening evaluated all eligibility criteria defined in **Table 2-1**. For unretrievable reports, we requested access to unpublished full manuscripts from authors via email on at least two separate occasions.

Two reviewers (MP and MA) independently extracted data from included studies. In registry studies, the estimates were calculated based on the reported number of patients tested, if available. Discrepancies were resolved by consensus or a third reviewer (BQ). The Joana Briggs Institute tool was used to assess the methodological and reporting quality of prevalence (96,97,101–103) by two independent reviewers (MP and MA). Overall low risk of bias was defined as low risk in the assessments of the sampling frame, sample size, population description and statistical methods. Data extraction was based on a pre-specified data dictionary piloted with 10 studies (**Appendix A-3**). We did not impute any missing data. In studies with unclear years of data collection, we assumed that data was obtained from before publication.

2.2.4 Data analysis

Data were analyzed with the meta and metafor packages using R studio and R version 4.1.1 (104–107). Risk of bias plots were produced with the robvis and ggplot2 packages (108,109), and tables with the flextable package (110). We pre-specified the use of random-effects models based on expected heterogeneity by study regions and dates. All meta-analytical models are generalized linear models with LOGIT transformed proportions (111–113). We summarized point prevalence (and annual prevalence) of NTM infection as a meta-analysis; annual prevalence was included because it provides the same outcome measure as registry reports. The remaining outcomes (period prevalence of NTM infection, incidence of NTM infection, point/ period prevalence of NTM-PD and incidence of NTM-PD) are reported in tables and text only. The period prevalence was not pooled as varying time intervals influence estimates, while the other outcomes were not suitable for pooling due to the low number of available studies. Heterogeneity was assessed by calculation of the I^2 index with a 95% confidence interval, the significance level for heterogeneity was established at $p<0.10$. Publication bias was explored graphically through funnel plots (114), using sample size as the predictor (X-axis) of small studies bias in the funnel plot (115).

We pre-specified subgroup analyses by study design, age category (pediatric vs adult), year of data collection (five-year intervals), geographical region (grouped as North America, Europe and others) and separate pooling for MABs and MAC infection. The pre-specified meta-regression model was built using optimization of maximum likelihood in a generalized linear model with LOGIT transformed proportions and a random-effects model. In a stepwise approach, we added pre-specified coefficients and evaluated the Akaike's information criteria against the pre-inclusion

value. Exploratory/unspecified analyses include subgroup analyses by region in MAC and MABs meta-analyses. Reporting is based on the recommendations of the Joanna Briggs Institute and the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist (96,116).

Sensitivity analyses include a subgroup analysis by study design and a subgroup analysis by years of data collection removing data from registry reports. As we could not control for the overrepresentation of registry reports, we decided to include only the last report (most representative) per registry.

2.3 Results

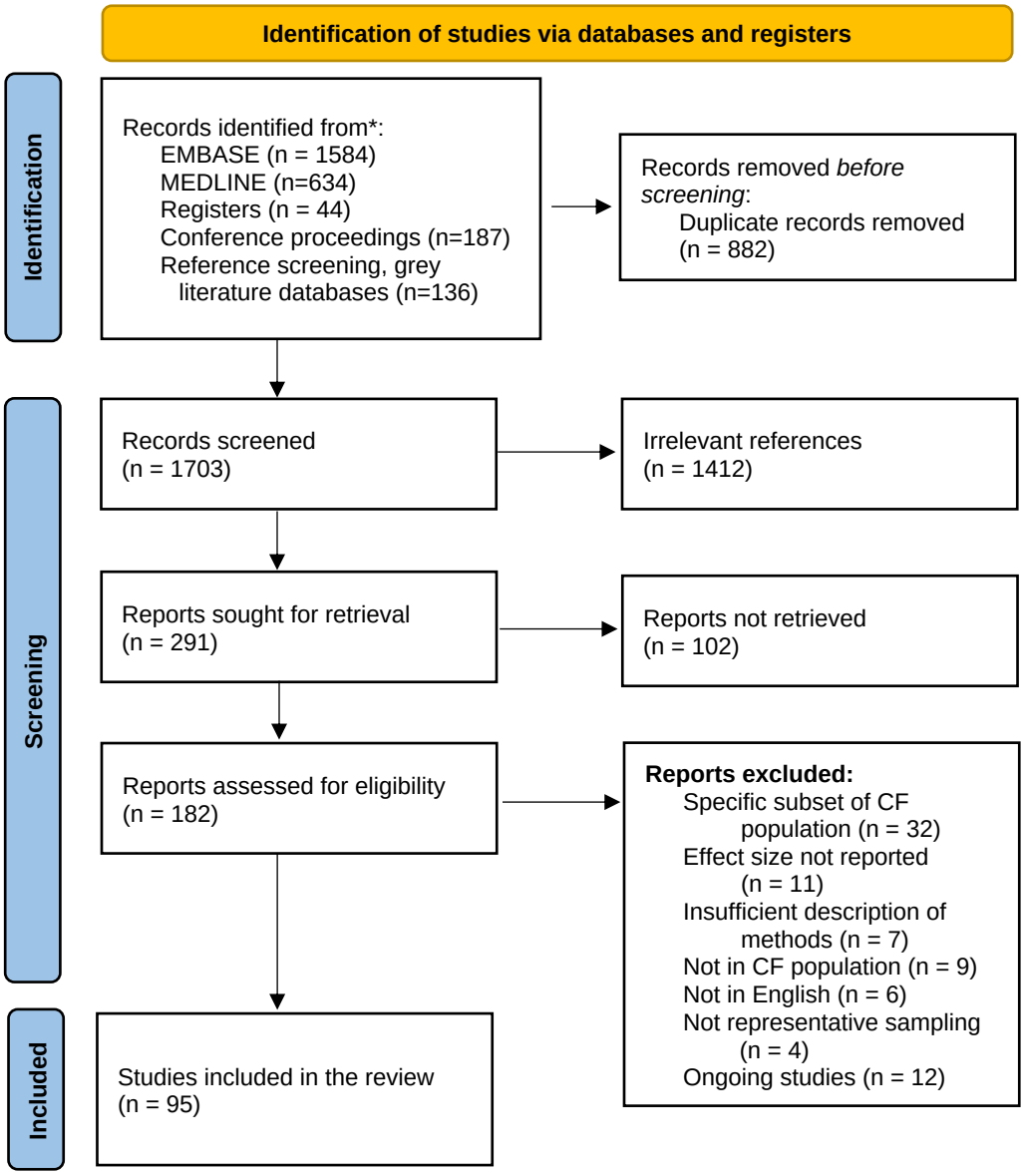
2.3.1 Description of studies

After removing duplicates, 1703 references were included for abstract screening, 291 were reviewed as full-text and 95 were included in the systematic review. The PRISMA flowchart in **Figure 2-1** summarizes the screening process. The abstract and full-text screening processes had a Cohen's kappa of 0.899 and 0.698, respectively. All disagreements were resolved by consensus.

The majority of the publications originated from Europe (42%) and North America (33%). The most common study design was cross-sectional registry (n=44, 46%), followed by cross-sectional non-registry (n=35, 37 %) and cohort (n=16, 17 %). A majority of studies (n = 75; 79%) included a mixture of pediatric and adult patients. The most represented period of data capture was 2010-2019 (n=66, 68.6%) where registry reports are available. Registry reports and studies using registry data had larger sample sizes (median 4278, range 1323 - 39667) compared to non-registry studies

(median 155, range 28 - 7122). **Tables 2-2 and 2-4 to 2-7** summarize the characteristics of the included studies according to the outcome reported. The figure in **Appendix A-4** shows the relationship between the sample size of non-registry studies and the first year of data collection, a trend towards larger sample sizes in recent years is observed.

Figure 2-1. PRISMA flowchart of screening process



By outcome, 67 studies reported point (or annual) prevalence of NTM infection while 43 reported period prevalence of NTM infection. The incidence proportion of NTM infection was reported in 5 studies while the incidence rate was not reported. NTM-PD point prevalence was reported in 2 studies and period prevalence in 13 studies. Some studies included multiple outcomes We did not retrieve any report of NTM-PD incidence.

Figure 2-2. Quality assessment summary of registry reports (n=44)

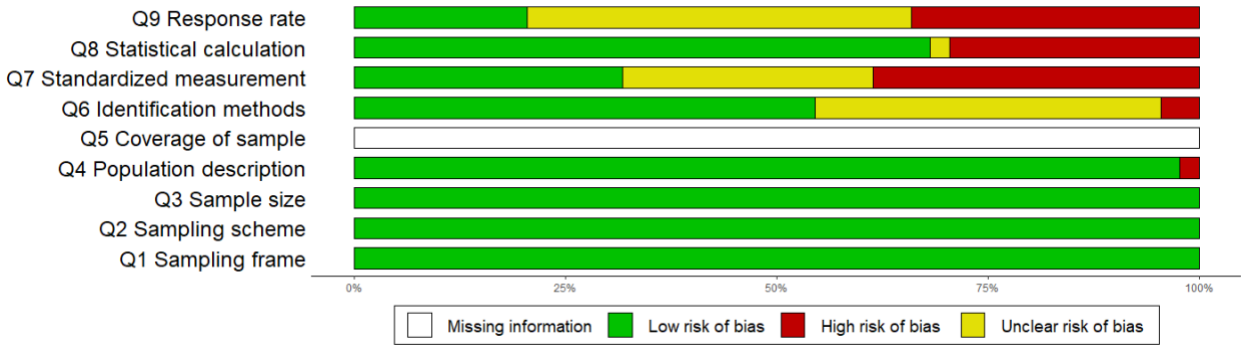
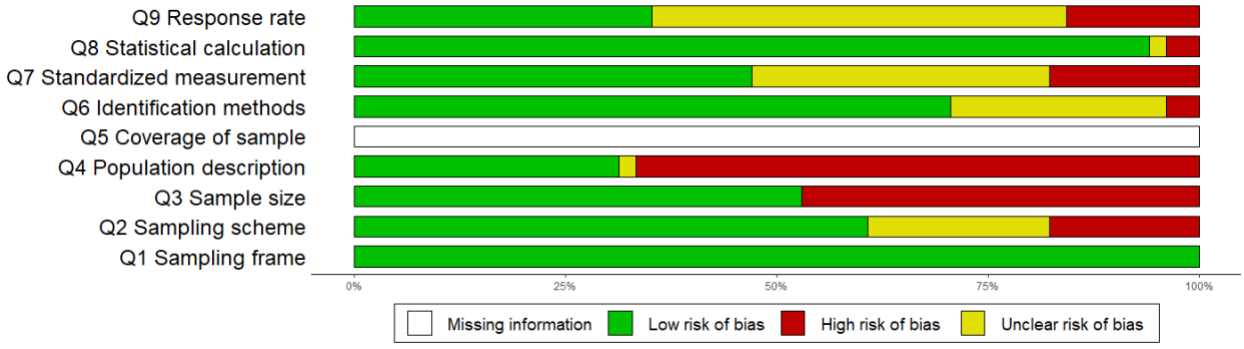


Figure 2-3. Quality assessment summary of non-registry reports (n=51)

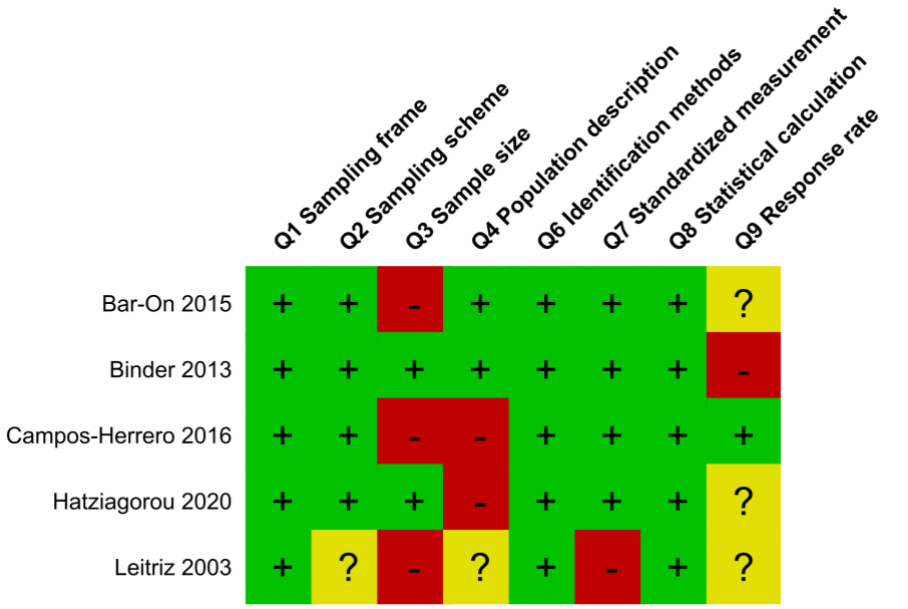


2.3.2 Quality assessment

The results of the Joanna Briggs Institute tool quality assessment are summarized in **Figures 2-2 and 2-3**. Registry reports had mostly low-risk scores on the domains of sampling frame, sampling

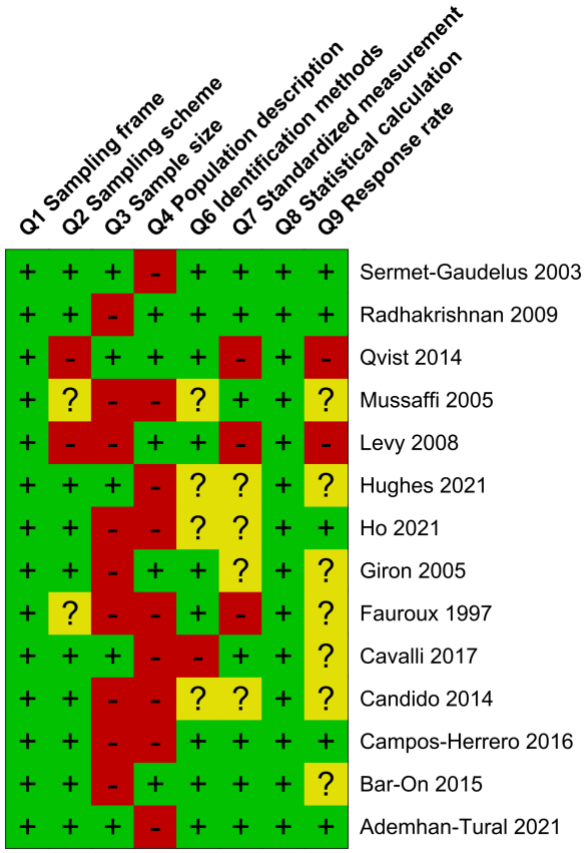
approach, sample size and population description. In contrast, registry reports had unclear risk in response rate (47%), standardized measurement (33%) and identification methods (33%). Question 5 (coverage of sample) applies mostly to survey studies and was not evaluated in our project.

Figure 2-4. Traffic light plot for quality assessment of studies reporting NTM infection incidence



Non-registry studies had a higher risk of bias scores in terms of sample size and population description. However, no difference between registry and non-registry studies was seen for identification methods, standardized measurement and response rate domains (**Figures 2-2 and 2-3**). By outcome, studies that reported incidence of NTM infection had, in general, low risk of bias scores for all questions except sample size. Also, studies reporting NTM-PD had high risk of bias scores for sample size and population description, but mostly low/unclear risk for the remaining domains (**Figures 2-4 and 2-5**).

Figure 2-5. Traffic light plot for quality assessment of studies reporting NTM-PD point and period prevalence (n=14)



2.3.3 NTM infection point (annual) prevalence: pre-specified analyses

Point prevalence and annual prevalence of NTM infection were summarized together in a meta-analysis. Annual prevalence was included because of its similarity with the estimates found in registry reports. We included only the last registry report of a region/country to avoid duplication of patients over the years. Also, n = 4 studies that used registry data between 2010 and 209 were excluded to avoid artificial duplication of data. Overall, n = 21 studies were included in the meta-analysis. The primary random-effects model with all studies produced an NTM infection prevalence estimate of 7.7% (95% CI 4.9 – 12 %), with a wide 95% prediction interval of 0.9 –

43.4% and substantial heterogeneity (I^2 = 99%). The characteristics of studies reporting point prevalence and annual prevalence of infection are summarized in **Table 2-2**.

Heterogeneity of results was explored through subgroup analyses. We did not explore age because 81% (17/20) of included studies had a mix of pediatric and adult populations without individual estimates for each group. No significant differences among subgroups were found according to the type of study design. However, less than 5 cross-sectional registry reports and cohort studies were included (**Figure 2 – 6**). Heterogeneity was large inside all subgroups (I^2 > 70%), likely due to the small number of studies per group. Exploratory subgroup analysis with registry vs not-registry data showed significant differences with a lower estimate in registry 3% (CI 1 -14%) vs non-registry data 11% (CI 8 – 15%) (**Appendix A-5**).

Figure 2-6. NTM infection prevalence – subgroup analysis by study design

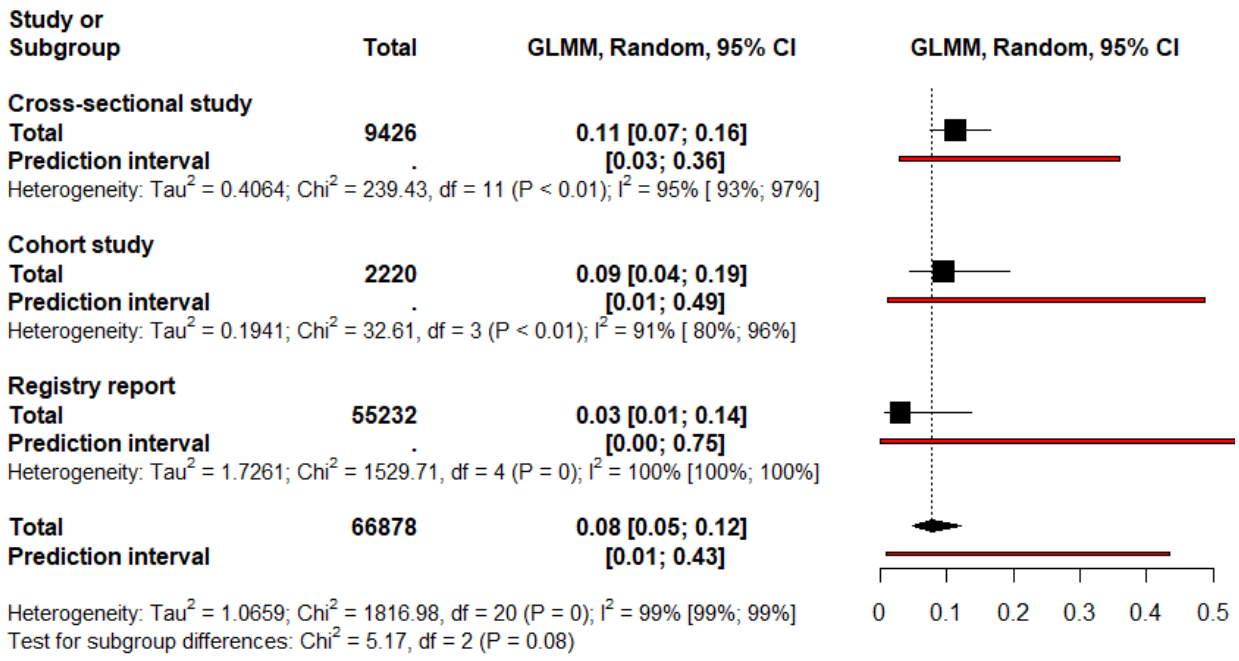


Table 2-2. Characteristics of studies reporting NTM infection point (or annual) prevalence

Study ID	Study design	Sample size	Location	Age (y)	Females	Specimen	Culture method	Speciation	Year or interval	Prevalence estimate
Abidin 2020** (117)	Cross-sectional	4,687	United Kingdom	9 (5 - 13) [median; IQR]	51.4%	NR	NR	NR	2016 2017 2018	3.5% 3.1% 3.6%
Adjemian 2014** (87)	Cross-sectional	10,527	United States	27 (12 - 82) [mean; range]	NA	NR	NR	NR	2010 - 2011	13.2%
Adjemian 2018 (118)	Cross-sectional	16,153	United States	12 to 18 - 23% 18 to 60 - 75% ≥60 - 2%	48%	NR. Annual screening (only 77% had 2/5 years of testing)	NR	NR	2010	11.0%
Aitken 1993 (119)	Cross-sectional	64	United States	17 - 50 [range]	**NTM +** 50% **NTM -** 57.1%	Sputum. Frequency NR	Auramine and Kinyoun stains. L-J, BACTEC 12B and 7H11	NR	Dec 1990 - Dec 1991	12.5%
Australia 2010 (120)	Registry	1,946 (tested*)	Australia	median: 17.6 mean: 19 Adults: 1500 (49%)	46.9% (n = 3,063)	Sputum, BAL. Frequency NR	NR	NR	2010	1.1%
Australia 2011 (121)	Registry	2,001 (tested*)	Australia	mean: 19.2 Adults: 1528 (49%)	47.3% (n = 3,133)	Sputum, BAL. Frequency NR	NR	NR	2011	1.2%
Australia 2012 (122)	Registry	2,182 (tested*)	Australia	median: 17.7 Adults: 1556 (49%)	47.1% (n = 3,156)	Sputum, BAL. Frequency NR	NR	NR	2012	1.5%
Australia 2013 (123)	Registry	2,206 (tested*)	Australia	median: 17.9 mean: 20 Adults: 1613 (50%)	47.1% (n = 3,235)	Sputum, BAL. Frequency NR	NR	NR	2013	1.9%
Australia 2014 (124)	Registry	2,021 (tested*)	Australia	median: 18.4 mean: 20.5 Adults: 1684 (51%)	47.0% (n = 3,294)	Sputum, BAL. Frequency NR	NR	NR	2014	2.5%
Australia 2015 (125)	Registry	2,047 (tested*)	Australia	median: 18.8 mean: 20.9 Adults: 1756 (52%)	46.8% (n = 3,379)	Sputum, BAL. Frequency NR	NR	NR	2015	2.8%

Australia 2016 (126)	Registry	1,769 (tested*)	Australia	median: 18.4 mean: 20.5 Adults: 1684 (51%)	46.61 (n = 3,422)	Sputum, BAL. Frequency NR	NR	NR	2016	2.6%
Australia 2017+ (127)	Registry	1,323 (tested*)	Australia	median: 19.6 mean: 21.7 Adults: 1684 (54%)	46.3% (n = 3,156)	Sputum, BAL. Frequency NR	NR	NR	2017	4.2%
Bar-On 2015 (92)	Cohort	110 (2011)	Israel	**2008** **NTM +** 17.8 (4.3 - 55.3**)** **NTM –** 15.2 (0.2 - 59.3) [median; range**]**	2008 **NTM -** 47.9% **NTM +** 35.3%	Sputum. Screened every 3-6 months	L-J and BACTEC MGIT. Monitored for 8 weeks	Mycobacteria Genotype kits	2003 2004 2005 2006 2007 2008 2009 2010 2011	5.1% 4.5% 4.4% 6.5% 7.3% 8.8% 12.4% 13.5% 14.5%
Brazil 2010 (128)	Registry	1,440	Brazil	12.9 (10.9) [mean ± sd]	47.6% (n = 1,798)	NR	NR	NR	2010	0.4%
Brazil 2011 (129)	Registry	1,440	Brazil	13.18 (10.9) [mean ± sd]	46.5% (n = 2,182)	NR	NR	NR	2011	0.3%
Brazil 2012 (130)	Registry	2,132	Brazil	13.49 (11.01) [mean ± sd]	46.9% (n = 2,669)	NR	NR	NR	2012	0.2%
Brazil 2013 (131)	Registry	2,238	Brazil	13.87 (11.8) [mean ± sd]	47.2% (n = 2,924)	NR	NR	NR	2013	0.4%
Brazil 2014 (132)	Registry	2,571	Brazil	13.57 (11.2) [mean ± sd]	47.2% (n = 2,924)	NR	NR	NR	2014	0.5%
Brazil 2015 (133)	Registry	2,961	Brazil	14.25 (11.95) [mean ± sd]	47.8% (n = 3,806)	NR	NR	NR	2015	0.4%
Brazil 2016 (134)	Registry	3,212	Brazil	13.84 (11.57) [mean ± sd]	48% (n = 4,654)	NR	NR	NR	2016	0.5%
Brazil 2017+ (135)	Registry	3,378	Brazil	14.58 (11.94) [mean ± sd]	48% (n = 5,128)	NR	NR	NR	2017	0.3%

Campos-Herrero 2016 (136)	Cross-sectional	44	Spain	**NTM+** 12 (5 - 59) [median; range]	**NTM +** 38.9%	Sputum. Frequency NR	BACTEC MGIT and on L-J	Phenotypic tests and/or nucleic acid hybridization assays	2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012	33.3% 24% 19.2% 12.5% 0% 12.5% 12.9% 13.3% 9.7% 8.8% 9.1%
Canada 2011 (137)	Registry	3,913	Canada	median: 20 mean: 21.8	47.3% (n = 3,913)	NR	NR	NR	2011	2.3%
Canada 2012 (138)	Registry	3,975	Canada	median: 21 mean: 22.3	47.1% (n = 3,975)	NR	NR	NR	2012	2.7%
Canada 2013+ (139)	Registry	4,077	Canada	median: 21.4 mean: 22.6	47.1% (n = 4,077)	NR	NR	NR	2013	2.8%
Canada 2014 (140)	Registry	4,128	Canada	median: 21.9	46.9% (n = 4,182)	NR	NR	NR	2014	3.5%
Canada 2015 (141)	Registry	4,192	Canada	median: 22.3	47.1% (n = 4,192)	NR	NR	NR	2015	3.9%
Canada 2016 (142)	Registry	4,246	Canada	median: 22.7	46.4% (n = 4,246)	NR	NR	NR	2016	4.5%
Canada 2017 (143)	Registry	4,309	Canada	median: 22.8	46.1% (n = 4,302)	NR	NR	NR	2017	6.5%
Canada 2018 (144)	Registry	4,371	Canada	median: 23.5	46.5% (n = 4,371)	NR	NR	NR	2018	6.1%
Canada 2019 (10)	Registry	4,344	Canada	median: 23.7	46.6% (n = 4,344)	NR	NR	NR	2019	6%
ECFS 2010 (145)	Registry	31,932	European countries	17.8 (0 - 80.1) [median; range]	47.7% (n = 32,248)	NR	NR	NR	2010	2.3%
ECFS 2011 (146)	Registry	26,700	European countries	mean: 19.6 17.9 (9.3 - 27.5) [median; IQR]	47.5% (n = 36,340)	NR	NR	NR	2011	2.5%
ECFS 2012 (147)	Registry	27,686	European countries	mean: 19.8 18.1 (9.3 - 28) [median; IQR]	47.4% (n = 37,404)	NR	NR	NR	2012	3.0%

ECFS 2013 (148)	Registry	28,596	European countries	mean: 20.1 18.4 (9.3 - 28.5) [median; IQR]	47.3% (n = 38,985)	NR	NR	NR	2013	3.3%
ECFS 2014 (149)	Registry	28,961	European countries	mean: 20.5 18.6 (9.4 - 29.2) [median; IQR]	47.37% (n = 35,582)	NR	NR	NR	2014	3.5%
ECFS 2015 (150)	Registry	31,763	European countries	mean: 20.7 18.8 (9.4 - 29.5) [median; IQR]	47.46% (n = 42,054)	NR	NR	NR	2015	3.3%
ECFS 2016 (151)	Registry	25,464	European countries	mean: 21 19 (9.5 - 30) [median; IQR]	47.45% (n = 44,719)	NR	NR	NR	2016	2.5%
ECFS 2017 (152)	Registry	39,667	European countries	mean: 20.8 18.5 (9.1 - 30) [median; IQR]	47.4% (n = 48,204)	NR	NR	NR	2017	3.6%
ECFS 2018+ (23)	Registry	30,957	European countries	mean: 19.8 18.5 (9.2 - 30.3) [median; IQR]	47.5% (n = 49,886)	NR	NR	NR	2018	4.1%
Gardner 2019** (153)	Cross-sectional	5,333	United Kingdom	6 (2 - 12) [median; IQR]	49.1% (n = 5,333)	NR. Annual screening.	NR	NR	2010 2011 2012 2013 2014 2015	1.3% 1.7% 1.8% 2.1% 3.6% 3.8%
Hatziagorou 2020 (24)	Cohort	41,101	European countries	NA	NA	NR	NR	NR	2011 2012 2013 2014 2015 2016	2.6% 3.1% 3.4% 3.5% 3.3% 3.3%"
Hjelt 1994 (154)	Cross-sectional	185	Denmark	15.3 (2.2 - 38.5) [mean; range]	NA	Sputum. Three samples in 3 months	L-J	Nucleic-acid hybridization or biochemical tests	1987 - 1988	7%
Mulherin 1990 (155)	Cohort	41 (tested*)	Rep. of Ireland	NA	NA	Sputum. Frequency NR	L-J	NR	1990 (uncertain)	2.4%
Olivier 2003 (74)	Cross-sectional	986	United States	23 ± 9 [mean ± sd]	47% (n = 986)	Sputum. Frequency NR	L-J and BACTEC MGIT	RGM by Hsp65 sequencing. Slow growers by PCR and restriction digest	1994 (uncertain)	13.0%

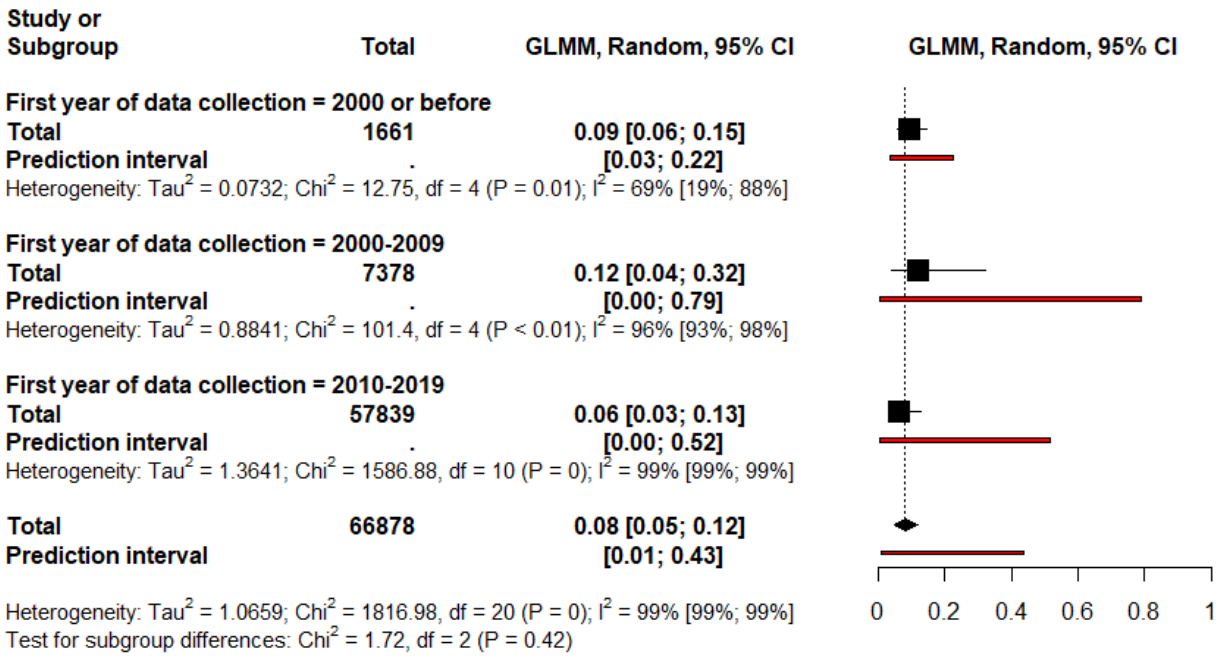
Paschoal 2007 (156)	Cross-sectional	54	Brazil	41.8 ± 17.2 [mean ± sd]	50%	Sputum. Frequency NR	NR	NR	2003 - 2004	16.7%
Pierre-Audigier 2005 (157)	Cross-sectional	385	France	12.0 ± 6.1 [mean ± sd]	47.3% (n = 385)	Sputum. Three times per year.	L-J up to 10 weeks.	RGM by biochemical techniques and hsp65 sequencing. MAC by PCR probes	2000	8%
Plongla 2017 (158)	Cohort	487	United States	14.9 (<1 - 71) [median; range]	53.6% (n = 487)	Sputum/tracheal aspirates, pharyngeal swabs, bronchial wash and BAL fluids. Frequency NR	MGIT L-J, RGM medium, and BCSA.	RGM by MALDI-TOF MS IVD system. Others by 16S rRNA sequencing.	Dec 2015 - Apr 2016	14.7%
Preece 2016 (159)	Cross-sectional	210	United Kingdom	<1 - 77 [range]	NA	Sputum. Less than 10% were regularly screened	RGM medium and BCSA	Sequencing of two genes among RPO-B, HSP65 and SOD-A	Feb - Sep 2014	9.5%
Radhakrishnan 2009 (160)	Cross-sectional	98	Canada	**NTM +** 15.1 ± 2.2 **NTM –** 14.0 ± 3.0 [mean ± sd]	**NTM+** 66.7% **NTM-** 53.3%	Sputum. Tested once in the year of study.	MGIT and L-J, up to 7 weeks	AccuProbe test for MAC and *M. gordonae.* Others by HP-LC	Mar - Nov 2004	6.1%
Raidt 2015 (93)	Cross-sectional	94	Germany	mean: 24.9	47.90%	Sputum or deep pharyngeal swab. Frequency NR	BCSA	GenoType Mycobacterium CM/AS assay	2011	7.4%
Roux 2009 (75)	Cohort	1,582	France	18.9 (0.33 - 82) [mean; range]	48.6% (n = 1,582)	Sputum. Frequency NR	MGIT and/or Lowenstein Colestos slants.	Sequencing of hsp65, 16S-23S intergenic region and rpoB (only MABs)	2014	6.6%
Salsgiver 2016 (60)	Cohort	**Total** 31,915 **Tested*** unknown	United States	NA	NA	Sputum or BAL (< 12 years). Frequency NR	NR	NR	2012	12.0%
Scohy 2018 (161)	Cross-sectional	124	Belgium	24.5 (6 - 68) [median; range]	47%	Sputum. Frequency NR	BACTEC MGIT and RGM medium	MALDI-TOF MS, Geno-Type NTM-DR and genotyping for MABs	Sep 2016 - Mar 2017	16.1%

Seddon 2013 (78)	Cross-sectional	7,122	United Kingdom	**Pediatric** 46.5% **Adults** 53.4%	NA	NR. 33/42 centers tested annually, 9 only by symptoms.	NR	NR	2008 - 2009	4.2%
Usa 2010 (162)	Registry	9,462	United States	17.2 (0 to 82) [median; range]	48.20%	NR	NR	NR	2010	9.9%
Usa 2011 (163)	Registry	10,848	United States	mean: 19.5 17.5 (0 to 81) [median; range]	48.20%	NR	NR	NR	2011	10.8%
Usa 2012 (164)	Registry	11,927	United States	mean: 19.8 17.7 (0 to 82) [median; range]	48.30%	NR	NR	NR	2012	11.8%
Usa 2013 (165)	Registry	12,873	United States	mean:20.2 median:17.2	48.50%	NR	NR	NR	2013	12%
Usa 2014 (166)	Registry	13,602	United States	mean:20.6 median:18.3	48.40%	NR	NR	NR	2014	12.2%
Usa 2015 (167)	Registry	14,225	United States	mean:20.9 median:18.6 Adults - 51.6%	48.40%	NR	NR	NR	2015	11.9%
Usa 2016 (168)	Registry	14,501	United States	mean:21.3 median:19	48.40%	NR	NR	NR	2016	12.7%
Usa 2017 (169)	Registry	15,041	United States	mean:21.7 median:19.3	48.40%	NR	NR	NR	2017	12.7%
Usa 2018 (170)	Registry	15,067	United States	mean:22.2 median:18.6	48.20%	NR	NR	NR	2018	13.6%
Usa 2019+ (18)	Registry	15,497	United States	mean:22.7 median:20.3	48.10%	NR	NR	NR	2019	13.9%
Valenza 2008 (171)	Cross-sectional	60	Germany	18 (6 - 41y) [median; range]	43.3% (n = 60)	Sputum. Frequency NR	MGIT	Sequencing of the 16S rRNA-gene	2006	13.3%
Viviani 2016** (77)	Cross-sectional	13,593	France, Sweden and UK	17.6 (0 - 82.5) [median; range]	47.4% (n = 13,593)	NR	NR	NR	2009	2.8%

BACTEC MGIT: Mycobacterial Growth Indicator Tubes.by BACTEC. **L-J**: Lowenstein-Jensen egg-based medium. **BAL:** Bronchoalveolar lavage. **RGM:** Rapid-growing mycobacteria (M. abscessus complex). **MALDI-TOF MS:** matrix-assisted laser desorption/ionization- time-of-flight mass spectrometry. **HP-LC**: High-performance liquid chromatography. **BCSA**: Burkholderia cepacia selective agar. **Tested*** specifies the actual number of at-risk patients tested for NTM in respiratory samples. ****** Excluded from meta-analysis as the data was duplicated with the registry reports. **+** Included in meta-analysis as the last available registry report from a region. The remaining reports from the region were excluded.

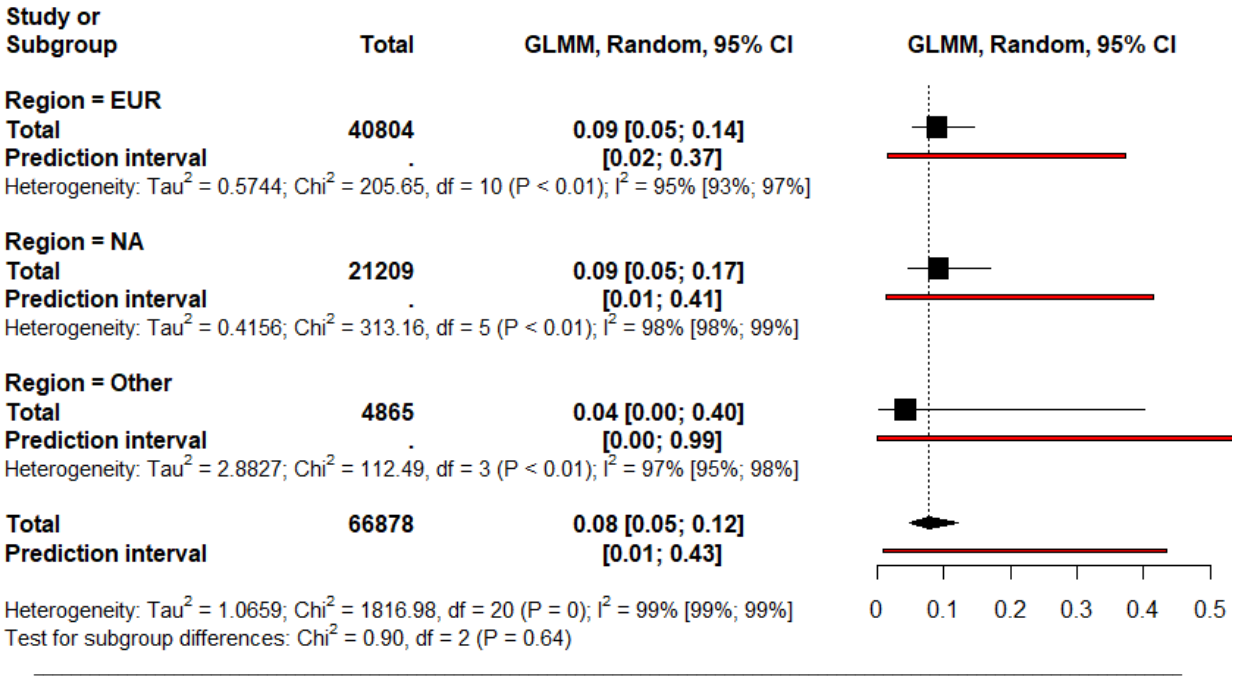
The pre-specified subgroup meta-analysis by the first year of data collection showed no significant differences among subgroups (p > 0.05), **Figure 2-7**. Three different time periods were evaluated, before 2000, 2000-2009 and 2010-2019.

Figure 2-7. Subgroup: NTM infection prevalence by years of data collection (non-registry studies)



In our subgroup analysis by geographical region **(Figure 2-8)**, we found no significant differences among subgroups. Heterogeneity was high in all subgroups (> 90 %). Also, studies conducted in other regions (Latin America and the Caribbean, Middle East, Africa and Australia) had a less precise estimate, 4% (95% CI 0 - 40), probably due to the low number of studies in the subgroup (n = 4).

Figure 2-8. Subgroup: NTM infection prevalence in non-registry studies by geographical region



Region = Other includes Australia, Asia, Africa and Latin America and the Caribbean

We summarized the NTM infection prevalence (point and annual prevalence) for infections with MAC or MABs separately (n =12 for both) using the same modeling approach described in the methods section (**Figures 2-9 and 2-10**). Interestingly, the variability was lower in these two estimates than in the analysis including all NTM species, although heterogeneity remained higher than 80%. The MAC estimate is 3.6% (95% PI of 0.7 - 16%) and the MABs estimate is 4.4 % (95 % PI of 1.2 – 15 %). In an exploratory subgroup analysis of MAC infection prevalence by geographical region (**Figure 2-11**), a significantly lower prevalence (annual and point) was seen in Europe (1.8 %; PI 1.2 – 2.6. I^2 = 25 %) compared to North America (7.8%, PI 3.1 – 18.1%. I^2 = 78 %). No differences were found in MABs infection prevalence by geographical region (**Figure 2-12**).

Figure 2-9. Meta-analysis of *M. avium* complex infection prevalence (point and annual prevalence)

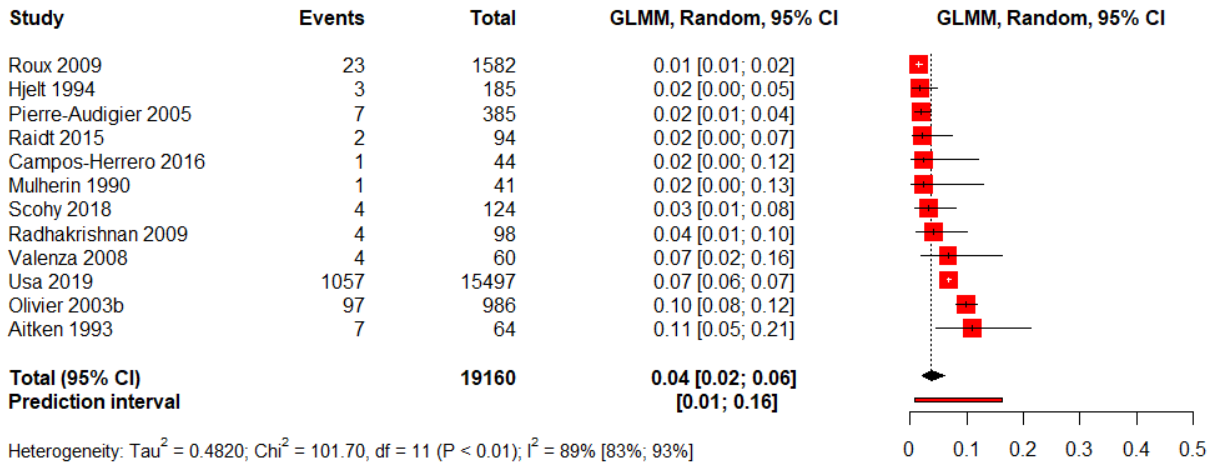


Figure 2-10. Meta-analysis of *M. abscessus* complex infection prevalence (point and annual prevalence)

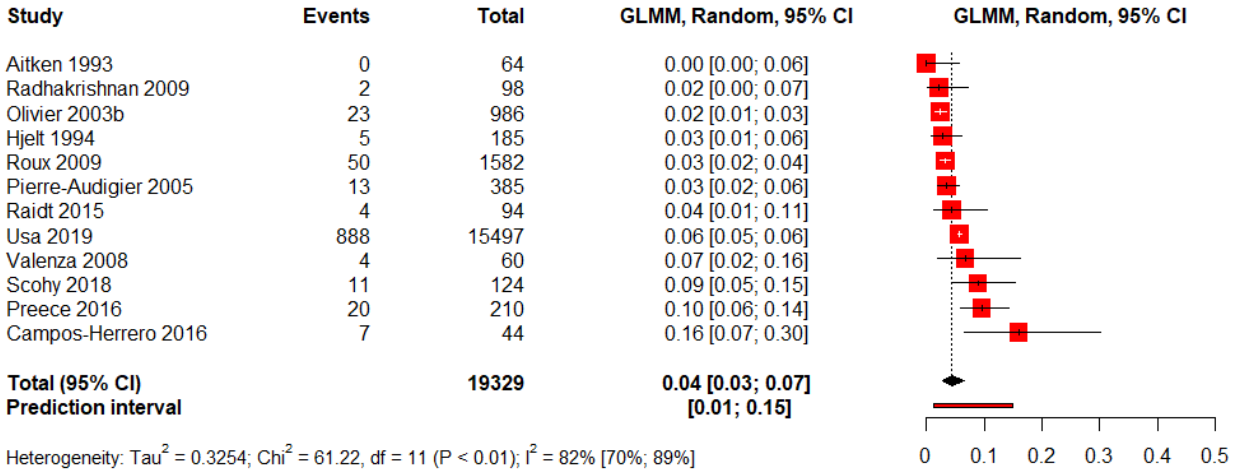


Figure 2-11. Exploratory subgroup of MAC infection (point and annual) prevalence by region

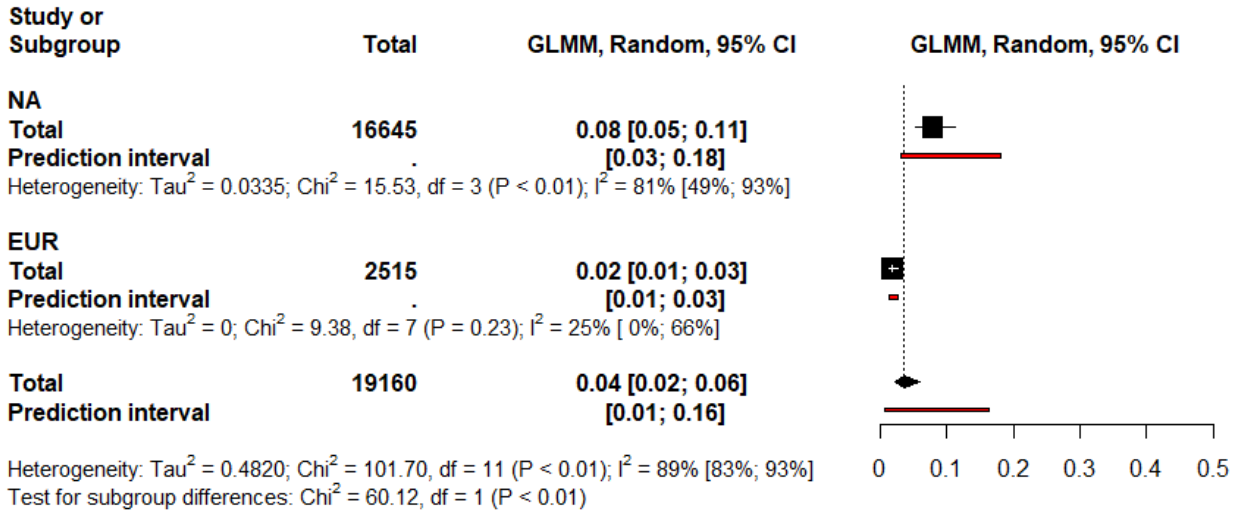
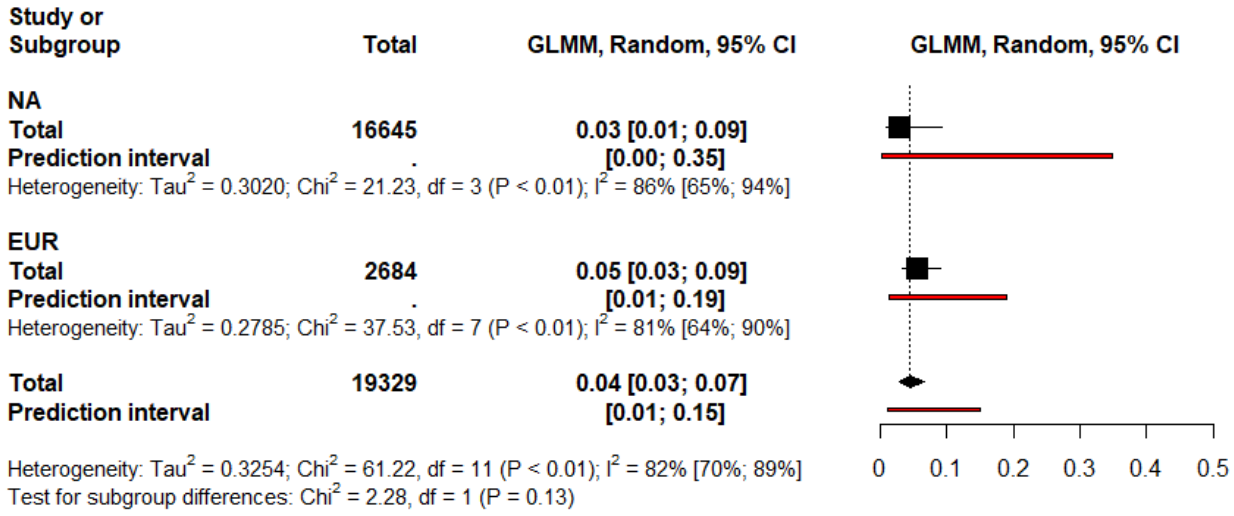


Figure 2-12. Exploratory subgroup of MABs infection (point and annual) prevalence by region



We also conducted a meta-regression to evaluate what conditions were significantly affecting the NTM infection prevalence while controlling for other covariates. The final model included study region, sample size category, year of data collection and study design. The age category was excluded because the numbers in pediatric and adult groups were too small. As seen in **Table 2-3**, only other geographical region and sample size < 1000 had a significant adjusted effect on the estimated LOGIT-prevalence ($p < 0.05$).

Table 2-3. Results of NTM infection point prevalence meta-regression

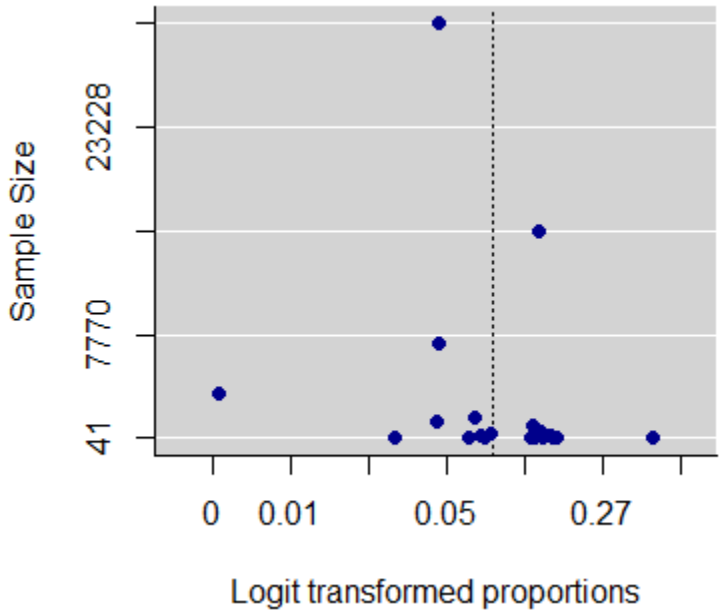
Coefficients	LOGIT-estimate	Std. error	p.value	CI-lower	CI-upper
Intercept	-3.1483	0.3812	0.0000	-3.8954	-2.4012
Design: Cross-sectional (non-registry)	-0.0563	0.9100	0.9507	-1.8399	1.7274
Design: cohort	-0.1569	0.7406	0.8322	-1.6084	1.2946
Sample size < 1000	**1.7381**	**0.6954**	**0.0124**	**0.3752**	**3.1010**
Sample size 1000 – 3000	1.0413	0.6650	0.1174	-0.2622	2.3447
European region	-0.2984	0.4043	0.4605	-1.0908	0.4940
Other regions	**-1.1369**	**0.5105**	**0.0259**	**-2.1374**	**-0.1364**
Before year 2000	-0.7475	0.4973	0.1328	-1.7222	0.2272
2000 - 2009	0.2826	0.5499	0.6074	-0.7953	1.3604

Reference categories are Design: Cross-sectional registry, Sample size >3000, North-American region and conducted between 2010-2019

The calculated proportions are obtained by back-transforming the LOGIT estimates [e^{coef} / $(1 + e^{coef})$]. The calculated estimate for the intercept (4.1% prevalence) provides the NTM infection prevalence (point and annual) for studies with all reference categories: cross-sectional registry studies with sample sizes > 3,000 conducted in North America between 2010 and 2019. Each coefficient shows the magnitude of change in the associated category while holding all other covariates constant. On average, studies conducted in other regions besides Europe and North America had a reduced estimate of NTM infection prevalence of 1.4% compared to those

conducted in North America while controlling for all other factors. Also, studies with sample sizes below 1000 had a larger estimate on average (19.6%) compared to those with sample sizes above 3000 while holding all other covariates constant.

Figure 2-13. Funnel plot of studies included in NTM infection prevalence meta-analysis



The funnel plot for small studies bias **Figure 2-13** was not further explored because in a single proportion meta-analysis the risk of bias according to positive results or low p-values is not relevant.

Potential sources of variability include differences in studied populations, microbial identification methods or bacterial distribution. Among the characteristics of the study population, biological sex is probably not contributing to heterogeneity as female representation was fairly homogeneous (median of 47.9%, range 43.3 – 56.2 %, n = 15). The majority of studies included mixed pediatric

and adult populations, but the lack of a unified reported measure (mean or median) prevented further exploration. Another important factor is the frequency of testing, which was reported only in 28.6% (6/21) of studies in the meta-analysis. Furthermore, a single study screened for NTM only in the presence of symptoms; a sensitivity analysis removing this study had no impact on the primary meta-analysis results (**Appendix A-6**). However, due to missing data, we could not evaluate if differences in ethnicity or lung disease severity (pulmonary function tests) are affecting our estimates. The identification method had several missing values among all studies reporting NTM infection point (and annual) prevalence (n = 35 missing for sample type and n = 44 for culture method). Among the ones included in the meta-analysis, n = 5 did not report the sample used and n = 7 failed to report the identification method. Sputum was the most commonly used specimen in 24/24 studies reporting NTM infection point/annual prevalence and 16/16 of those in the meta-analysis. Mycobacterial growth indicator tubes (MGIT) and Lowenstein-Jensen (L-J) medium are recommended for mycobacterial culturing and were the most frequently used methods: 12/14 studies among all studies for this outcome and 10/12 of those in the meta-analysis (172). However, the length of incubation, method of speciation and the decontamination procedures varied significantly among studies. Particularly, registry studies, which represent around 40% of studies for the NTM infection point/annual prevalence outcome, did not report primary identification methods or screening approaches.

2.3.4 NTM infection period prevalence

Table 2-4 summarizes the characteristics of studies that reported period prevalence of NTM infection (n = 43). A majority of them were cross-sectional non-registry studies (n = 31, 72.1%) conducted in Europe (n = 26, 60.5%) with a mixed pediatric and adult populations (n = 26, 60.5%).

Typically, studies collected data spanning two years (n = 12, 34.3%) while the longest study period was fourteen years (173). Among these studies, five were secondary analyses of registry data. Estimates of period prevalence ranged from 1.7% (4/233) in a 7-year interval study (174) to 40% (18/44) in an 11-year timeframe (136). As seen in **Figure 2-14**, studies with an evaluation period of ≤ 2 years had less variability in their estimate compared to the rest. In summary, most estimates of NTM infection period prevalence were between 6.8% and 16.4% (IQR). No meta-analysis was conducted due to diverging study periods. The median sample size was 210 with a range between 28 and 30,896, and only 18 studies had sample sizes larger than 300 participants.

Figure 2-14. Boxplots of NTM infection period prevalence estimates according to categories of study length

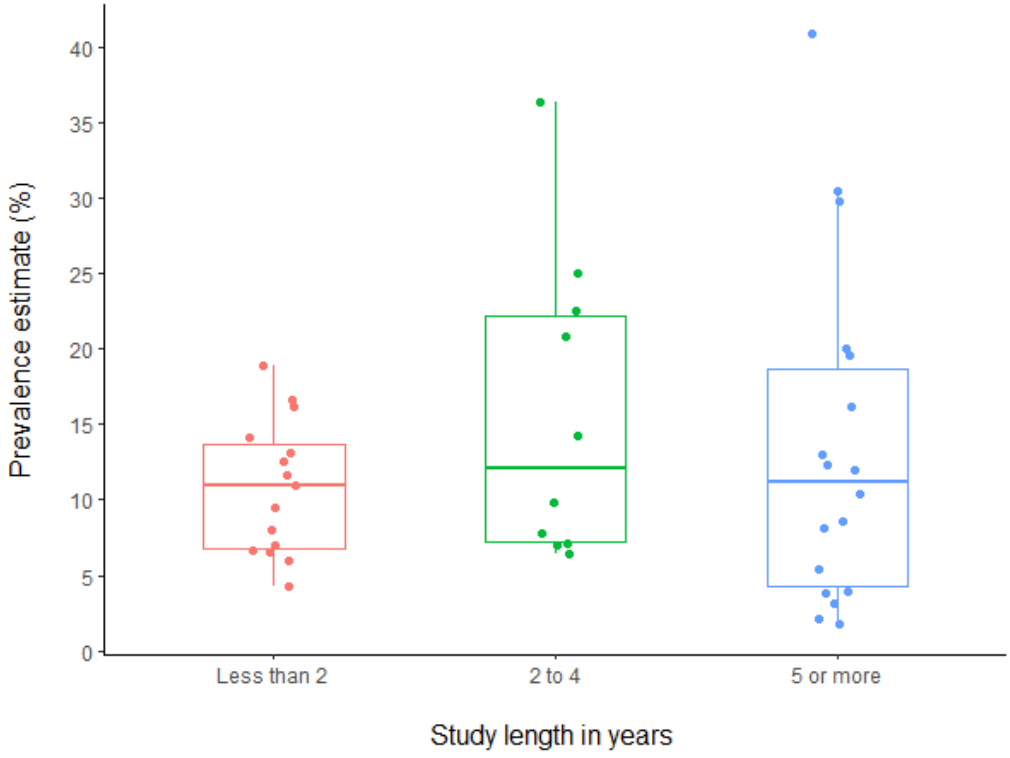


Table 2-4. Characteristics of studies reporting the period prevalence of NTM infection

Study ID	Study design	Sample size	Location	Age(y)	Females	Specimen	Method culture	Speciation	Period	Prevalence estimate
Abidin 2021 (117)	Cross-sectional	4,687	United Kingdom	9 (5 - 13) [Median; IQR]	51.4%	NR	NR	NR	2016 - 2018	6.5%
Ademhan-Tural 2021 (175)	Cohort	485	Turkey	**NTM+** 19 (8 - 27) [median; range]	**NTM+** 30% (n=10)	Sputum, BAL. Annual screening.	MGIT and L-J.	Commercial reverse hybridization assays	2012 - 2020	2.1%
Adjemian 2014 (87)	Cross-sectional (US registry)	10,527	United States	27 (12 - 82) [mean; range]	NA	NR. Annual screening in 60% of states	NR	NR	2010 - 2011	13.2%
Adjemian 2018 (118)	Cross-sectional	16,153	United States	12 to <18 - 23% 18 to <60 - 75% ≥60 - 2%	48%	NR. Annual screening (77% had 2/5 years of testing)	NR	NR	2010 - 2014	20.8% 19.9% (No *M. gordonae*)
Ahmed 2019 (176)	Cohort	42	United Kingdom	**NTM +** 12.7 ± 3.4 **NTM -** 11.2 ± 3.7 [mean ± sd]	45.2%	Induced sputum. Annual screening.	L-J and BACTEC MGIT. Incubated up to 12 weeks	NR	Jan 2012 - Dec 2016	14.3%
Aiello 2018 (177)	Cross-sectional	117	Brazil	**NTM +** 21 (9 - 56) [mean ± sd]	**NTM+** 42.8%	Sputum or BAL. Annual screening	BACTEC MGIT, up to 42 days of incubation	PCR-restriction enzyme analysis	Jan 2014 - Dec 2015	6%
Aitken 1993 (119)	Cross-sectional	64	United States	17 - 50 [range]	56.2%	Sputum. Frequency NR	L-J, Middlebrook, 7H11, and BACTEC 12B media	NR	Dec 1990 - Dec 1991	12.5%
Bange 2001 (178)	Cross-sectional	214	Hannover, Germany	NR	NA	Sputum, tracheal aspirates, and BAL. Frequency NR.	BACTEC MGIT	PCR amplification of 16S rRNA gene and sequencing	Sep 1997 - Mar 1999	7%
Bar-On 2015 (92)	Cohort	180	Israel	2008 **NTM +** 17.8 (4.3 - 55.3) **NTM –** 15.2 (0.2 - 59.3) [median; range]	2008 **NTM -** 47.9% **NTM +;** 35.3%	Sputum. Screened every 3-6 months	L-J and BD BACTEC MGIT. Monitored for 8 weeks	Mycobacteria Genotype kits	Jan 2002 - Dec 2011	18.9%

Campos Herrero 2016 (136)	Cross-sectional study	44	Gran Canaria, Spain	12 (5 - 59) [median; range]	**NTM+** 38.9%	Sputum. Frequency NR	BACTEC MGIT 960 and L-J medium	Phenotypic tests and/or nucleic acid hybridization assays	2002 - 2012	40.9%
Candido 2014 (179)	Cross-sectional	129	Brazil	NR	NA	Sputum. Frequency NR	L-J	Hsp65 PCR restriction analysis and partial sequencing of the RpoB gene	Jun 2009 - Mar 2012	7.8%
Cavalli 2017 (180)	Cohort	401	France	18.9 ± 7.4 [mean ± sd]	42%	Sputum. Annual screening	NR	Hsp65 sequencing	1997 - 2002	8.6% (n = 139)
Esther 2005 (181)	Cross-sectional	431 114 (BAL)	United States	**NTM+** 7.7 ± 3.8 [mean ± sd]	47%	Sputum and BAL. Screened by symptoms.	L-J (8 wk) an BACTEC 7HB12 vial (4 wk)	NR	1993 - 2002	3.9%
Esther 2010 (76)	Cross-sectional	829	United States	NR	NA	Sputum, BAL. Frequency NR.	NR	Biochemical methods and Hsp65 sequence analysis after 2007	2000 - 2007	13.7%
Fauroux 1997 (182)	Cohort	106	France	1 - 18y [range]	57.1%	Sputum. Screened twice per year	L-J	Biochemical methods	May 2012 - Dec 2013	6.6%
Fernandez-Caso 2020 (183)	Cross-sectional	92	Madrid, Spain	29.1 ± 9.5 [mean ± sd]	48.9%	Sputum. Frequency NR	NR	MALDI-TOF MS and PCR followed by reverse hybridization	2010 - 2017	30.4%
Gardner 2019 (153)	Cross-sectional	5,333	United Kingdom	6 (2 - 12) [median; IQR]	49.1%)	NR. Annual screening.	NR	NR	2010 - 2015	5.4%
Giron 2005 (184)	Cohort	28	Spain	25.3 ± 6.7 [mean ± sd]	42.8%	Sputum. Frequency NR	Coletsos and liquid MGIT 960 with modified 7H9 broth	NR	Jan 1996 - Dec 1999	25%
Hjelt 1994 (154)	Cross-sectional	185	Denmark	15.3 (2.2 - 38.5) [mean; range]	NA	Sputum. Sampled 3 times in 3 months.	L-J	Nucleic-acid hybridization kit or biochemical tests	1987 - 1988	7%
Ho 2021 (173)	Cross-sectional	171	Tropical French Reunion Island, Africa	**NTM +** 16 (10 - 23) [median; range]	55%	Sputum and BAL. Annual screening.	NR	16S rRNA gene sequencing after ruling out MTBC using the AccuProbe MTB DNA probe kit	2002 - 2015	29.8%

Hughes 2021 (185)	Cross-sectional	567	United Kingdom	**MABs** 11.8 (3.2 – 17.3) **MAC** 12.7 (3.6 – 16.7) **Other NTM** 11.6 (7.4 – 15.9) [median; range]	**NTM +** 63.5% (n = 63)	Sputum and BAL. Frequency NR.	NR.	NR.	2011 - 2018	10.4%
Kilby 1992 (186)	Cross-sectional	87	United States	**NTM +** 25.8 ± 4.6 [mean ± sd]	70.6%	Sputum. Tested by clinical symptoms.	L-J and BACTEC 7H12	Biochemical techniques and DNA probes for MAC	1981 - 1990	19.5%
Kopp 2015 (187)	Cross-sectional (US registry)	30,896	United States	<18y - 55.7% ≥18y - 44.3%	48.1%	NR	NR	Biochemical methods	2007 - 2012	8.1%
Leitriz 2004 (188)	Cohort	91	Munich, Germany	17.8 ± 9.2 [mean ± sd]	58.2%	Sputum/BAL. Frequency NR	BACTEC modified 7H12, L-J. Incubated for 8 weeks.	Nucleic acid probes, 16S rRNA sequencing, and biochemical tests	Jan 1999 - Dec 2000	11%
Levy 2008 (189)	Cross-sectional	186	Israel	20.5 ± 10.4 [mean ± sd]	60.2%	Sputum. Frequency NR	MB/BacT bottle, L-J and Middlebrook 7H11 plate, up to 7 weeks.	Biochemical methods and drug susceptibility patterns. MAC confirmed by RNA/DNA probes	Jul 2001 - Jul 2003	22.6%
Mussaffi 2005 (190)	Cross-sectional	139	Israel	2 - 52 [range]	NA	Sputum. Frequency NR.	NR	NR	1997 - 2002	8.6%
Oliver 2001 (191)	Cohort	37	Spain	21 (4 - 48) [mean; range]	NA	Sputum. Sampled twice in a week for study.	Coletsos, L-J and ESP liquid medium for 56 days.	Biochemical tests, and hybridization probes for MAC	2001 (uncertain)	16.2%
Olivier 2003 (74)	Cross-sectional	986	United States	23 ± 9 [mean ± sd]	47%	Sputum. Frequency NR	L-J and BACTEC MGIT.	RGM by Hsp65 sequencing. Slow growers by PCR and restriction digest	1994 (uncertain)	13%
Paschoal 2007 (156)	Cross-sectional	54	Brazil	41.8 ± 17.2 [mean ± sd]	50%	Sputum. Frequency NR	NR	NR	2003 - 2004	16.7%
Phelippeau 2015 (192)	Cohort	354	France	≥18 y - 235 <18y - 119	56.2%	NR	MGIT and Coletsos slant	Partial *rpo B* sequencing	Jan 2010 - Sep 2014	7.1%

Pierre-Audigier 2005 (157)	Cross-sectional	385	France	12.0 ± 6.1 [mean ± sd]	47.3%	Sputum. Thrice in a year.	L-J up to 10 weeks	RGM by biochemical methods and hsp65 sequencing. MAC by PCR probes	2000	8%
Plongla 2017 (158)	Cohort	487	United States	14.9; <1 - 71 [median; range]	53.6%	Pharyngeal swabs, sputum/tracheal aspirates, bronchial wash and BAL. Frequency NR.	BACTEC MGIT, L-J, RGM medium and BCSA	RGM by MALDI-TOF MS. Partial sequenc*ing of the 16S rRNA* for slow growers and others	Dec 2015 - Apr 2016	14.2%
Preece 2016 (159)	Cross-sectional	210	United Kingdom	<1 - 77 [range]	NA	Sputum. No regular screening (< 10% of cohort sampled)	RGM medium and BCSA	Sequencing of two genes among RPO-B, HSP65 and SOD-A	Feb 2014 - Sep 2014	9.5%
Qvist 2014 (193)	Cohort	198	Denmark	NR	NA	Sputum, laryngeal aspirates or BAL. Annual screening.	L-J and BACTEC MGIT, incubated for 8 weeks. BCSA for 14 days.	MALDITOF and 16S rRNA sequencing locally.	May 2012 - Dec 2013	11.6%
Qvist 2015 (194)	Cross-sectional	1,270	Denmark, Norway and Sweden	19(13 - 22) [median; IQR]	**NTM+** 26.7%	Sputum, BAL, laygneal suction. Annual screening.	L-J, BACTEC MGIT or BCSA	16-23s spacer/*rpoB/hsp65* sequencing, biochemical tests, hybridization, GenoType Mycobacterium CM and/or growth on L-J	2000 - 2012	12.4%
Roux 2009 (75)	Cohort	1,582	France	18.9 (0.3 - 82) [mean; range]	48.6%	Sputum. Frequency NR	BACTEC MGIT and/or Lowenstein Colestos slants.	Hsp65 and 16S-23S intergenic gene region sequencing. MABs by rpoB sequencing	Jan 2014 - Dec 2014	6.6%
Satana 2014 (195)	Cross-sectional	130	Turkey	12.1 ± 3.1 [mean ± sd]	47.6%	Sputum. Frequency NR	BACTEC MGIT and L-J for 10 weeks.	GenoType Mycobacterium CM/AS assay	Apr 2003 - Nov 2008	3.1%
Scohy 2018 (161)	Cross-sectional	124	Belgium	24.5 (6 - 68) [median; range]	47%	Sputum. Frequency NR.	BACTEC MGIT and RGM medium	MALDI-TOF MS, Geno-Type NTM-DR and whole genome sequencing for MABs	Sep 2016 - Mar 2017	16.1%

Seddon 2013 (78)	Cross-sectional	7,122	United Kingdom	Pediatric - 46.5% Adults 53.4%	NA	NR. Annual screening in 33/42 centers, by symptoms in 9.	NR	NR	2008 - 2009	4.2% **Adult** 5% (n=3805) **Pediatric** 3.3% (n = 3317)
Sermet-Gaudelus 2003 (196)	Cross-sectional	296	France	11.3 (0.2 - 32) [mean; range]	53.4%	Sputum. Annual screening	L-J with 10 wks of incubation	RGM by biochemical methods/hsp65 sequencing. MAC through PCR probes	Jan 1996 - Dec 1999	9.8% MABs - 5.1%
Smith 1984 (174)	Cross-sectional	223	United Kingdom	**NTM +** 21 (17 - 29) [mean; range]	**NTM+** 50%	Sputum. Screened by symptoms.	NR	Biochemical methods	1978 - 1984 (uncertain)	1.7%
Torrens 1998 (197)	Cross-sectional	372	United Kingdom	16.1 ± 4.5 [mean ± sd]	**NTM+** 28.6%	Sputum. Frequency NR	L-J	NR	1989 - 1997 (uncertain)	3.8%
Yan 2020 (198)	Cross-sectional	99	Melbourne, Australia	**MABS+** 13 (6 - 17) [mean; range]	40.9%	Sputum, BAL. Tested annually	NR	NR	Jan 2013 - Mar 2017	36.4% [screened 99/238]

NTM: Nontuberculous mycobacteria. **MGIT:** Mycobacterial Growth Indicator Tubes. **L-J:** Lowenstein-Jensen egg-based medium. **BAL:** Broncho-Alveolar Lavage. **RGM:** Rapid growing mycobacteria (*M. abscessus* complex). **MAB:** *M. abscessus* complex. **MAC:** Mycobacterium avium complex. **PCR:** Nucleic acid amplification by polymerase chain reaction. **MALDI-TOF MS:** matrix-assisted laser desorption/ionization- time-of-flight mass spectrometry. **NR:** Not reported. **BCSA:** *Burkholderia cepacia* selective agar. **MTBC:** *Mycobacterium tuberculosis*.

Table 2-5. NTM infection incidence proportion

Study ID	Study design	Sample size	Location	Age(y)	Females	Specimen	Culture method	Incidence definition	Years	Incidence proportion
Bar-On 2015 (92)	Cohort (retrospective)	110	Israel	2008 **NTM +** 17.8 (4.3–55.3) **NTM –** 15.2 (0.2–59.3) [median;range]	2008 **NTM +** 35.3% **NTM -** 47.9%	Sputum. Frequency NR	L-J and BACTEC MGIT. Incubated up to 8 wks.	Percentage of patients with a new NTM positive sputum / all clinic patients at the end of that year (includes those with a different strain)	2003 2004 2005 2006 2007 2008 2009 2010 2011	1.4% 1.3% 2.2% 3.3% 4.3% 3.1% 5.5% 5.2% 8.7%
Binder 2013 (199)	US registry Cohort (retrospective)	5,403	United States	**MAC** 25 ± 13 **MABs** 23 ± 13 [mean ± sd]	49.3% (n = 5212)	NR	NR	Incident cases: patients with positive mycobacterial culture in 2011 and negative culture in 2010	2011	3.5%
Campos-Herrero 2016 (136)	Cross-sectional	44	Gran Canaria, Spain	**NTM +** 12 (5-59) [median;range]	**NTM +** 38.9%	Sputum. Frequency NR	BACTEC MGIT 960 and L-J	Percentage of patients with a NTM positive culture for the first time during each calendar-year	2002 2003 2004 2005 2006 2007 2008 2009 2010:2012	14.3% 4 % 7.7 % 4.2% 0 % 12.5% 6.5% 6.7% 0 %
Hatziagorou 2020 (24)	Cohort (prospective)	41,101	European countries	NR	NR	NR	NR	Incident case is a patient that reports a first-time positive culture for Mycobacteria spp. with negative cultures in prior two years; excluded from later years	2011 2012 2013 2014 2015 2016	1.4% (n = 15,308) 1.3% (n = 19,350) 1.3% (n = 22,173) 1.8% (n = 22,952) 1.5% (n = 23,536) 1.4% (n = 24,137)
Leitriz 2004 (188)	Cohort (prospective)	91	Munich, Germany	17.8 ± 9.2 [mean±SD]	58.2%	Sputum/Broncho-Alveolar lavage. Frequency NR.	BACTEC 460 12B and L-J. All specimens for 8 wks.	New cases over the number of study population at risk (total population minus prevalent cases)	Jan 1999 - Dec 2000	8%

MGIT: Mycobacterial growth indicator tubes. **L-J:** Lowenstein-Jensen egg-based culture medium. **NTM:** nontuberculous mycobacteria

2.3.5 NTM infection incidence

Incidence was reported as incidence proportion in five studies, with no reports of incidence rate (24,92,136,188,199). The characteristics and estimates of these studies are summarized in **Table 2-5**. Besides secondary registry analyses (Hatziagorou 2020 and Binder 2013), all studies had small sample sizes (110 or less). Bar-On 2015 was conducted in Israel, Binder 2013 in the US and the remaining three in Europe. The annual estimates of incidence proportion per year were typically below 10%. The highest estimate (14.3% - 2002) was reported in Campos-Herrero 2016, the study with the smallest sample size (n = 44) (136). In contrast, the study with the largest sample size (Hatziagorou 2020) had estimates of around 1% over the years (24).

2.3.6 NTM pulmonary disease

Point prevalence of NTM-PD was reported in only 2 studies. The first, Radhakrishnan 2009 (160) had a 1/98 (1.0%) prevalence using ATS 2007 criteria in 2004 (200). The second, Bar-On 2015 evaluated annual prevalence in Israel between 2002 and 2011 using ATS 2007 criteria and showed a prevalence between 2.5 % and 11.3%, see **Table 2-6** (92)**.** Both of these studies had small sample sizes, BarOn with n = 110 in 2011 and Radhakrishnan with n = 98.

NTM-PD period prevalence was reported in 13 studies, with estimates ranging between 1.0% (3-year period) and 22.7% (10-year period), see **Table 2-7** (136,179). Most studies were conducted in Europe (8/13), with the remaining ones in Israel or Brazil. Most of them applied the ATS 2007 criteria (n=7), but 4 studies failed to report the criteria used to define NTM-PD. Only three studies had sample sizes above 300 included participants. No reports of NTM-PD incidence were identified.

Table 2-6. Characteristics of studies reporting NTM-PD point prevalence

Study ID	Study design	Sample size	Location	Age(y)	Females	Specimen	Culture method	NTM-PD criteria	Years	Point prevalence
Bar-On 2015 (92)	Cohort (retrospective)	70 (2002) 110 (2011)	Israel	**2008** **NTM +** 17.8 (4.3–55.3) **NTM -** 15.2 (0.2–59.3) [median;range]	**2008** **NTM +** 35.3% **NTM -** 47.9%	Sputum. Screened every 3-6 months	L-J and BACTEC MGIT. Incubated at 37 °C incubator up to 8 wks	ATS 2007	2003 2004 2005 2006 2007 2008 2009 2010 2011	2.5 % 3.4 % 3.3 % 4.3 % 7.3 % 8.8 % 11.3 % 7.7 % 5.5 %
Radhakrishnan 2009 (160)	Cross-sectional	98	Canada	**NTM +** **1**5.1 ± 2.2 **NTM -** 14.0 ± 3.0 [mean±sd]	**NTM +** 66.7% **NTM -** 53.3%	Sputum. Annual screening in study period	BACTEC MGIT and L-J. Incubated at 37°C for up to 7 wks	ATS 2007	Mar 2004 - Nov 2004	1.0 %

MGIT: Mycobacteria growth indicator tube. **RGM**: Rapid-growing mycobacteria. **L-J:** Lowenstein Jensen egg-based medium. NTM: nontuberculous mycobacteria

Table 2-7. Characteristics of studies reporting NTM-PD period prevalence

Study ID	Study design	Sample size	Location	Age(y)	Females	Specimen	Culture method	NTM-PD criteria	Years	Period prevalence
Ademhan-Tural 2021 (175)	Cohort	485	Turkey	**NTM+** 19 (8 - 27) [median; range]	**NTM+** 30% (n = 10)	Sputum, BAL. Annual screening.	MGIT and L-J	ATS 2007	2012 - 2020	1.0%
Bar-On 2015 (92)	Cohort	180	Israel	2008 **NTM +** 17.8 (4.3 - 55.3) **NTM -** 15.2 (0.2 - 59.3) [median; range]	2008 **NTM –** 47.9% **NTM +** 35.3%	Sputum. Screened every 3-6 months	L-J and BACTEC MGIT for 8 wks	Unknown	Jan 2002 - Dec 2011	9.4%
Campos Herrero 2016 (136)	Cross-sectional	44	Gran Canaria, Spain	12 (5 - 59) [median - range]	**NTM+** 38.9%	Sputum. Frequency NR	BACTEC MGIT and L-J	ATS 2007	2002 - 2012	22.7%
Candido 2014 (179)	Cross-sectional	129	Brazil	NA	NA	Sputum. Frequency NR	L-J	ATS 2007	Jun 2009 - Mar 2012	0.8%
Cavalli 2017 (180)	Cohort	401	France	18.85 ± 7.4 [mean ± sd]	42%	Sputum. Annual screening	Not specified	ATS 2007	1997 - 2002	3.7%
Fauroux 1997 (182)	Cohort	106	France	1 - 18y [range]	57.1%	Sputum. Screened twice per year.	L-J medium	Unknown	May 2012 - Dec 2013	1.9%
Giron 2005 (184)	Cohort	28	Spain	25.3 ± 6.7 y [mean ± sd]	42.8%	Sputum. Frequency NR	Coletsos and liquid MGIT 960 with modified 7H9 broth	Unknown	Jan 1996 - Dec 1999	8%
Ho 2021 (173)	Cross-sectional	171	Tropical French Reunion Island, Africa	**NTM +** 16 (10 - 23) [median; range]	55%	Sputum and BAL. Annual screening.	NR	Unknown	2002 - 2015	7%
Hughes 2021 (185)	Cross-sectional	567	United Kingdom	**MABs** 1.8 (3.2 – 17.3) **MAC** 12.7 (3.6 – 16.7) **Other** 11.6 (7.4 – 15.9) [median; range]	67.8% (n = 59)	Sputum and BAL. Frequency NR.	NR	ATS 2007	2011 - 2018	6.2%

Levy 2008 (189)	Cross-sectional	186	Israel	20.5 ± 10.4 [mean ± sd]	60.2%	Sputum. Frequency NR	MB/BacT, L-J, and Middlebrook 7H11. Up to 7 wks	ATS 2007 and ATS 1997	Jul 2001 - Jul 2003	6.4% and 10.8%
Mussaffi 2005 (190)	Cross-sectional	139	Israel	2 - 52 [range]	NA	Sputum. Frequency NR.	Not described	ATS 1997	1997 - 2002	4.3%
Sermet-Gaudelus 2003 (196)	Cross-sectional	296	France	11.3 (0.2 - 32) [mean - range]	53.4%	Sputum. Annual screening	L-J up to 10 wks	ATS 1997	Jan 1996 - Dec 1999	1.4%
Qvist 2014 (193)	Cohort	198	Denmark	NA	NA	Sputum, laryngeal aspirates or BAL. Annual screening	L-J slants and MGIT for 8 weeks. BCSA for 14 days	ATS 2007	May 2012 - Dec 2013	9.6%

BAL: Broncho-Alveolar Lavage. **ATS:** American Thoracic Society. **MGIT:** Mycobacteria growth indicator tube. **RGM:** Rapid-growing mycobacteria. **L-J:** Lowenstein Jensen egg-based medium. **PCR:** Polymerase chain reaction assay. **MALDI-TOF:** matrix-assisted laser desorption/ionization- time-of-flight. **NR:** Not reported

2.4 Discussion

Our systematic review represents a large and comprehensive overview of the literature on the prevalence/incidence of NTM infection and NTM-PD in the CF population. The estimated prevalence (annual and point) of NTM infection in CF was 7.7% (95% CI 5 – 12%; 95% PI 1 - 43%) based on a meta-analysis of all non-registry and registry studies. Individual estimates for infection with the most common mycobacteria in CF followed a similar pattern: MABs 4.4% (PI 95% 1.2 – 15) in 20 studies, and MAC 3.6% (95% PI 0.7 – 16) in 12 studies each. NTM-PD had only two reports of point prevalence, and estimates of period prevalence were usually below 10%, despite variable interval length per study (n = 13). In general, all included studies had high quality in the appraisal of sampling and statistical methods, but lower scores for microbiological methods and screening approaches.

We employed meta-regression to elucidate the adjusted contributors to heterogeneity in the meta-analysis of NTM infection prevalence (point and annual). The results show that studies with different categories of sample size and geographical region produce significantly different estimates, even after adjusting by covariates. In an exploratory subgroup analysis (**Appendix A-5**), registry studies had a significantly lower estimate of NTM infection prevalence (point/annual) than other study types. However, as seen in the meta-regression, this may be the effect of larger sample size in registry studies.

Differences in NTM infection prevalence by geographical region could be explained by environmental factors and NTM species distribution. Previous studies in CF and non-CF populations have shown different risks according to geographical region (75,76,196,201). In

meta-regression, there was a significantly different estimate for studies conducted outside of Europe and North America. However, only 4 studies were included in this group and it may not be representative of each geographical region. Also, in an exploratory analysis, we observed a lower MAC infection prevalence in European studies with significantly reduced heterogeneity (I^2 = 25%). Interestingly, some studies from Western Europe have reported a predominance of MABs infection in contrast to the MAC predominance seen in North America (75,202–204). Differences by region could not be explored for NTM-PD due to the limited amount of data retrieved. Thus, we believe some of the heterogeneity in the meta-analyses could be associated with differences in species distribution.

Previous reports on CF and the non-CF population point towards an increase in the NTM burden (76,80,92,180,205). Our analyses did not show significant differences in prevalence by years of data collection. Given the possible methodological variability between studies to evaluate temporal trends, we explored the longitudinal report of NTM infection (point) prevalence inside individual registries. An increasing trend of prevalence was observed in all but the Brazilian registry (**Table 2-2**), which has poor coverage of screening. Improvements in screening rates over the years, novel detection methods, initial inconsistent reporting by primary centers and increased awareness may also explain this increase (82). Overall, the increase in NTM infection was found in individual registries, where methods are standard throughout the years.

We also explored other potential sources of heterogeneity and found apparent good concordance in microbiological identification methods (culture and specimens) in non-registry studies. Heterogeneity due to included population characteristics could not be evaluated due to the

differences in primary data reporting of summary (mean, median) and distribution (median, mean, IQR, range) statistics. A major and common flaw was the limited reporting of screening frequency, a variable that was notoriously absent in registry reports. To obtain comparable estimates, harmonization of screening practices is necessary (82). Adherence to published reporting guidelines for observational studies (i.e., STrengthening the Reporting of OBservational studies in Epidemiology) and harmonization of registry reporting standards will facilitate comparisons across geographic regions and over time (206,207).

Only a few studies have evaluated NTM infection incidence measures (n=5) or NTM-PD prevalence measures (n = 13 in period prevalence and n = 2 for point prevalence). From this limited set of studies, the incidence proportion of NTM infection seems to be less than 10% per year in European populations (24,136), without sufficient data from North America to make meaningful conclusions. Moreover, the conversion rate to NTM-PD (incidence) after NTM infection remains unclear. Hopefully, ongoing studies like the NCT02073409 trial, which is evaluating a standardized approach to NTM-PD diagnosis in CF, will contribute to determine an approximate risk of progression (208).

Overall, the results from this systematic review present a clear picture of the known burden of NTM in CF while pointing out gaps in knowledge and relevant research topics (94). However, a lack of reported data in primary studies did not allow further exploration of sources of heterogeneity beyond the ones already described. Another limitation was the large variability and wide predictive intervals of our NTM infection meta-analysis, which limits their utility for decision-making. Once CFTR modulators are widely implemented, their impact on infection

prevalence is likely to change and our results can serve as a baseline to measure this impact on NTM and NTM-PD.

Finally, moving forward, we advocate for stronger emphasis on reporting standards for registry and non-registry studies (207). A significant and relatively low-cost way to build upon this work is to create a living systematic review of the NTM burden in CF; which could be updated annually with new registry and observational data (209).

Chapter 3: Evaluation of host genetic susceptibility to predict nontuberculous mycobacterial pulmonary disease in patients with cystic fibrosis

3.1 Introduction

The clinical course of an NTM infection in CF is highly variable. Its outcomes include isolated single growth (transient growth), chronic infection without overt complications (persistent infection), and pulmonary NTM disease (NTM-PD), sometimes characterized by a rapid deterioration in lung function (76,81,210). Regardless of clinical course, both NTM infection and NTM-PD can negatively affect eligibility for a lung transplant (211). According to current CF consensus guidelines, a combination of repeated microbiological isolation, clinical deterioration, and characteristic radiological findings are needed for NTM-PD diagnosis (82). However, CF patients have underlying symptoms and radiological changes that overlap with NTM-PD. Thus, is recommended to rule out alternative causes before committing to anti-NTM therapy, because treatment courses are prolonged (>12 months), poorly tolerated, and have microbiological clearance rates as low as 50% (82).

Risk factors or biomarkers that predict progression to NTM-PD would be extremely valuable for CF clinicians to identify high-risk patients and focus interventions on them. NTM infection in CF has been linked to specific age groups (MABs in children and MAC in adolescents/adults), history of allergic bronchopulmonary aspergillosis, infection with *Aspergillus* spp., and chronic exposure to immunomodulatory drugs like macrolides and steroids (77,82,189,203,212–214). However, fewer studies have evaluated the determinants of progression from infection to NTM-PD in CF.

Among them, Caverly et al. showed the relationship between changes in the microbiome composition (*Rhotia* taxa abundance) and outcomes of NTM infection in a small sample (n = 25) (215). Also, Martiniano et al. described a prospective cohort of 96 patients where those who progressed to NTM-PD had lower baseline FEV1 and a faster lung function decline in the year prior to infection (81). However, there is no validated tool, biomarker or algorithm for clinicians to approach a CF patient with an initial growth of NTM, and the current paradigm is watchful waiting and exploration of alternative diagnoses in cases of deterioration.

In the CF population, the relationship between host response to NTM infection and the development of NTM-PD is largely unknown. In the non-CF population, various genetic polymorphisms have been associated with NTM-PD. The *rs109592* polymorphism in the *CHP2* (cofactor for plasma membrane sodium/hydrogen ion exchangers) locus was associated with NTM-PD in Japanese, Korean and European populations (216). Cowman et al. evaluated whole blood transcriptomics in a cohort of patients with underlying lung disease and found a significantly reduced expression of genes involved in lymphocyte effector functions and Interferon-γ production in those with NTM-PD patients (217). **Table 3-1** summarizes the gene polymorphisms and gene expression results associated with NTM-PD. However, no studies have examined genetic or genomic biomarkers in the CF population. Furthermore, the intrinsic structural and immune changes in the CF lung microenvironment may be susceptible to NTM in different ways. Here, we conducted an exploratory whole blood gene expression study using RNAseq to evaluate predictive biomarkers of NTM-PD in CF patients with positive growth of NTM.

Table 3-1. Candidate genes reported in non-CF populations with NTM-PD

Candidate(s)	Sample size and study type	Reference
IL-10	Targeted genotyping of laboratory identified variants NTM-PD (n = 79) vs controls (n = 188).	Affandi 2013 (218)
CHP2, PRKCB	GWAS using Japanese, Korean and European ancestry populations Discovery cohorts of pulmonary MAC disease (n > 400)	Namkoong 2021 (216)
STK17A	GWAS study using Korean population Discovery cohort, NTM-PD (n > 400)	Cho 2021 (219)
NRAMP1	Targeted genotyping case-control designs **Koh** - Korean population: NTM-PD (n = 41) vs healthy controls (n = 50) **Tanaka** – Japanese population: MAC disease (n = 111) vs healthy controls (n = 177)	Koh 2005 and Tanaka 2007 (220,221)
Negative regulation of α-β T cell proliferation	Differential gene expression microarrays Patients with structural lung damage. NTM-PD vs not NTM-PD (n = 52)	Cowman 2018 (217)

IL-10: Interleukin 10. ***CHP*2**: Calcineurin Like EF-Hand protein 2. ***PRKCB***: Protein Kinase C-Beta. STK17A: Serine/Threonine Kinase 17a**.** ***NRAMP1***: Solute Carrier Family 11 Member 1

3.2 Methods

3.2.1 Study population and clinical data

This is a secondary analysis of a prospective cohort study "CF Biomarkers" with providence health ethics review board accession numbers H12-00910 and H12-00835. Patients were recruited from the adult CF clinic at St. Paul's Hospital in Vancouver, Canada between 2012 and December 2019. Inclusion criteria required enrollment in the CF biomarkers study, consent for the future use of samples and data, a positive respiratory culture for NTM, and availability of stored whole blood RNA (in PAXgene® tubes). Lung transplant recipients and subjects without a definite diagnosis of CF based on published criteria were excluded (222). In the primary study, samples were collected longitudinally, at visits with clinically stable or pulmonary exacerbation status, and

stored at -70°C until processing. The sample closest to the first positive growth of NTM was used. The protocol was reviewed and approved by the local ethics board (H20-00117).

Clinical data was extracted from the clinical charts using a case report form including demographic characteristics, anthropometric measurements, pulmonary function tests, genotype, microbiology laboratory results, evidence of comorbidities and radiological reports. If patients had a growth of NTM in the pediatrics clinic, we extracted baseline demographic and clinical data from that period. Missing values were searched in registry data, no imputation was performed. The progression to NTM-PD was defined independently by two expert clinicians, based on current guidelines, and disagreements were resolved by consensus (82). Lung function measurements were standardized to sex, ethnicity and anthropometric measurements using the 2012 Global Lung Function Initiative equations (223). CT chest images were reviewed by a chest radiologist and summarized according to the Brody score in the domains of mucus plugging, bronchiectasis, airway wall thickening and parenchymal compromise; air trapping could not be measured as most CT scans were inspiratory (224).

3.2.2 Clinical and demographic data analysis

No sample size calculation was performed due to a lack of prior data. Clinical and demographic differences were explored according to NTM-PD outcomes using univariate statistics. Characteristics of the population were summarized using frequencies and central tendency measures. All analyses were performed using R studio and R version 4.1.1 with the tidyverse package collection (104,107,225), and plots were produced using the ggplot and ggpubr packages (109,226).

3.2.3 RNA extraction and RNA sequencing experiments

Total RNA extraction was performed in five batches. The PAXgene Blood micro-RNA Kit was used with the manufacturer's (QIAGEN) instructions, omitting the DNA depletion steps (227). For quality control purposes, extracted RNA was evaluated with the NanoDrop™ spectrophotometer at 260/280 nm wavelengths, and the mean concentration was 117.4 ± 73.2 (SD). Also, RNA Integrity Number (RIN) was evaluated using a 2100 Bioanalyzer instrument from Agilent. Both quality control approaches were repeated at the Genome Quebec sequencing facility and a threshold of Ribonucleic acid Integrity Number ≥ 7 was required for sequencing. Two samples failed bioanalyzer quality control and were re-extracted.

RNAseq libraries were prepared in a single batch with 250ng per sample. Strand-specific library preparation was performed using Nextera NEB mRNA kit with adapters AGATCGGAAGAGCA CACGTCTGAACTCCAGTCAC and AGATCGGAAGAGCGTCGTG TAGGGAAAGAGTGT. A NovaSeq 6000 S4 PE100 (Illumina) platform with automatic base calling (RTA3) and an initial concentration of 200pM per library was used for sequencing. All samples were multiplexed in a single flow cell using specific barcoded dual sequences. The sequencing run generated 150 bp paired reads with a minimum average Phred+33 score of 36/40; the median number of reads per sample was 65 x 10^6 (range: 36 - 151 x 10^6).

3.2.4 RNAseq data analysis

Raw reads in FASTQ format were exported to the University of British Columbia Sockeye High-Performance computational cluster (CentOS 7 Linux). Fast-QC was used to evaluate the quality of raw reads before alignment (228). The primary human assembly GRCh38.p13 v38 (May 21,

2021) from GENCODE was the reference for alignment of untrimmed paired reads using STAR v 2.7 (229). RSEM v1.3.3 with default parameters was used to produce bam formatted files with gene-level quantification (230). Quality control of alignment was performed using Picard tools and Fast-QC (231). All quality assurance reports were summarized using MultiQC (232).

Unnormalized count data was imported to R version 4.1.1 using tximport (233). Annotation was performed using Bioconductor's annotation hub and the Ensembl database version 104 (234–236). Hemoglobin subunits and genes showing unusually large expression counts ($>7*10^7$) were filtered out. Also, based on the principal component analysis of DESeq2 (v 1.3.2) variance stabilized count data, we removed an outlier sample (CFB2006) and genes from non-autosomal chromosomes. No further separation was evident in PCA. Count data was corrected for extraction batches using the ComBat-seq algorithm (237). Differential gene expression of NTM-PD vs not NTM-PD outcomes was performed using DESeq2 with a cut-off FDR of 0.3 and no fold-change threshold (238). Finally, gene set enrichment analysis of the molecular signatures database (human hallmark pathways) was performed in fgsea using fold change ranked results from DESeq2 (239–242).

The cell population compositions were explored using gene expression deconvolution in cibersortX and compared with complete blood cell counts taken from study participants at the same time of study sampling. The LM22 (immune cell types) signature matrix, with bulk mode batch correction and 100 iterations were used for deconvolution. Our sample size was insufficient to calculate cell population-specific gene expression (243). Also, previously described candidate genes were explored for trends in gene expression among our outcome groups: NTM-PD and no NTM-PD.

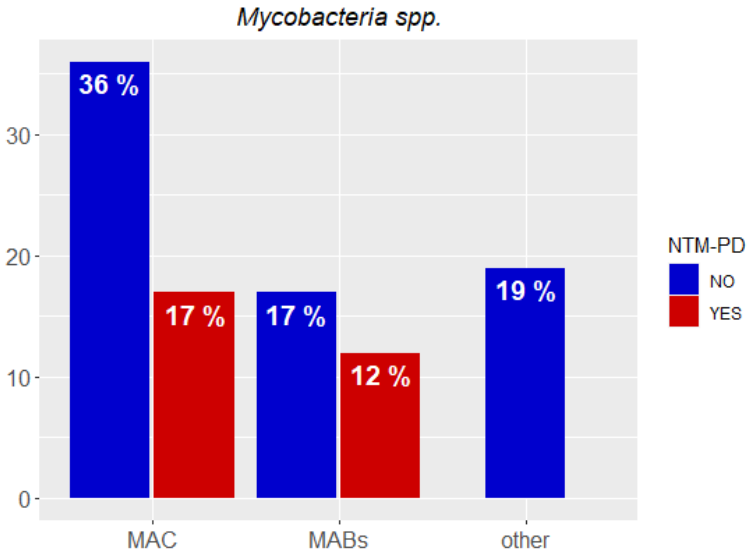
3.3 Results

3.3.1 Characteristics of the study population

Among 189 participants included in the CF biomarkers study, 53 had positive growth for NTM and 42 fulfilled all eligibility criteria and were included in the study. Exclusion criteria included unclear CF diagnosis (n = 1), lung transplant recipient (n = 1) and no whole blood sample available (n = 9). Our main outcome, NTM-PD or not NTM-PD, was defined independently by two expert clinicians with Cohen's kappa of 90%, and disagreements were solved by consensus. Overall, 12 out of 42 included patients progressed to pulmonary NTM disease (NTM-PD) during the recorded follow-up (until December 31, 2019). The median follow-up was 54.3 months (range 0 - 224) and the median interval between first NTM growth and diagnosis of NTM-PD was 14.3 months (range 0 – 199). **Figure 3-1** shows the distribution of NTM species in the cohort. No statistically significant differences were seen at baseline. Only patients infected with MAC or MABs progressed to NTM-PD in our cohort.

Included participants were a median of 25 years of age (range 12 – 59 years), were mostly of male sex (n = 29, 69%) and 83% had at least one copy of F508del. Common comorbidities including pancreatic insufficiency and CF diabetes were similarly distributed among outcome groups. **Table 3-2** summarizes the demographic and clinical characteristics at baseline for the study cohort.

Figure 3-1. Distribution of *Mycobacteria* spp. in the study cohort



Distribution of mycobacterial species in the cohort; the category of other includes *M. gordonae* (n=3), *M. fortuitum* (n=2) and *M. cosmeticum*, *M. peregrinum* and *M. simiae* (n=1 of each). No significant differences were observed in univariate analyses.

No differences were found in exposure to azithromycin (prior three months) or to oral steroids (prior month) between patients who progressed or did not to NTM-PD at the time of first NTM growth. Furthermore, baseline clinical characteristics including body mass index, forced expiratory volume in 1 second (FEV1), and sputum microbiology were not significantly different for the outcome groups. **Figure 3-2** shows the rate of chronic positivity for these CF pathogens according to the outcome group (n = 4 with missing data). No significant differences were found among rates of chronic infection at baseline for any pathogen.

Table 3-2. Demographic and clinical characteristics of included patients at baseline

	Total (n = 42)	**No NTM-PD (n=30)**	**NTM-PD (n=12)**
Age - median (range)	28.5 (18 – 60)	29.5 (18 - 60)	24 (20 - 49)
Females - n (%)	13 (31.0)	9 (30.0)	4 (33.3)
Genotype - n (%)			
F508del/F508del	17 (40.5)	11 (36.7)	5 (41.7)
F508del/other	18 (42.9)	13 (43.3)	6 (50)
Others	7 (16.7)	6 (20)	1 (8.3)
Pancreatic insufficiency - n (%)	33 (78.6)	23 (76.7)	10 (83.3)
CF diabetes - n (%)	10 (23.8)	7 (23.3)	3 (25.0)
Body mass index* mean ± SD	22.1 ± 2.6	22.3 ± 2.7	21.5 ± 2.3
FEV1 [% predicted] mean ± SD	75.3 ± 21.4	75.1 ± 22.3	75.4 ± 21.0
Oral steroids exposure - n (%) *	3 (7.1)	2 (6.7)	1 (8.3)
Macrolide exposure - n (%) *	14 (33.3)	11 (36.7)	3 (25)
Follow-up time in months median(range)	54.3 (0 – 223.9)	69.2 (10.1 - 224)	14.8 (0 - 199)

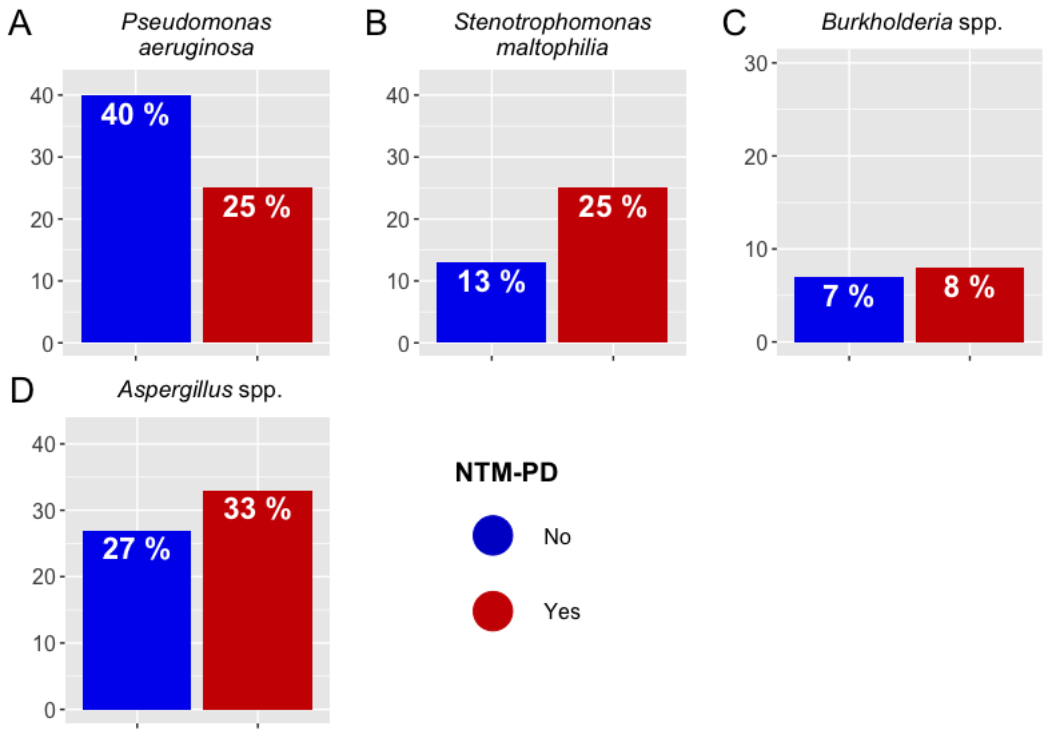
* n = 41, missing data for 1 patient

Despite the prospective follow-up and longitudinal collection of clinical specimens in the CFB cohort, the window between the first NTM growth and the available whole blood RNA was highly variable. Among the 42 patients included, 12 (29%) had less than 6 months between sampling and first NTM growth while 22 (52%) had 18 months or more. Besides, the RNA sample from 5/42 patients was obtained before NTM growth and 6/12 after NTM-PD diagnosis.

In an exploratory analysis, we evaluated CT scans taken less than 18 months apart from the time of first NTM growth and before the diagnosis of NTM-PD (n = 18). Longer intervals were considered too long to have a relationship with baseline status. A majority of the 18 CTs had less

than 6 months from the growth (n = 12, 66.7%). The mean overall Brody score was similar in both outcome groups, 63 for NTM-PD and 51.3 for no NTM-PD (p = 0.61). No significant differences were seen among specific domains either, see **Table 3-3**. A cavity was found in a single subject who did not progress to NTM-PD.

Figure 3-2. Distribution of chronic colonization with respiratory pathogens at baseline



Plots showing the percentage of patients with chronic respiratory growth of four common pathogens in CF. **A.** *Pseudomonas spp.* **B.** *Stenotrophomonas spp.* **C.** *Burkholderia spp.* **D.** *Aspergillus spp.* No significant differences were observed in univariate analyses.

Finally, using complete blood cell counts (CBC) processed when the whole blood RNA sample was collected, we explored baseline differences in white blood cell populations; 39/42 (93%) participants had a CBC available within three days of sampling. No significant differences in absolute counts of leukocytes, neutrophils, monocytes or lymphocytes were found among NTM

outcome groups using the Wilcoxon rank-sum test for unpaired samples (**Figure 3-3).** Furthermore, principal component analysis of all CBC parameters explained more than 50% of the variance in the first two components, but did not separate NTM outcome groups (**Figure 3-4-A).** In **Figure 3-4-B,** we can see that the absolute WBC, neutrophil counts and neutrophil percentage contribute the most to the first two principal components.

Table 3-3. Radiological findings at baseline, Brody score*

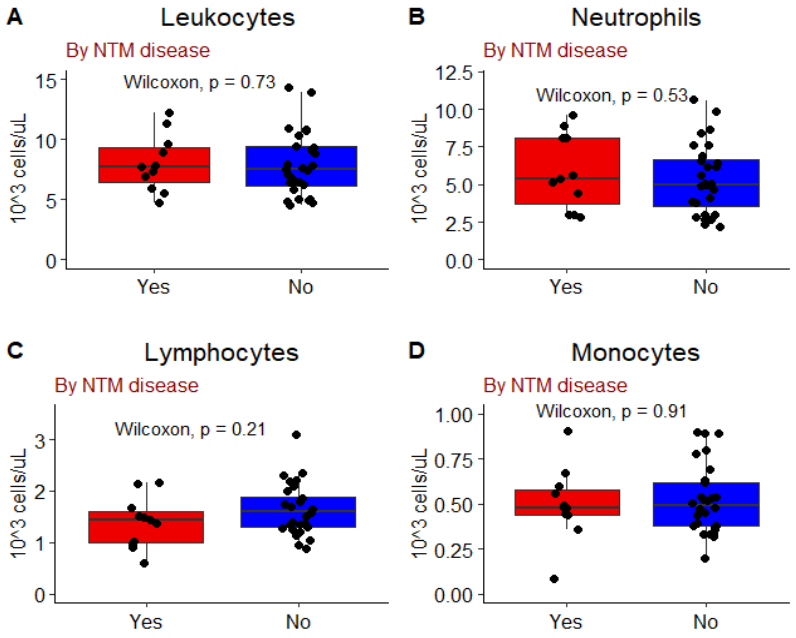
CT finding	All patients (n = 18)	No NTM-PD (n = 13)	NTM-PD (n = 5)
Global Brody Score	54.6 ± 29.8	51.3 ± 25.8	63.2 ± 40.6
Bronchiectasis	20.1 ± 14.4	17.7 ± 12.2	26.2 ± 19.3
Mucus plugging	12.5 ± 6.14	12.1 ± 5.99	13.6 ± 7.09
Airwall thickening	19.6 ± 10.3	19.1 ± 9.91	20.9 ± 12.2
Parenchymal compromise	2 (0 – 8)	2 (0 – 8)	2 (0 - 6)

* Air trapping was not evaluated due to the lack of expiratory CT images in most cases. Values were summarized as mean ± SD, except for parenchymal compromise which was summarized as median (range)

3.3.2 RNAseq results

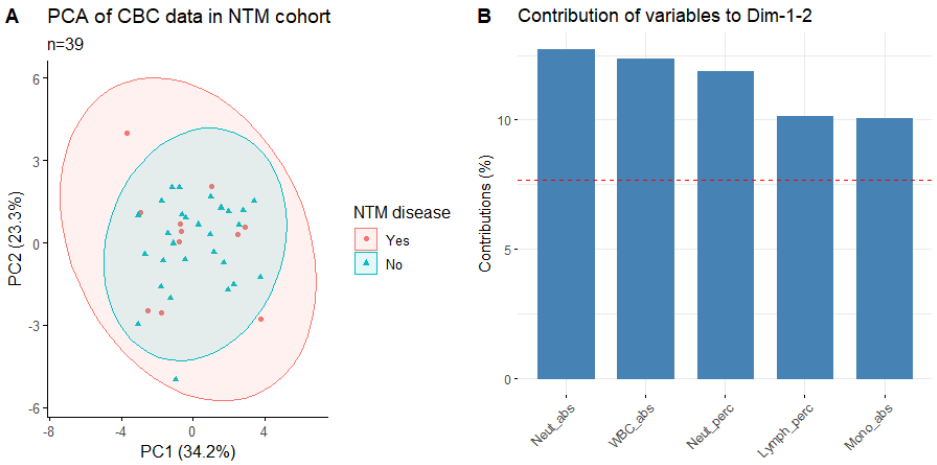
Initial quality control of raw sequencing reads did not show significant bias in nucleotide distribution, sequencing depth or base calling quality. Still, a significant proportion (68 – 93% per sample) of reads were overrepresented in whole blood human RNA; as expected, these sequences resembled hemoglobin genes in a nucleotide Basic Local Alignment Search Tool. Following alignment, Picard tools quality control for RNAseq showed a minimum alignment per sample of 94.6% with at least 49.1% aligning to proteincoding regions, see **Appendix B.1.**

Figure 3-3. Absolute whole blood cell population counts in samples used for RNAseq



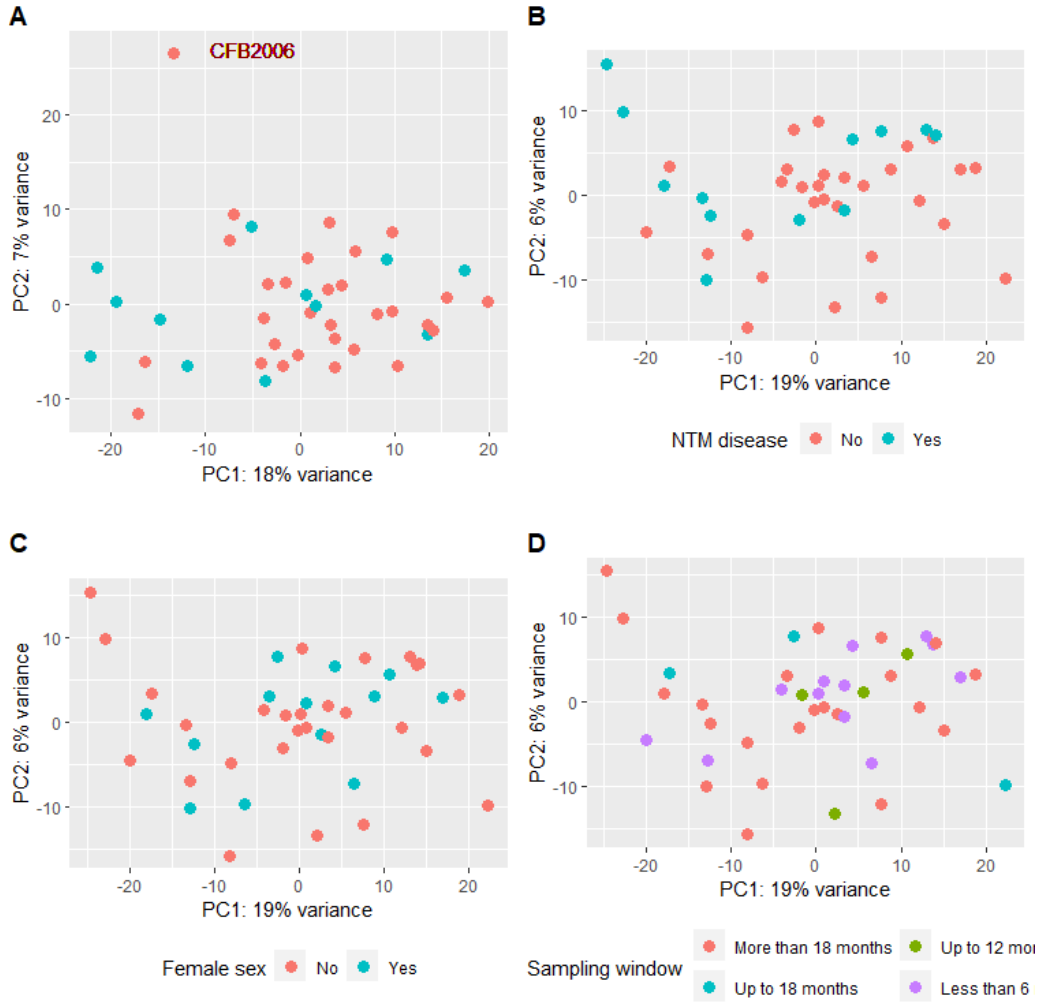
Boxplots with the distribution of absolute values for leukocytes, neutrophils, lymphocytes and monocytes in complete blood counts at the time of sample procurement for RNAseq. Compared using unpaired Wilcoxon rank-sum test.

Figure 3-4. Principal component analysis of CBC values



A. PCA plot of 39 samples included in the project. PC1 explains 34.2% of the variability in the sample while PC2 explains 23.3%. No separation Is seen according to NTM-PD outcome groups. **B.** Contribution of different CBC variables to principal components 1 and 2. The dotted line represents the expected contribution by random chance.

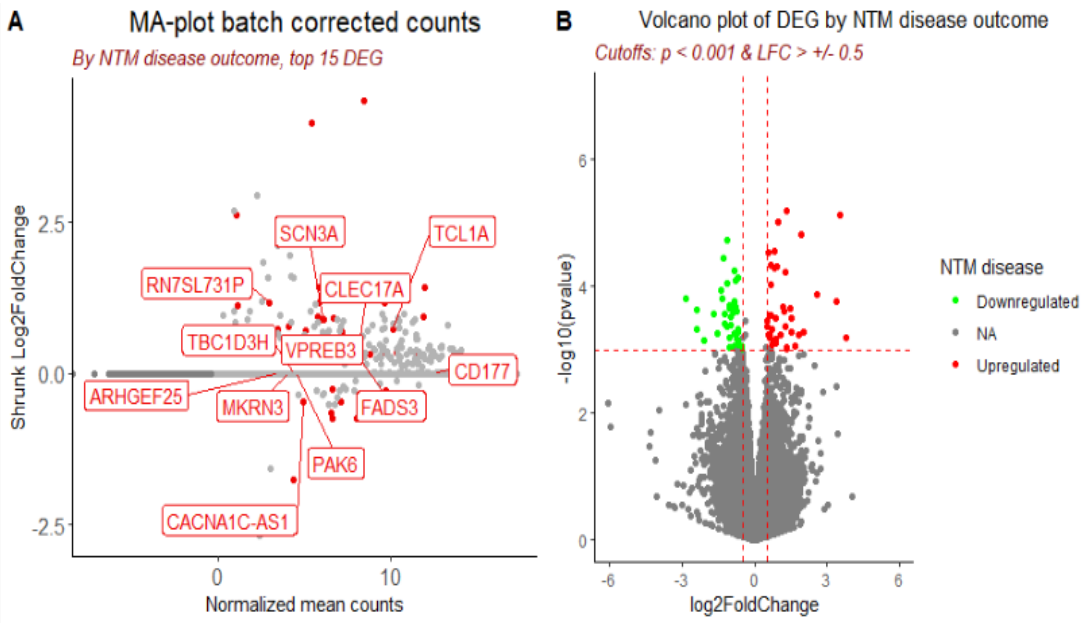
Figure 3-5. Exploratory principal component analyses of count data



PCA plots of the first two principal components using variance stabilized count data, all but the first panel depict batch corrected count data. **A.** PCA of included samples prior to batch correction showing outlier sample CFB2006. **B.** PCA according to NTM-PD outcome. **C.** PCA according to biological sex. and **D.** PCA according to the time interval between first NTM growth and whole blood sample collection.

During exploratory principal component analyses (PCA) of batch corrected and variance stabilized gene counts, we found no particular grouping by biological sex, mycobacterial species, or interval between blood sampling and first NTM growth. **Figure 3-5** shows the results of these exploratory PCA, **Figure 3-5-A** shows the location of the outlier (CFB2006).

Figure 3-6. Volcano plot and MA plot of DESeq2 differential expression analysis



A. Bland-Altman plot of normalized mean counts and shrunk (apeglm) Log2 Fold-changes. The top 15 differentially expressed genes (DEG) are labeled, remaining DEG are colored red. **B.** Volcano plot of differential gene expression analysis with cut-offs for significance of p< 0.001 and Log2 Fold-Change higher than absolute 0.5. Highlighted in red are genes with higher mean expression in patients who progressed to NTM-PD.

Differential gene expression with DESeq2 identified 111 differentially expressed genes (DEG) at an FDR cut-off of < 0.3. The top 30 differentially expressed genes are summarized in **Table 3-4** in descending order of adjusted p-value. The Bland–Altman plot (**Figure 3-6-A)** shows no outliers in shrunk Log2 Fold-change estimates according to mean expression. The volcano plot in **Figure 3-6-B** summarizes genes with p values < 0.001 and absolute Log2 Fold-change > 0.5.

Table 3-4. Top 30 differentially expressed genes by progression to NTM outcome in DESeq2 analysis

SYMBOL	padj	stat	DESCRIPTION
TBC1D3H	0.000	-10.849	TBC1 domain family member 3H
TCL1A	0.054	4.426	TCL1 family AKT coactivator A
CD177	0.054	4.477	CD177 molecule
NA	0.054	4.504	novel transcript, antisense to TCL1A
RN7SL731P	0.069	4.322	RNA, 7SL, cytoplasmic 731, pseudogene
ARHGEF25	0.070	-4.279	Rho guanine nucleotide exchange factor 25
VPREB3	0.084	4.187	V-set pre-B cell surrogate light chain 3
FADS3	0.084	4.174	fatty acid desaturase 3
MKRN3	0.089	-4.133	makorin ring finger protein 3
SCN3A	0.095	4.053	sodium voltage-gated channel alpha subunit 3
CLEC17A	0.095	4.067	C-type lectin domain containing 17A
NA	0.095	4.063	novel transcript, antisense to HS3ST1
PAK6	0.095	-4.023	p21 (RAC1) activated kinase 6
NA	0.095	4.016	POM121 membrane glycoprotein-like 1 pseudogene
CACNA1C-AS1	0.109	-3.967	CACNA1C antisense RNA 1
CTSW	0.117	-3.934	cathepsin W
NA	0.117	-3.919	novel transcript
NIBAN3	0.121	3.897	niban apoptosis regulator 3
DDX11L2	0.136	-3.856	DEAD/H-box helicase 11 like 2 (pseudogene)
RPL13P12	0.150	3.820	ribosomal protein L13 pseudogene 12
RBPMS2	0.158	-3.784	RNA binding protein, mRNA processing factor 2
NA	0.158	-3.783	novel transcript
RIMBP2	0.163	3.753	RIMS binding protein 2
NA	0.163	-3.753	zinc finger protein 726 pseudogene 1
GZMK	0.176	-3.660	granzyme K
KLRC1	0.176	-3.653	killer cell lectin like receptor C1
CLDN12	0.176	-3.698	claudin 12
CMBL	0.176	3.687	carboxymethylenebutenolidase homolog
KRT72	0.176	-3.720	keratin 72
SERF1A	0.176	3.709	small EDRK-rich factor 1A

The top differentially expressed genes did not share an easily identifiable function in gene ontology enrichment. Thus, we performed gene set enrichment analysis to infer the more relevant biological pathways in our results. The Hallmark pathways from the molecular signatures database were used to minimize the redundancy observed in other dictionaries. Overall, we found a positive significant enrichment (higher in NTM-PD outcome) for several pathways of immune function, including Interferon-α response (Adjusted p = 2.5 e $^{-09}$), Interferon-γ response (Adjusted p = 2.5 e^{-09}) and IL6 - JAK - STAT3 signaling (Adjusted p = 1.88 e^{-05}). **Table 3-5** includes all enriched pathways with an adjusted p-value below 0.001.

Table 3-5. Pathways enriched with adjusted p value <0.001

Pathway	p.value	Adjusted p	NES	Size
Interferon Alpha Response	1.00 e-10	2.50 e-09	2.98	96
Interferon Gamma Response	1.00 e-10	2.50 e-09	2.65	199
IL-6 JAK STAT3 signaling	1.51 e-06	1.88 e-05	2.05	81
Heme metabolism	4.47 e-10	7.46 e-09	2.04	188
Tumor Necrosis Factor-α signaling via NFK-β	2.29 e-06	2.29 e-05	1.74	198
Inflammatory response	2.58 e-05	2.15 e-04	1.66	194
Protein secretion	6.50 e-03	3.61 e-02	1.53	86
Oxidative phosphorylation	5.18 e-04	3.24 e-03	1.51	188
E2F targets	1.03 e-04	7.37 e-04	-1.60	193

3.3.3 Complementary analyses

To corroborate that the observed CBC counts represented the cell composition in the RNA-sequencing experiment, we used cibersortX deconvolution to impute the immune blood cell proportions using a reference signature matrix containing 22 different blood cell types. A high level of agreement, calculated by Pearson correlation coefficient (> 0.8), was observed for

lymphocytes and neutrophils. A moderate correlation (R = 0.4) was observed for monocytes, **Figure 3-7**.

The mean expression of genes previously associated with NTM-PD in the non-CF population was also explored. None of these reported genes were differentially expressed in our DESeq2 analysis. We also evaluated trends in their expression according to our NTM outcomes. As seen in **Figure 3-8** no significant differences were observed in expression values for any gene according to outcome groups (Wilcoxon rank-sum test for unpaired samples). No differences were observed if we divide our sample into three different NTM categories, transient growth, persistent growth and NTM-PD (data not shown). The remaining candidate genes can be seen in **Appendix B.2.**

Figure 3-7. CibersortX deconvolution of cell percentages vs. ground truth reference values in CBC

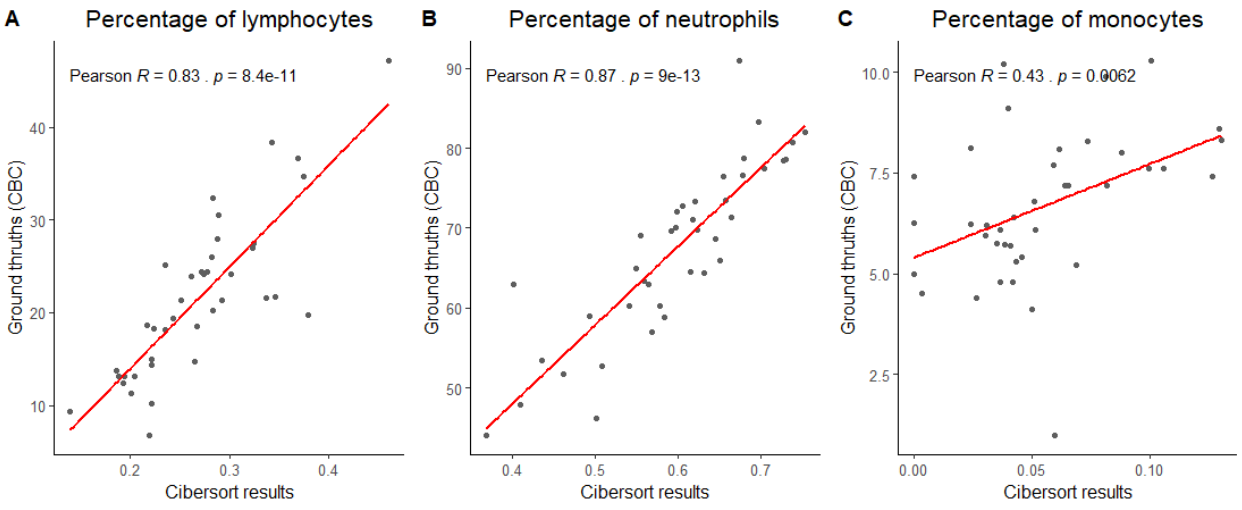
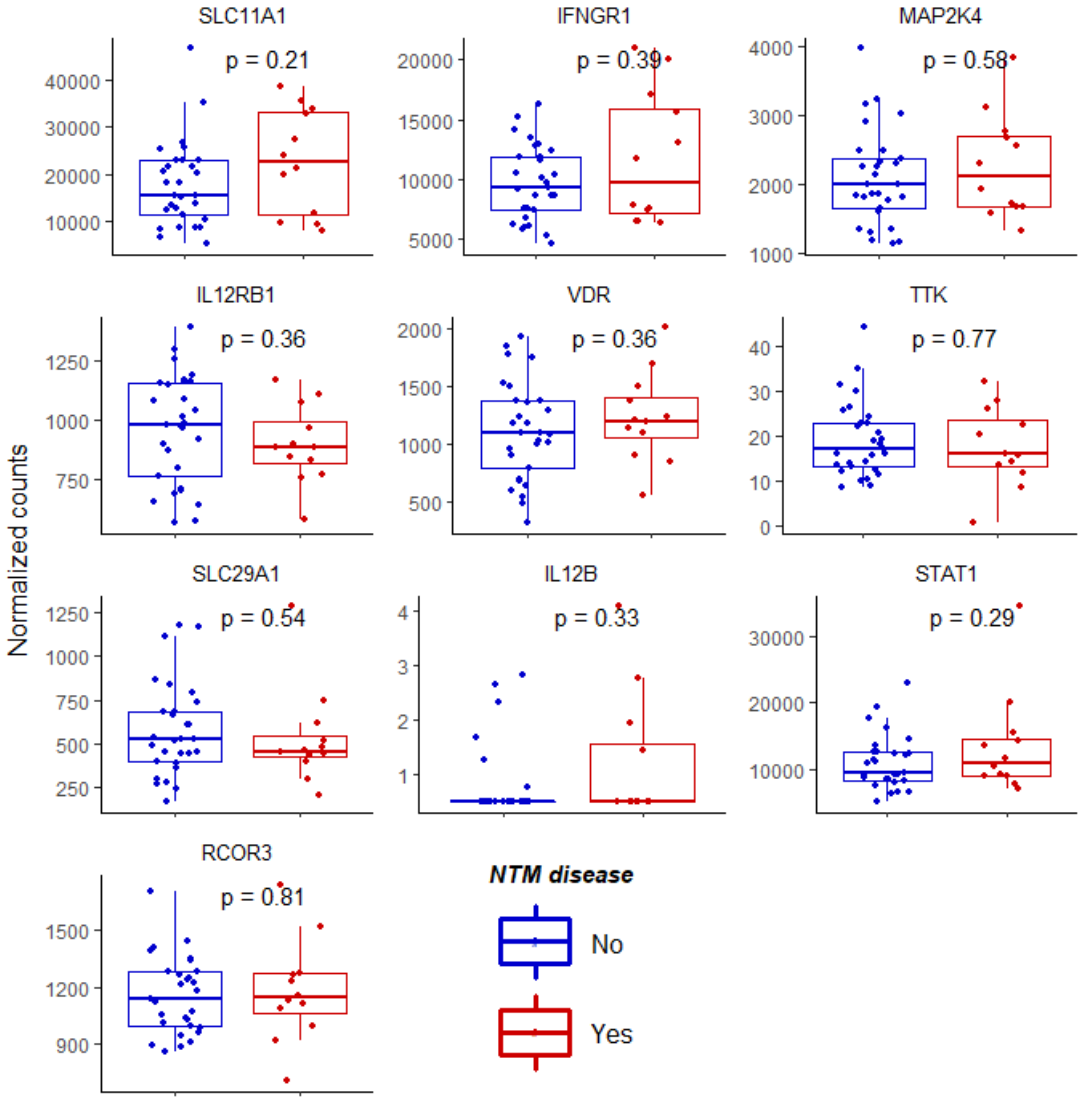


Figure 3-8. Mean expression values per NTM outcome group in selected genes previously reported as associated with NTM susceptibility in non-CF populations



3.4 Discussion

Based on prior literature evaluating NTM-PD in the non-CF population, we hypothesized that a lower expression of genes involved in lymphocyte and monocyte responses is associated with NTM-PD in CF patients. Thus, we evaluated whole blood RNA expression in a cohort of patients

with positive growth for NTM, using a sample collected close to the time of first isolation. Our results show, based on gene set enrichment analysis, that interferon and tumor necrosis factor-α responses, as well as the IL6-JAK-STAT3 pathway, were positively enriched in participants who went on to develop NTM-PD. These results are opposite to the findings of Cowman et al., the only transcriptomics study published so far, which had significantly lower expression of genes related to Interferon-γ production and lymphocyte activation in the NTM-PD group (217). Other studies in the non-CF population describe similar results to Cowman *et al.* (244–246). Interestingly, genes associated with NTM infection and NTM-PD in previous studies (non-CF populations) were not differentially expressed in our cohort, **Table 3-1** (216,218,220,221,247). Our exploratory results provide preliminary evidence for biomarkers of NTM-PD in CF (248). In contrast to the results of non-CF studies, an exaggerated pro-inflammatory response may be more in line with the baseline inflammation that characterizes the CF lung microenvironment, and it could be exacerbated by particularly virulent or more abundant bacteria.

Phagocytic cells, particularly macrophages, are the main responders against mycobacterial infection (249,250). No significant differences were apparent in monocyte or neutrophil counts between NTM-PD outcome groups using CBC data at baseline. Yet, the concordance of monocyte CBC counts with deconvolution was not high ($r = 0.43$) (243). The enrichment results showed an increased inflammatory response in pathways that are associated with phagocyte responses. The detailed composition of our whole blood could not be sorted in our bulk-RNAseq analysis. However, based on CBC counts, we infer that functional and not quantitative differences in phagocytes may be playing a role in susceptibility to NTM. The next step in the discovery pipeline

will be to use single-cell RNA-sequencing to evaluate changes in our enriched pathways in particular cell populations.

NTM-PD has been associated with multiple immune deficits. Monogenic susceptibility to NTM-PD is associated with loss of function mutations in the Interferon Gamma Receptor 1, Interleukin-12 subunits and STAT1 (245). However, it is more likely that small but coordinated changes are promoting susceptibility to NTM in the CF population. Particularly because the CF lung suffers from sustained inflammation that favors tissue damage with minimal microbicidal control. Overall, reports in the non-CF population represent the loss of protective mechanisms against intracellular pathogens (217). However, the CF pathology already predisposes to bacterial infections and other factors may better separate those at higher risk of NTM-PD. The increased pro-inflammatory state observed in our results could be due to a higher tissue abundance of NTM or the presence of more virulent strains in those that developed NTM-PD. As shown in animal models, CFTR dysfunction already decreases mycobacterial killing and the exaggerated response in our analysis may be a futile attempt to eliminate the NTM (251,252). The role of changes in virulence in infecting NTM populations could explain the appearance of delayed exaggerated immune responses. To verify this, a prospective evaluation of isolates is necessary (81). Finally, the dynamics of the lung microbiome may play a role in the virulence and proliferation of NTM and could contribute to the pro-inflammatory gene expression we observed (215).

Our study had divergent intervals between whole blood sampling, first NTM growth and development of NTM-PD. A subset of patients was sampled before growing the bacteria and half of those who developed NTM-PD were tested after diagnosis. Thus, the temporal relationship

between exposure to NTM and gene expression cannot be established (**Appendix B.3**). We did not anticipate this as our initial hypothesis was that sustained impaired immune responses, similar to the non-CF population, were related to NTM-PD. As an initial study, we have a small sample size, and further validation requires higher numbers. No other transcriptomics subsets were available for validation, so another cohort is the only option for external validation (10). We also explored CT findings at baseline as possible candidate biomarkers. However, the results were not informative as a large proportion of our cohort had no radiologic data at baseline. Looking forward, the prospective recruitment of CF patients will limit the variability in window sampling seen in our secondary data analysis (248). As other microbial colonizers could also be playing a role in the CF lung microenvironment, the microbial dynamics should also be evaluated (251). Finally, future studies must include a baseline reference gene expression by sampling participants before NTM growth or evaluating a group of CF patients without NTM infection.

Nevertheless, our most significant contribution is the narrowing of differential gene expression to pathways involved in pro-inflammatory innate immune responses. Novel studies will benefit from focusing efforts on these pathways and can use our cohort for sample size calculation and external validation.

Chapter 4: Conclusion

4.1 Overall summary and main results

In this thesis, we examined the burden of NTM in the CF population and explored the role of host responses in the progression towards pulmonary NTM disease (NTM-PD) in infected patients.

In our second chapter, we used a systematic review approach and included all available data regarding the prevalence and incidence of different outcomes related to NTM infection in the CF population. Prior studies focused on single-center or national data using registry reports. With our approach, we provide the most accurate estimate of the burden of NTM infection and NTM-PD in the cystic fibrosis population, not limited to countries where registries are available. Furthermore, we identified that differences in sample sizes, years of data collection and geographical region may affect the estimates of NTM infection prevalence; although other sources of heterogeneity cannot be ruled out due to missing data. Our results summarize the most updated information about NTM infection and NTM-PD in the CF population. The results of this project will inform future research priorities in the cystic fibrosis community. Finally, they will also serve as reference material for historical comparison of interventions and the design of novel studies.

In our third chapter, we began the discovery pipeline for biomarkers of progression to NTM-PD in CF. Most reported studies using human samples were conducted in the general population or populations with structural lung damage, but never in a CF population. As an exploratory study, we aimed to explore what genes or functional pathways may be driving the differential progression of patients after NTM infection. At conception, we expected a similar result to what was reported

in non-CF populations. However, we found that in stark contrast to other reports, patients with worse outcomes had positive enrichment of inflammatory pathways, particularly those of the innate immune response. Our results will help other research groups to conduct validation studies, calculate sample size, and design their biomarker discovery studies.

4.2 Limitations and future steps

In our systematic review, we were not able to conduct statistical pooling for several outcomes. The period prevalence was not considered for pooling as the variable length of follow-up in studies is an inherent bias for prevalent measures. Furthermore, the number of retrieved studies for incidence in general and measures of NTM-PD was insufficient for pooling. This information was summarized narratively and in tables. Our results highlight the lack of available data for the risk of progression to NTM-PD and incidence in general; only prospective trials can help us elucidate these conundrums. Furthermore, among included studies, the amount of missing data prevented us from exploring other causes of heterogeneity beyond the ones reported in the results. Particularly, the lack of adequate reporting of microbial identification methods and screening approaches are important determinants of epidemiological outcomes in infectious diseases and could affect the generalizability of results. As the most important sources of clinical and epidemiological data for the CF community, we encourage registry managers to facilitate access to methodological data not readily available in reports and to harmonize reporting standards globally.

Our RNAseq results should be considered pilot in nature and hypothesis-generating, thus requiring validation. In particular, the sampling window between whole blood acquisition and first NTM growth was variable and dependent on the sampling scheme of the primary study. As our initial

hypothesis was that diminished gene expression in immune response pathways would be associated with NTM-PD (as observed in the non-CF populations) and related to intrinsic genetic factors of the host, we did not anticipate that timing of blood sampling would be critical. However, we observed an exacerbated immune response which might be explained by other factors. Also, as there are no other datasets for validation, our results must be validated in a prospective cohort that evaluates the dynamics of our pathways of interest through the development of NTM-PD. Finally, in our bulk RNAseq results, we cannot pinpoint the cellular populations that drive the differences in gene expression. Future studies will benefit from using single-cell RNAseq to generate novel hypotheses about the responsible cell population(s).

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Appendices

Appendix A - Complementary methods and tables for systematic review chapter

A.1 Systematic review search strategies

Database: OVID Inc. MEDLINE(R) and Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Daily and Versions(R)
Search Strategy:

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1 exp Cystic Fibrosis/ (35441)
2 (cystic adj3 fibrosis).mp. (51651)
3 CFTR.mp. (11167)
4 or/1-3 (52236)
5 exp Nontuberculous Mycobacteria/ (11647)
6 exp Mycobacterium Infections, Nontuberculous/ (35039)
7 ((abscessus or avium or atypic* or gordonae or kansasii) adj5 mycobacteri*).mp. (16561)
8 (non*tuberculosis or non*tuberculous or NTM or "mycobacteria other than tuberculosis" or MOTT).mp. (14149)
9 or/5-8 (52341)
10 4 and 9 (576)

Database: Embase
Search Strategy:

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1 cystic fibrosis/ (71509)
2 (cystic adj3 fibrosis).mp. (84692)
3 CFTR.mp. (17470)
4 or/1-2 (84692)
5 atypical mycobacteria/ (4439)
6 atypical mycobacteriosis/ (5361)
7 ((abscessus or avium or atypic* or gordonae or kansasii) adj3 mycobacteri*).mp. (24844)
8 (non*tuberculosis or non*tuberculous or NTM or "mycobacteria other than tuberculosis" or MOTT).mp. (9445)
9 or/5-8 (29259)
10 4 and 9 (1427)

A.2 Grey literature sources

Additional sources of grey literature reviewed

Resource	URL
Canadian Institute for Health Information (CIHI). Quick Stats	https://www.cihi.ca/en/quick-stats
IQVIA	https://www.iqvia.com/
Institute for Clinical Evaluative Sciences (ICES). Publications	http://www.ices.on.ca/Publications.aspx
Institute of Health Economics (IHE). Database of Online Health Statistics	http://www.ihe.ca/health-statistics-database)
New Brunswick Ministry of Health, Office of the Chief Medical Officer of Health. Epidemiology and Surveillance	http://www2.gnb.ca/content/gnb/en/departments/ocmoh/epidemiology_surveillance.html
Public Health Agency of Canada (PHAC). Public Health Infobase	http://infobase.phac-aspc.gc.ca/index-en.html
Statistics Canada. Diseases and physical health conditions.	https://www150.statcan.gc.ca/n1/en/subjects/health/diseases_and_physical_health_conditions
Center for Disease Control (USA). National center for health statistics.	https://www.cdc.gov/nchs/
The Organisation for Economic Co-operation and Development	https://data.oecd.org/
World Health Organization – Global health observatory.	https://www.who.int/data/gho
BMC proceedings	https://bmcproc.biomedcentral.com/)

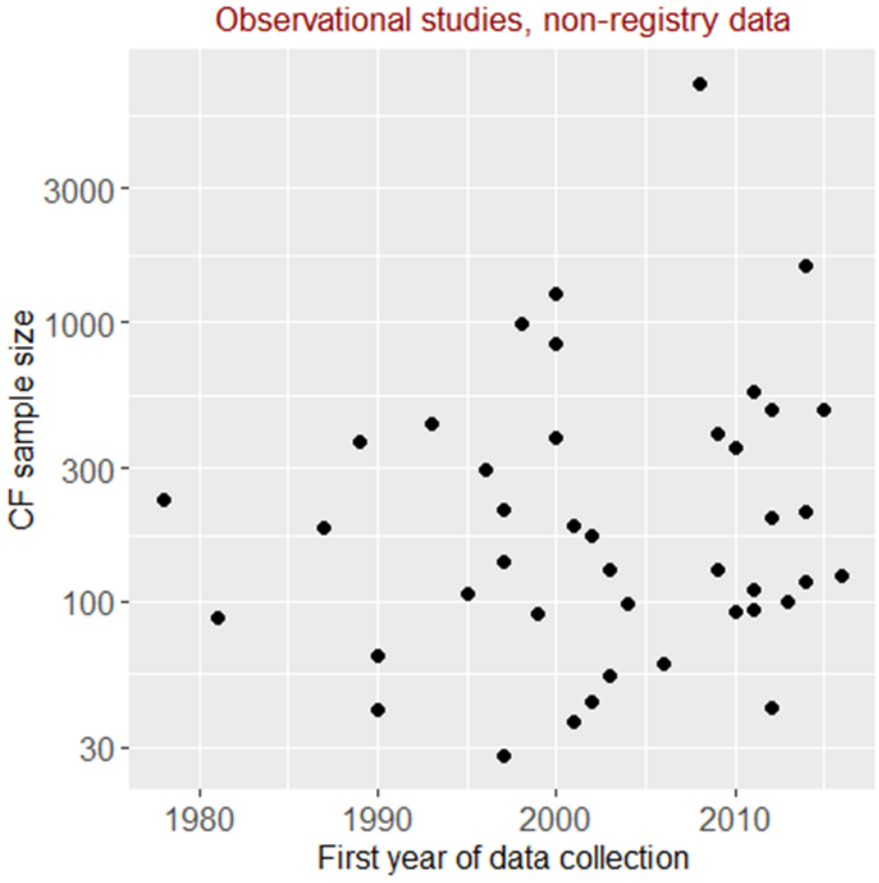
DOI of articles used in forward and backward reference search	
DOI: 10.1164/rccm.200207-678OC	DOI: 10.1097/MCP.0b013e328365ab33
DOI: 10.1128/CMR.00068-09	DOI: 10.1002/ppul.23825
DOI: 10.1164/ajrccm/147.5.1271	DOI: 10.1128/AAC.00861-10
DOI: 10.1136/thoraxjnl-2015-207360	DOI: 10.1164/ajrccm.185.2.231
DOI: 10.1002/ppul.24913	DOI: 10.1128/JCM.01257-09
DOI: 10.1513/AnnalsATS.201709-727OC	DOI: 10.1016/j.jcf.2007.06.006
DOI: 10.1378/chest.126.2.566	DOI: 10.3201/eid1403.061405
DOI: 10.1164/rccm.200604-571ST	DOI: 10.1378/chest.102.1.70
DOI: 10.1126/science.aaf8156	DOI: 10.1136/thoraxjnl-2017-210927
DOI: 10.1016/j.jcf.2009.12.001	

A.3 Data dictionary for extraction in systematic review

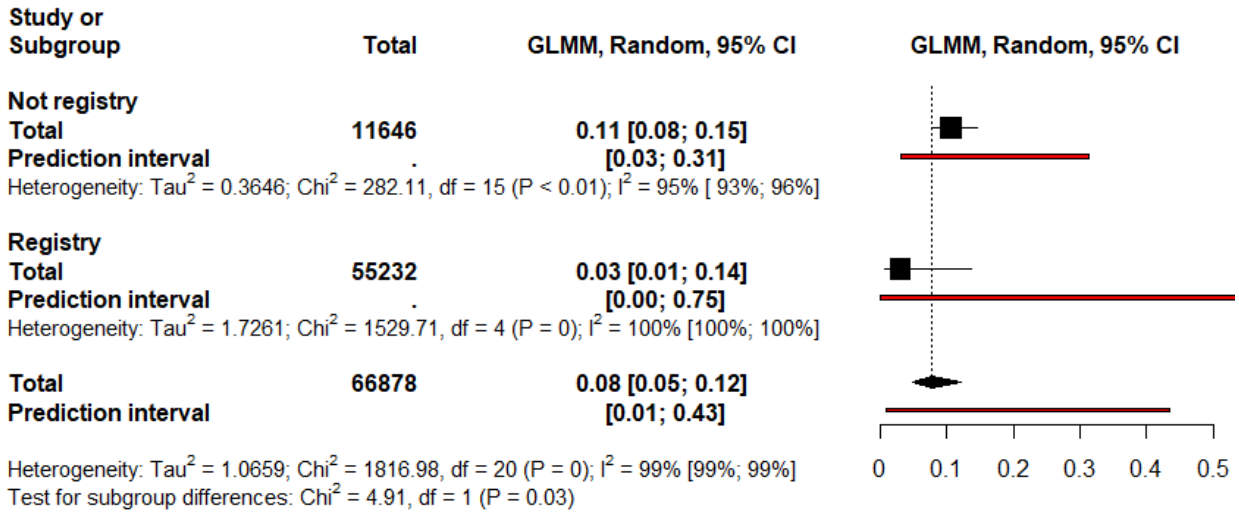
variable_name	Question
id	First author surname + year of publication
title	Full title of the study/report
publication_date	Annotate the year of publication of the primary report
study_design	Which study design was used? (cross-sectional, cohort study, registry, etc.)
eligibility	Which inclusion and exclusion criteria were used/reported in the study?
sample_size	How many individuals were included in each group?
reference_population	Which was the sampling frame for recruitment?
region	Continent where the study was conducted
country	Country(ies) where the study population was recruited
study_funding	What is the source of funding for the study?
study_aims	What is the explicit aim of the manuscript? As reported by the authors, even if it is not aimed at prevalence/incidence
conflicts_interest	Are there perceived or reported conflicts of interests?
cf_definition	What is the criteria for definition of cystic fibrosis used in the study?
age	What is the age distribution among included participants? (only those tested for NTM)
females	What is the distribution of females among included participants? (only those tested for NTM)
ethnicity	What is the ethnicity of included participants? (only those tested for NTM)
lung_function	What is the distribution of lung function measures in the study?
genotype	What is the distribution of CF genotype among included participants?
bmi	What is the distribution of body mass index in participants tested for NTM?
testing_freq	What is the reported testing frequency for NTM in the study?
infection_definition	How was pulmonary NTM infection defined?
disease_definition	How was pulmonary NTM disease defined?
ntm_specimen	Which sample(s) type were used to test for NTM? (sputum, saliva...)
ntm_technique	What type of decontamination technique was used prior to NTM detection?
ntm_molecular	What molecular method was used to detect NTM?
ntm_culture	What type of media and technique was used to culture the NTM?
ntm_speciation	How was the species of infecting NTM identified?
mabc_distribution	What is the distribution of Mycobacterium abscessus complex bacteria in the study population?
avium_distribution	What is the distribution of Mycobacterium avium complex bacteria in the study population?
ntm_other_distribution	What is the distribution of NTM species in the study (M. avium, M. abscessus, M. gordonae, etc.)
point_infection	What is the reported **point prevalence** for NTM infection?
point_disease	What is the reported **point prevalence** for **NTM-PD?**
year_point	In which year was the point prevalence calculated?
period_infection	What is the reported **period prevalence** of **NTM infection?**
period_infdisease	What is the reported **period prevalence** of **NTM-PD?**
period_years	In which years was the period prevalence calculated?

incidence_calculation	Briefly describe how the estimate of incidence was calculated.
incidence_rate	Incidence reported as a rate (longitudinal studies): number of cases over the adjusted follow-up period
incidence_proportion	Incidence reported as **new cases of NTM-PD** during a period of follow up over the at risk patients
fac_corticosteroid	Percentage of the population at risk and those with positive NTM infection/disease that are in corticosteroid therapy
fac_aspergillus	Percentage of patients with presence of *Aspergillus spp.* in respiratory cultures at time of NTM positivity
fac_ABPA_diagnosis	Percentage of the population at risk and those with positive NTM infection/disease that have an ABPA diagnosis
fac_macrolide	Percentage of the population at risk and those with positive NTM infection/disease that are receiving macrolides

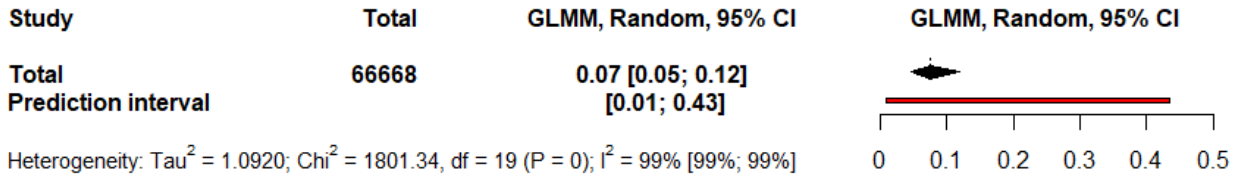
A.4 Scatter plot of sample size and first year of data collection in non-registry studies



A.5 Exploratory subgroup analysis in NTM infection meta-analysis comparing registry and not-registry data

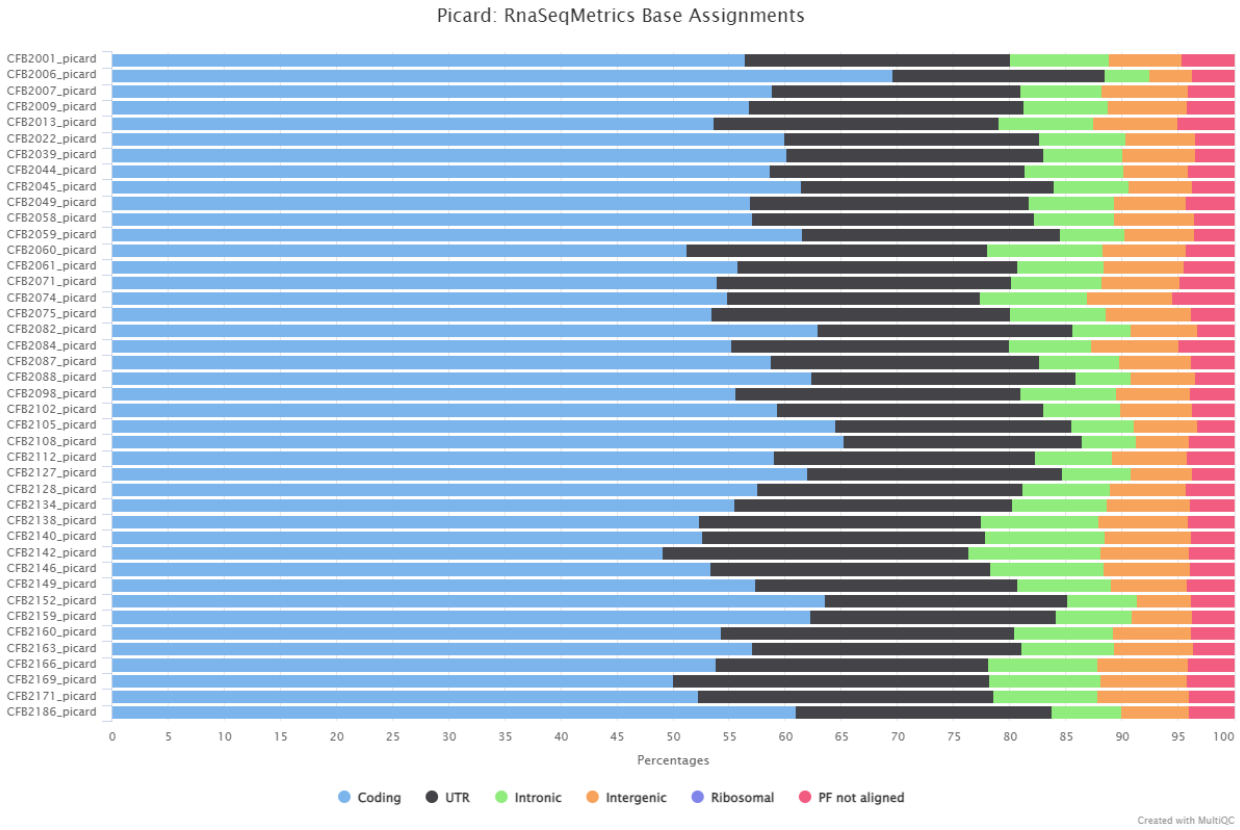


A.6 Meta-analysis of NTM infection point (and annual) prevalence excluding Preece 2016

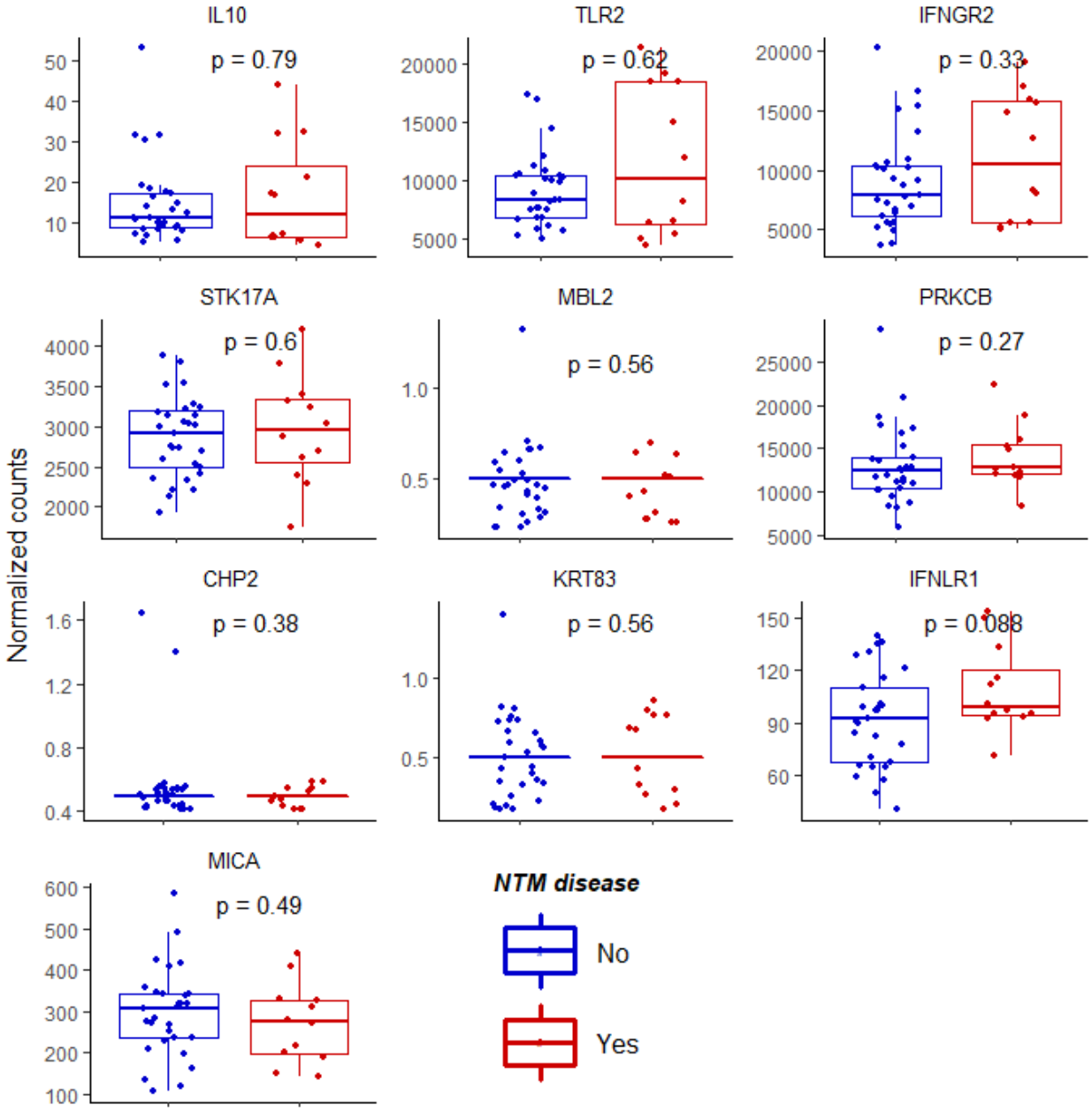


Appendix B - Supplementary material for biomarker discovery study

B.1 Picard tools assignment of reads to genomic regions



B.2 Expression values of previously described candidate genes according to three groups of NTM outcomes



B.3 Principal component analysis showing grouping by interval between RNA sample and NTM-PD (PermANOVA p=0.003)