Nontuberculous mycobacteria: new insights in epidemiology and clinical relevance

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Nontuberculous mycobacteria: new insights in epidemiology and clinical relevance

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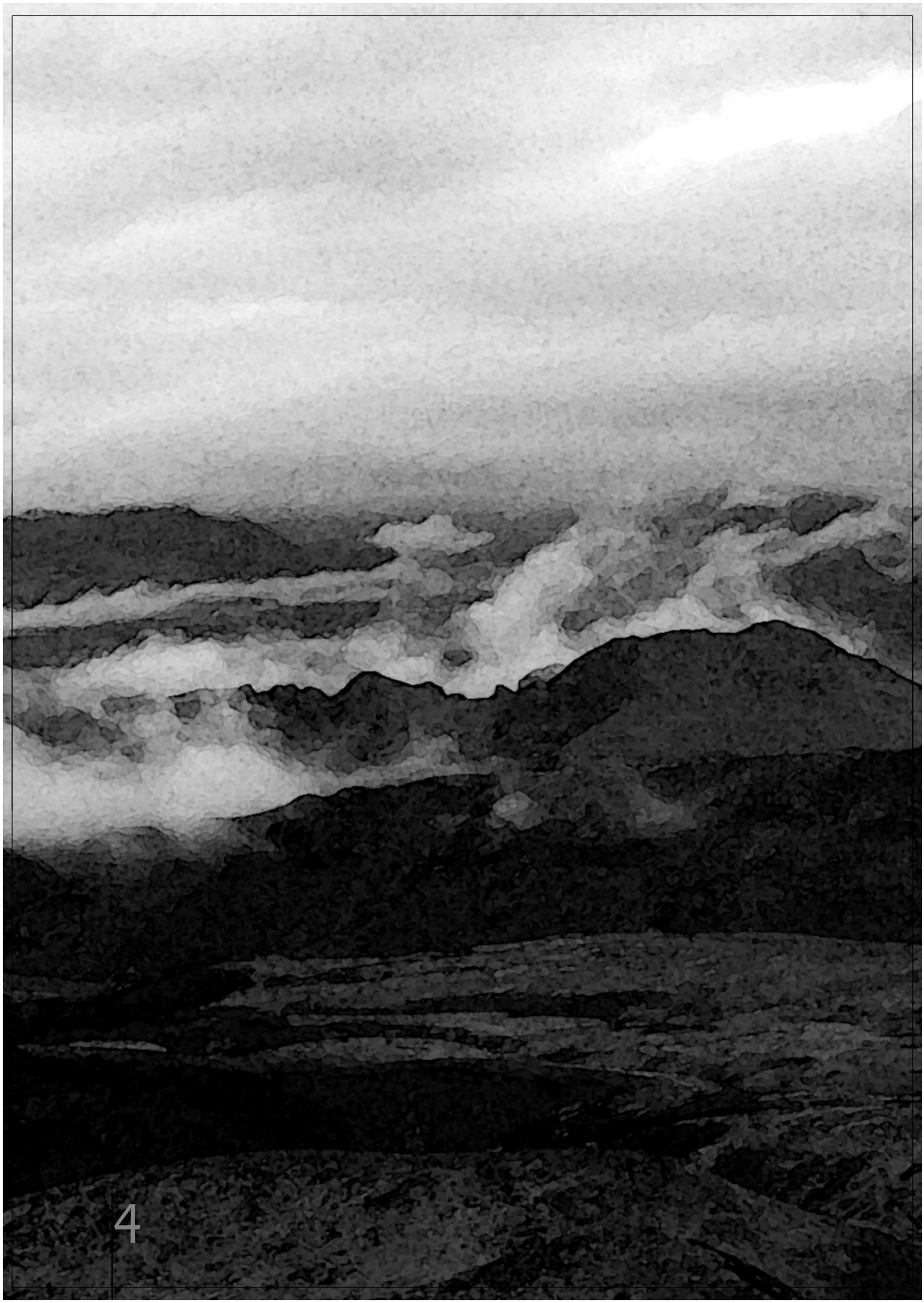


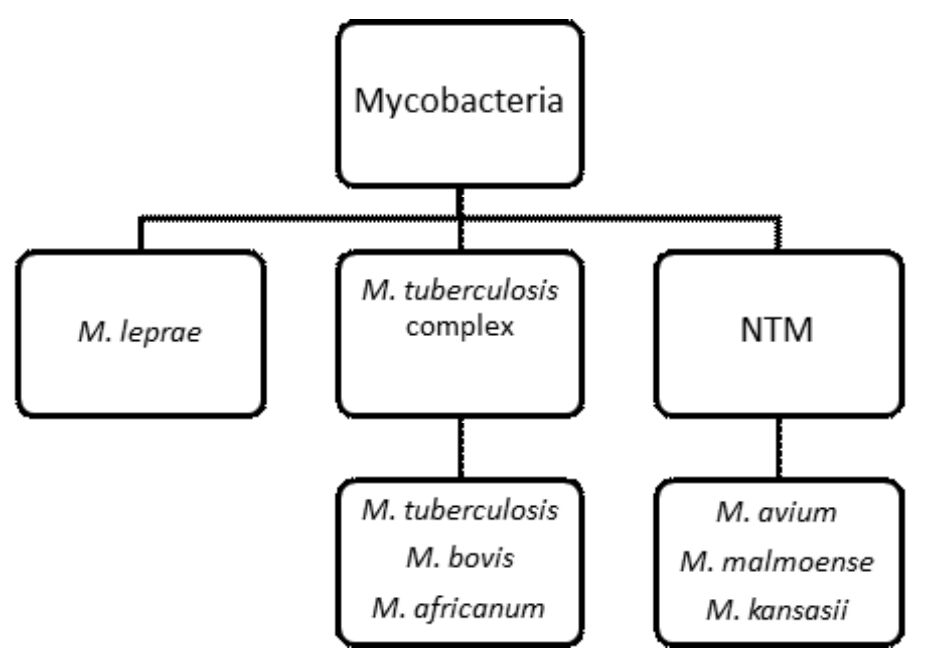
Chapter 1

General introduction

Mycobacteria

The genus *Mycobacterium can* be divided in three groups; *Mycobacterium tuberculosis* complex, *M. leprae* and the nontuberculous mycobacteria (NTM) (Figure 1). *M. tuberculosis* complex and *M. leprae* cause disease (tuberculosis and leprosy) in healthy immunocompetent individuals; NTM are opportunistic pathogens.[1] NTM are also known as environmental mycobacteria due to their widespread presence in, for example, water and soil. NTM disease most commonly presents at pulmonary sites in humans. Chronic obstructive pulmonary disease (COPD) and bronchiectasis are important pre-existing conditions and known risk factors for NTM pulmonary disease.[2-5] NTM are also known to cause disseminated disease in immunocompromised hosts, including patients with HIV, haematological malignancies, solid organ transplants, and those with inherited immune disorders.[2] Human transmission of NTM has never been proven. This is an important epidemiological difference compared to mycobacteria belonging to the *Mycobacterium tuberculosis* complex. The total number of identified and officially recognised NTM species is increasing. This increase is probably mainly due to improved laboratory techniques, especially molecular identification techniques. In 2008, 128 different species had been published (http://www.bacterio.cict.fr/m/mycobacterium.html). Improved knowledge of the divergence among the NTM species is important to improve understanding of the epidemiology and pathogenesis of disease caused by NTM.

Figure 1: Taxonomy of mycobacteria; 128 different NTM were validly published by Euzeby's in 2008. NTM = nontuberculous mycobacteria. The *M. tuberculosis* complex furthermore comprises *M. canetti, M. orygis*, *M. bovis* BCG, *M. pinnipedii, M. mungi*, and *M. microti*



Mycobacteria: from bacteriology to clinical disease

NTM have been recognized as different mycobacteria not long after Robert Koch discovered *Mycobacterium tuberculosis* in 1882. Mycobacteria isolated from the environment, but also from animals were indicated as atypical *M. tuberculosis.* These atypical, or anonymous mycobacteria were divided by Runyon, in 1959, in four groups based on their pigmentation after light exposition and grow rate: (1) photochromogens, i.e. those producing a pigment of exposition to light, slow growing; (2) scotochromogens, i.e. those producing pigment irrespective of light exposure, slow growing; (3) non-chromogens, i.e. those not producing pigments, slow growing and (4) rapidly growing mycobacteria (i.e. growth in <7 days from subculture).[6] After the discovery of NTM, it took almost 50 years until the first large case series were published related to pulmonary disease caused by these mycobacteria.[7-12] Buhler and Polack identified a 'yellow bacillus' that was capable of causing tuberculosis-like disease in humans in the 1950s.[7] This bacillus produced a yellow pigment after exposure to light, hence its name. This *Mycobacterium* is now known as *M. kansasii*. Other NTM were identified, based on differences in their phenotypic properties and growth rates that were capable of causing chronic pulmonary disease. It became clear that NTM disease showed some important epidemiological differences with tuberculosis. In a retrospective case-control study by Kamat 1961,[9] 57 patients with NTM isolates were compared with 57 tuberculosis patients. Miners were significantly more represented in the NTM group than in the tuberculosis group. Also the NTM patients had significantly more pneumoconiosis on chest X-ray examination.
Furthermore, patients with NTM were found to be of older age and more common of the Caucasian race compared to tuberculosis patients. In a large retrospective epidemiologic survey, NTM pulmonary disease was found more commonly among the low socio-economic stratum.[13] Although not systematically recorded, Kamat reported chronic bronchitis and emphysema as commonly mentioned in the medical files of the NTM group. Pneumoconiosis and chronic obstructive pulmonary disease (COPD) among middle aged male patients were from then consistently reported in case series. Wolinsky described the average NTM case to be a middle-aged white man with long standing lung disease, such as COPD or silicosis, who presents with a 3-month history of productive cough, night sweats, moderate weight loss, low grade fever, and a chest roentgenogram with upper-lobe cavitation (Figure 2A).[1]

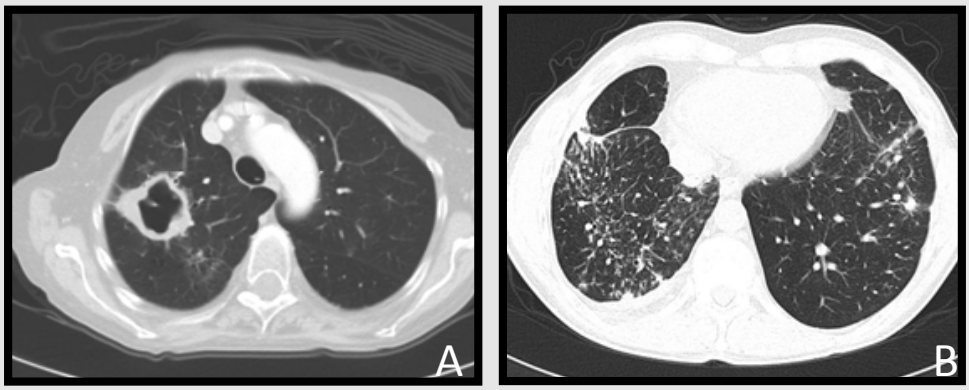
Pre-existing lung disease including COPD and bronchiectasis are still associated with NTM pulmonary disease.[2-5] NTM disease in patients with pre-existing pulmonary disease, especially COPD, is likely the most frequent NTM disease entity worldwide. However, little is known about the pathologic background of COPD –or other underlying lung diseases- and the association with NTM pulmonary disease. For example, there is no evidence of an influence of NTM isolation/disease on COPD exacerbations or disease progression.

In the late 1980s a case series was published describing 21 patients, of whom 17 were females, with *M. avium* complex (MAC) lung disease without any apparent underlying (pulmonary) disease.[14] Bronchiectasis and nodular abnormalities, especially affecting the lingula or middle lob, were the radiographic abnormalities seen in this patient group (Figure 2B). Reich and Johnson[15] later described the same category of patients and hypothesized that these women could have had the habit of voluntary suppression of cough, responsible for the inability to clear the secretions from the lingula and middle lobe. This habit results

in a focus of inflammation in these areas, which in turn predisposes to MAC infection. They named this condition Lady Windermere syndrome after Oscar Wilde's Victorian-era play *Lady Windermere's Fan* to suggest the fastidious behavior. Recently, it was found that a genetic background is more likely to be responsible based on an associated body habitus (scoliosis, pectus excavatum, and mitral valve prolaps), together with more frequently found mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) in these women.

Hypersensitivity pneumonitis is another form of pulmonary NTM disease, also called hot-tub lung since aerosols from hot-tubs containing NTM (mainly *M. avium* complex) cause an inflammatory reaction in the lung.[16] Non-necrotizing granulomas are found in lung tissue of these patients. More recently, pulmonary NTM disease emerged as a severe and possibly emerging opportunistic infection in cystic fibrosis (CF) patients. MAC and M. abscessus are the most important causative agents of NTM disease in CF patients, the latter most frequently isolated in Europe.[17-19]

Figure 2: Radiographic abnormalities seen in NTM pulmonary disease.
A: upper lobe cavitation;
B: nodular-bronchiectatic disease.



During the HIV epidemic, NTM (especially *M. avium* complex and *M. kansasii*) were increasingly recognised as causing disseminated disease , especially among patients with low CD4 counts (below 50 cells/µL).[20-21] Other recognized risk groups for NTM disseminated disease were patients with other forms of immunosuppression (caused by drugs or certain disease states, i.e. hematologic malignancies) (Table 1).
Cervical lymphadenitis caused by NTM is an important disease entity seen in children aged below 9 years of age. Lymph node involvement is a form of extra-pulmonary disease, and interestingly not associated with immunosuppressive disorders. In developed countries, NTM cervical lymphadenitis is more commonly observed than tuberculosis lymphadenitis.[22]
Other forms of localised, extra-pulmonary NTM disease are NTM infections of skin, soft tissue and bone. Skin infections after skin wounds or in tattoos are most common in which *M. marinum* and rapid growing mycobacteria are frequently the causative agents.[2, 23] In rain forest areas, *M. ulcerans* is frequently encountered as the pathogen causing serious and treatment resistant necrotic lesions.[24]

Box 1: Summary of the 2007 American Thoracic Society diagnostic criteria[2]

American Thoracic Society Diagnostic Criteria of Nontuberculous Mycobacterial Lung Disease

Clinical criteria

1. Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or an HRCT scan that shows multifocal bronchiectasis with multiple small nodules.

and

2. Appropriate exclusion of other diagnoses.

Microbiological criteria

1. Positive culture results from at least two separate expectorated sputum samples. (If the results from the initial sputum samples are nondiagnostic, consider repeat sputum AFB smears and cultures.)

Abbreviations: CT = Computed tomography, HRCT = High resolution computed tomography

Table 1: NTM disease characteristics

NTM disease location	Clinical disease	Common causative NTM	Predisposing conditions
Pulmonary	Cavitary disease	MAC, *M. malmoense, M. kansasii*	COPD
	Nodular-bronchiectatic (lingular or middle lobe predominance)	MAC, *M. abscessus*	CF Female patients
	Hypersensitivity pneumonitis	MAC, *M. immunogenum*	
Extra-pulmonary	Cervical lymphadenitis	MAC, *M. haemophilum, M. malmoense*	Children
	Skin, soft tissue and bone infections	*M. marinum, M. ulcerans, RGM*	Wounds, tattoos, trauma
Disseminated	Bone marrow, liver, blood, skin	MAC, *M. kansasii, M. genavense*, RGM (skin)	HIV, immunosuppressive drugs, malignancies

MAC = M. avium complex, COPD = chronic obstructive pulmonary disease, CF = cystic fibrosis, RGM = rapid growing mycobacteria

Epidemiology and global aspects of NTM isolation

The epidemiology of NTM disease is difficult to assess since most of the studies available are based on voluntary reporting of NTM isolation. A great number of studies reported the frequencies of NTM isolation rather than the number of patients with true NTM disease. Epidemiological data regarding the number of patients with NTM disease are expected to be lower compared to epidemiological data concerning patients from whom a NTM has been isolated (see the Diagnosis of NTM disease).
Marras and Daley[25] reviewed the literature on epidemiology of pulmonary NTM isolation and disease. They identified several studies worldwide that showed an increase in pulmonary NTM isolation and disease together with a decrease in tuberculosis incidence, a trend that started soon after the introduction of successful chemotherapy for tuberculosis. NTM disease prevalence in North America was found to vary between 0.1-2/100.000. In specific populations, such as mine workers, extremely high rates of NTM disease were found for example in the Czech republic and South Africa (up to 78/100.000). In Queensland, Australia, NTM isolation is a condition that needs to be formally notified. This gives more representative information on NTM isolation and disease for this region. In a recent study from Australia, an increase in the prevalence of clinical relevant NTM pulmonary disease was observed of 2.2/100.000 in 1999 and 3.2/100.000 in 2005.[26] In Ontario, Canada, isolation prevalence increased significantly between 1997 and 2003.[27] A recent study from the United States found a minimum prevalence rate of pulmonary NTM disease of 8.6/100.000 in the general population.[28] In elderly subjects this figure rises to a minimum of 20.4/100.000. If these figures are representative for developed countries than NTM pulmonary disease is nowadays a larger health threat than pulmonary tuberculosis. However, these figures are far above the estimated NTM disease incidence of 1.7/100.000 in the Netherlands in 2008.[29]

Isolation frequencies of different NTM species depend on the area studied.[25, 30] Although MAC is the most common NTM isolated worldwide, in some areas other NTM are the predominant NTM isolated, and after MAC, the variation in isolation frequency differs significantly. In the Czech Republic and Turkey, *M. kansasii* and *M. fortuitum* were the most predominant NTM isolated, respectively.[30] In South Africa, *M. kansasii* was observed to be predominant among mine workers.[31] Since NTM isolation and disease is increasing in many countries, together with the observation that NTM isolation can differ between regions and countries, epidemiological studies remain important to understand and monitor NTM disease. A thorough understanding of NTM isolation and disease, together with information regarding differences in geographical isolation, may enhance knowledge about risk factors associated with NTM transmission to humans and thus prevention of NTM disease.

Diagnosis of NTM disease

The diagnosis of NTM disease is a challenge. Because of the environmental nature of the NTM, isolation of NTM from normally non-sterile sources, mainly the respiratory tract, has to be related to clinical and radiographic data to distinguish true mycobacterial disease from contamination from an environmental source. This contamination likely reflects occasional presence of NTM in the airways after environmental exposure which lasts until the NTM are either neutralized by the immune system or mechanically removed by

sputum expectoration.[32 33] Contamination can also be the result of, for example, laboratory procedures or contamination of bronchoscopes via bronchoscope washers, as well as sputum contamination because of tap water mouth rinsing prior to sputum expectoration.[32 33] The distinction between contamination and true infection is important, since diagnosis of infection with an NTM-correct or incorrect- may lead to a long period of treatment with anti-mycobacterial drugs. In the 1950s, Runyon proposed criteria for diagnosis of NTM disease.[6] At that time, the widespread environmental nature of the NTM, and thus the possibility of contamination was not clearly recognized. However, criteria were established because of the observation that, in contrast to tuberculosis, NTM isolation from respiratory specimens was not always related to clinical disease. One of the first proposed diagnostic criteria that incorporated clinical data and microbiological criteria was published by Ahn and co-workers in 1982, based on a cohort of 271 patients with *M. kansasii* isolates and 226 patients with MAC.[34] It was stated that colonization is an important issue to be considered, especially in patients with pre-existent chronic respiratory diseases and multiple NTM isolates but without cavitary lesions. Airway hygiene in these patient groups (with or without a short course of anti-mycobacterial drug therapy) often showed to be sufficient to clear the NTM from the airways.

The first American Thoracic Society (ATS) statement on NTM pulmonary disease was published in 1990 and incorporated clinical criteria as well.[35] For cavitary lesions , clinical relevant NTM isolation was considered to be present when (1) two or more sputa are acid-fast bacilli smear-positive and/or result in moderate to heavy growth of NTM on culture and (2) other diseases have been excluded. In 1997, a revised ATS statement was published in which the criteria for non-cavitary isolation were deleted since the increased use of computed tomography (CT) scanning revealed that multiple NTM isolation is often related to other lesions related to NTM pulmonary disease not seen on a conventional chest radiograph (i.e. nodular-bronchiectatic lung disease).[36] Colonization of NTM was believed to be uncommon. Fibrocavitary NTM pulmonary disease on one hand, and nodular-bronchiectatic disease on the other hand (Figures 2A and 2B) are still important in the most recently ATS criteria on NTM disease published in 2007 (Box 1).[2] The bacteriologic criteria in the previous statements were not supported by scientific evidence; the microbiological criteria in the current guideline are supported by one study. In this landmark study, Michio Tsukamura demonstrated that radiologic evidence of disease (infiltrates or cavitary lesions) and progression was found in 98% of the patients who had two or more positive sputum cultures for *M. avium* complex, compared to just 2% in those with a single positive culture during 12 months of observation. For 97% of patients, the first two positive cultures grew from the initial three sputum specimens.[37] The microbiological criteria are more difficult to apply to nodular-bronchiectatic disease because not all patients produce sputum and bacterial loads in sputum are generally lower than in cavitary disease.[2]

Treatment of NTM disease

Even if a diagnosis of NTM disease is made, the decision to start treatment creates a moment of weighing several arguments. Co-morbidity and resulting drug use, alcohol abuse and severe weight loss interfere with the toxic antimycobacterial drugs that generally have to be taken for at least 18 months depending on the NTM species and type of disease.[2] In addition, because of a lack of clinical trials, doubts about clinical efficacy often prevents or

delays the initiation of antimycobacterial drug treatment. These doubts are understandable, as relapses and treatment failures are frequent in studies of treatment outcome, especially in pulmonary NTM disease.[38] The first randomised clinical trial concerning NTM pulmonary disease was published in 2001 by the British Thoracic Society (BTS).[38] Patients with MAC, *M. xenopi,* and *M. malmoense* pulmonary disease were randomised between rifampicin plus ethambutol (RMP + EMB), and RMP/EMB plus isoniazid (INH). Failure and relapse rates were lower in the RMP/EMB/INH-group for patients with MAC disease, but RMP/EMB/INH was associated with increased mycobacterial disease related death for all three species combined. Outcome figures at five years showed a 33% cure rate for all species combined, 17% failure of treatment or relapse, and a 33% all-cause mortality rate. In 2008, the next comparative trial was published by the BTS: a RMP/EMB regimen combined with either ciprofloxacin or clarithromycin was not superior to the observed outcome of RMP/EMB only in the previous BTS trial for the species studied combined (MAC, *M. malmoense, M. xenopi*).[39] However, for MAC, relapse rates were lower for RMP/EMB plus clarithromycin compared to RMP/EMB plus ciprofloxacin. Furthermore, while the relapse rate of RMP/EMB was found to be 41% in the first BTS trial, RMP/EMB plus clarithromycin showed a relapse rate of 13%. These results are in line with previous observational studies performed in the US in which clarithromycin was found to be effective against MAC pulmonary disease, yielding sputum culture conversion rates of 92% at 12 months follow-up after completion of at least 6 months of a clarithromycin based multi-drug regimen.[40] Tanaka and colleagues revealed that culture conversion rate of the triple drug regimens RMP/EMB and a macrolide with adjunctive aminoglycosides during the first months of therapy was 71.8% overall.[41] In patients with MAC isolates that showed clarithromycin resistance, poorer sputum conversion rates were observed in both studies.

Drug susceptibility testing is useful for MAC lung disease (with only clarithromycin susceptibility predicting treatment outcome in these patients), in *M. kansasii* disease (RMP resistance), and in disease caused by some of the rapid growing mycobacteria.[42] Since ***in vitro*** drug susceptibility of other species, or of drugs other than clarithromycin and rifampicin for MAC and *M. kansasii* respectively, has never been correlated with clinical outcome, the interpretation of drug susceptibility testing should be made with caution.

The outcomes of treatment differs strongly by species Outcomes are favourable in *M. kansasii* disease, with long term culture conversion rates up to 100%[43-45]; outcomes are poor in pulmonary disease caused by e.g. the rapid grower *M. abscessus* at only 28%.[46] In general, there are few clinical trials and thus randomized controlled trials are needed including new drugs and drug combinations.[47] In addition to adequate chemotherapy, surgery may be considered in all patients with localized pulmonary NTM disease as it is known to improve sputum conversion rates in selected patient groups.[46,48] Surgical resection in patients for whom drug treatment is unlikely to be successful, e.g. those with *M. abscessus* disease will benefit from surgery especially.[2] However, only a minority of patients have NTM pulmonary disease suitable for surgery. Since outcome of surgical intervention is found to be inversely related to local experience with NTM surgery, referral to an expertise center is strongly advised.[49] Whether treatment with airway clearance interventions (bronchodilators, mucolytics, mechanical aids such as oscillatory positive expiratory pressure devices) are as effective as in non-CF bronchiectasis has to be addressed in future studies.

Research questions and thesis outline

Research of the epidemiology of NTM isolation and disease must have priority on the agenda. In clinical practice, isolation of NTM frequently raises questions about the association of NTM isolation and clinical symptoms. Sputum samples are generally taken because of (change in) clinical symptoms and signs related to the underlying condition itself rather than to mycobacterial disease which make interpretation even more difficult. In this thesis the focus is on NTM epidemiology, pulmonary NTM isolation, clinical relevance, and NTM disease.

Chapter 2 focuses on geographical aspects of pulmonary NTM isolation. Physicians are increasingly confronted with NTM isolates and the difficult task of establishing their clinical significance in individual patients. The scientific literature is of limited help , as both the species distribution and their clinical relevance seem to differ by geographical area. In this chapter the goal is to give a snapshot of worldwide pulmonary NTM isolation. Gaining knowledge on NTM species distribution can help in identifying factors associated with human NTM infection like climate differences, population density, or host factors. The epidemiology of NTM isolation in the Netherlands is studied in more detail in chapter 3. This study was carried out especially to explore the factors, other than improved laboratory techniques, associated with the observed increase in NTM isolation in the Netherlands.

In chapter 4 a prospective cohort study is described in which patients presenting with an acute exacerbation of COPD are screened for NTM in their sputum. COPD is a well-known risk factor for NTM disease, however, little is known about the pathogenesis of NTM isolation and disease in this patient group. The question raised in this study is whether NTM can be isolated from a COPD patient without clinical signs of NTM disease and to explore the possible risk factors of this NTM presence.

In chapter 5-7, we focus on *M. malmoense*, a NTM species strongly associated with clinical (pulmonary) disease in humans in The Netherlands. First, in chapter 5, we review the literature on epidemiology, clinical relevance, treatment, and outcome of *M. malmoense* worldwide. Because of the observed rise in incidence of *M. malmoense* isolation in recent years, together with a high percentage of patients showing definite clinical *M. malmoense* disease in Northern Europe, we performed a retrospective medical file study for all known *M. malmoense* isolates received at the national mycobacterial reference laboratory in the Netherlands; the National institute for Public Health and the Environment (RIVM) (chapter 6).
In this study, we identified two patients with strong epidemiological links and pulmonary *M. malmoense* disease in whom we studied the possible inter-human transmission of the causative *M. malmoense* strain (chapter 7).

We have reviewed the NTM database of the RIVM and observed that the infrequently isolated NTM *M. genavense*, especially known to cause disseminated disease in HIV positive patients with low CD4 counts, was isolated especially from sterile body sites like bone marrow and liver. In this era of successful HIV treatment, we expected a change in its epidemiology and etiology, which had not been studied in detail before. In chapter 8, we reviewed the medical files of all patients with *M. genavense* disease in the RIVM database to determine clinical relevance, predisposing conditions, treatment, and outcome.

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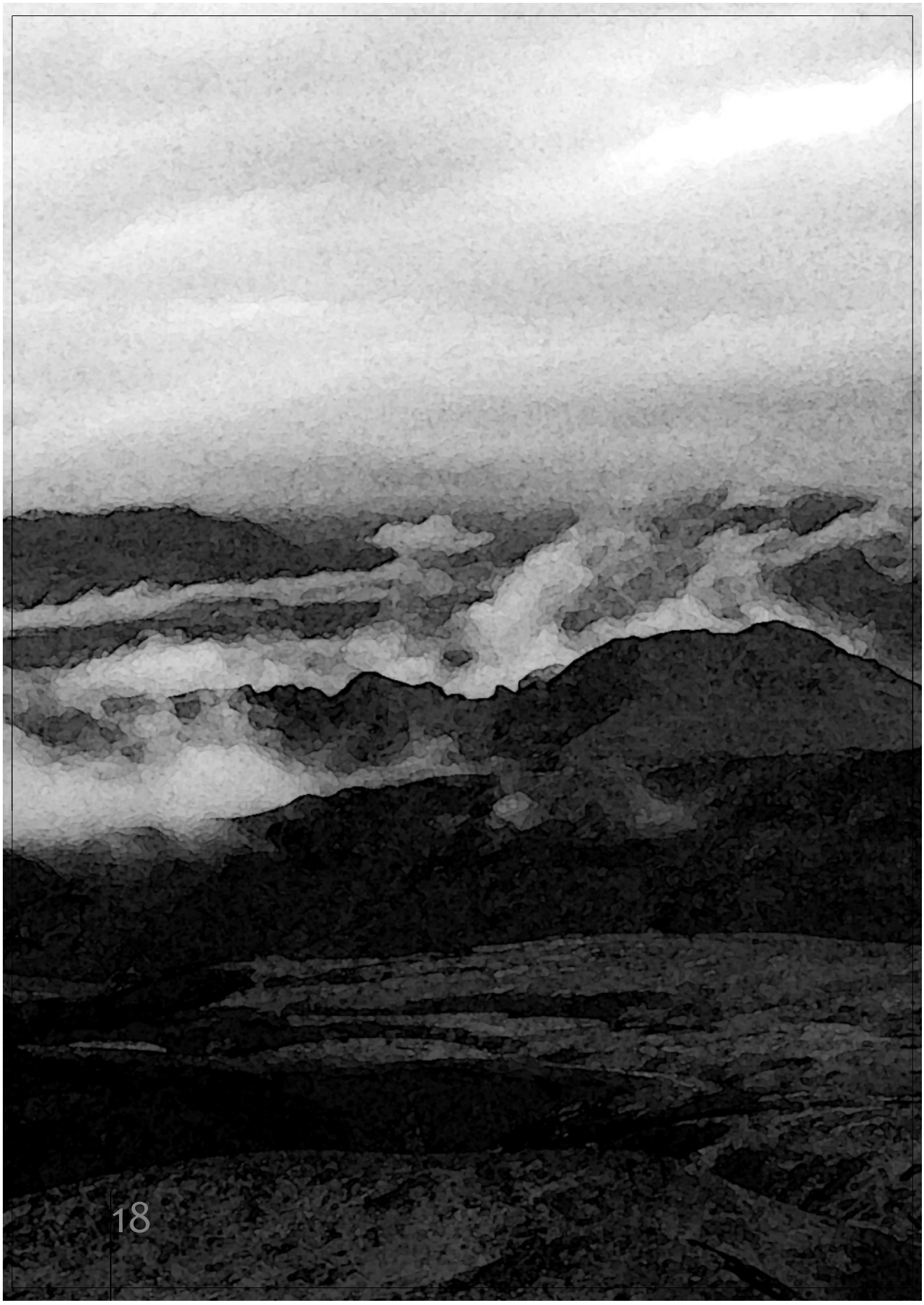

Chapter 2

The geographic diversity of nontuberculous mycobacteria isolated from pulmonary samples:

A NTM-NET collaborative study

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Abstract

A significant knowledge gap exists concerning the geographical distribution of nontuberculous mycobacteria (NTM) isolation worldwide.
To provide a snapshot of NTM species distribution, global partners in the NTM-NET framework (www.ntm-net.org), a branch of TBNET, provided identification results of the total number of patients in 2008 in whom NTM were isolated from pulmonary samples. From these data, we visualized the relative distribution of the different NTM found per continent and country.
We received species identification data for 20182 patients, from 62 laboratories in 30 countries across six continents. Ninety-one different NTM species were isolated. *Mycobacterium avium* complex (MAC) bacteria predominated in most countries, followed by *M. gordonae* and *M. xenopi*. Important differences in geographical distribution of MAC species as well as *M. xenopi, M. kansasii*, and rapid growing mycobacteria were observed.
This snapshot demonstrates that the species distribution among NTM isolates from pulmonary specimens in the year 2008 differed by continent and differs by countries within these continents. These differences in species distribution may partly determine the frequency and manifestations of pulmonary NTM disease in each geographical location.

Introduction

Disease caused by nontuberculous mycobacteria (NTM) has gained increased attention, in part because of an assumed increase in its incidence.[1,2] With the emergence of case reports and series from diverse countries and regions, it has become clear that the distribution of NTM species that are isolated from clinical samples differs strongly by region.[3] Yet, this geographic diversity has never been systemically studied. Increased understanding of this diversity is important, as it can provide important clues on the impact of geographical or climatic differences on NTM distribution and observed discrepancies in clinical relevance and treatment outcome.[3,4] In this study we have collected pulmonary NTM isolation and identification results from laboratories in different regions in the world, collaborating within the NTM-NET network (www.ntm-net.org, a branch of TBNET), from the same time period, to gain further insight on the geographical distribution of NTM species cultured from respiratory samples at a single time point.

Methods

Global partners in the NTM-NET framework were contacted and invited to provide data of the total number of patients from whom NTM were isolated from pulmonary samples in their hospital, regional or reference laboratory in the year 2008, as well as the species identification results and details of the identification methods used. Partners were eligible to contribute to the database if the number of patients with pulmonary NTM isolates per year exceeded 30 to ensure sufficient experience and interpretability of results; one isolate per species per patient was eligible for analysis. We have chosen pulmonary samples in an effort to minimize selection bias. Isolation of NTM from normally sterile sites such as blood or lymph nodes usually indicates definite disease and this may present a strong selection bias, as not all species are equally capable of causing such diseases.

We calculated total number of mycobacteria per continent, the relative contribution of the *Mycobacterium avium* complex for each continent or country, and studied the differences in the relative contribution of other NTM between countries and continents by generating pie-charts. Data of the respondents were plotted on a world map. Since most NTM-NET members are located in Europe, data from European participants were assessed in greater detail, with a focus on north-south differences; we considered Denmark, Norway, Sweden, Finland, the Netherlands, Belgium, Germany, England, Ireland and Poland as Northern Europe; all countries to the south of these countries were considered Southern Europe.
Within this study we did not assess the clinical relevance of these isolates. Ethical approval was waived for this retrospective laboratory database study.

Results

Overview

Sixty-two centres from 30 countries across six continents participated in this study. 17 national reference laboratories provided data representative for their whole country. For other countries, data was provided by a certain number of labs not covering the whole country. A total of 20182 patients had NTM species cultured from pulmonary samples in these centers in 2008; 91.3% (n= 18418) of the isolates were identified to species/complex level; the remaining 1765 isolates (8.7%) were not identified beyond Mycobacterium species other than *M. tuberculosis* complex.

A total of 91 different NTM species were encountered in this survey. The most commonly used identification assays were the GenoType CM/AS (n=28; Hain Lifescience, Nehren, Germany), AccuProbe assays (n=9; Gen-Probe, San Diego, USA), hsp65 PRA (n=6), Inno-LiPA Mycobacteria v2 (n=3; Innogenetics, Ghent, Belgium), in-house methods (n=6) or combinations thereof; mostly, these were supplemented by 16S rDNA sequencing. The six most frequently isolated NTM were *M. avium* complex (9421 isolates; 47%), *M. gordonae* (2170; 11%), *M. xenopi* (1605; 8%), *M. fortuitum* complex (1322; 7%), *M. abscessus* (664; 3%), and *M. kansasii* (720; 4%) (Figure 1). These six species accounted for 80% of all mycobacteria identified.

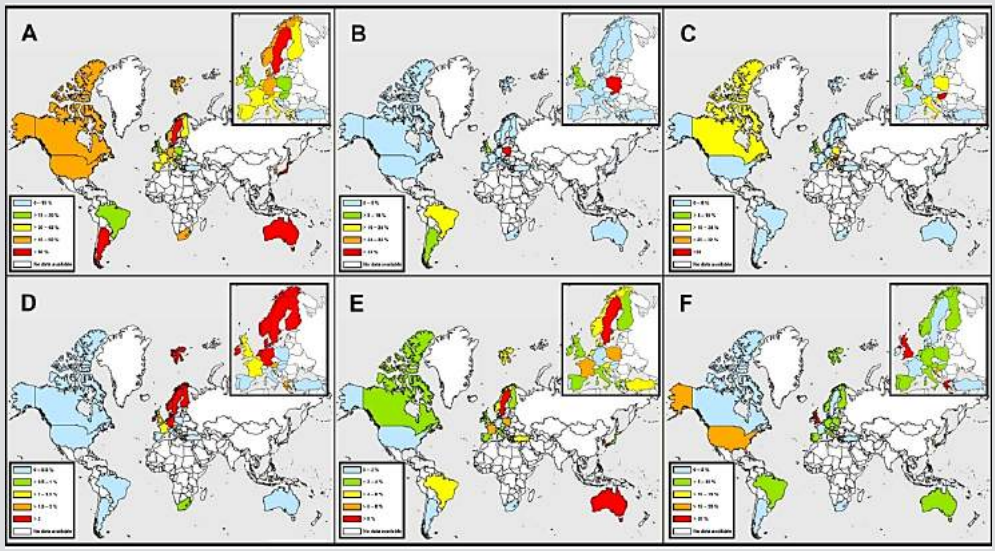


Figure 1: Worldwide distribution of different nontuberculous mycobacteria from pulmonary samples in 2008. A: *M. avium* complex; B: *M. kansasii;* C: *M. xenopi*; D: *M. malmoense*; E: *M. abscessus*; F: *M. fortuitum*.

Note: the data presented in this figure are not per se representative for each country. Species diversity may differ per country.

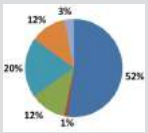
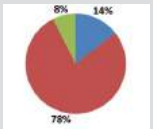
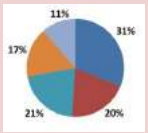
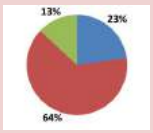
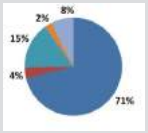
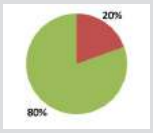
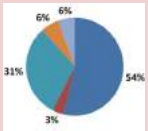
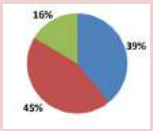
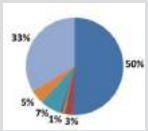
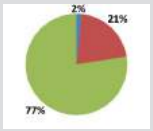
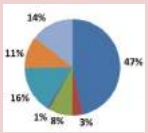
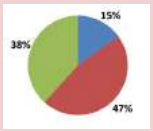
Continent	Number of labs	Number of patients with NTM isolated	Distribution of NTM MAC *M. kansasii* *M. xenopi* *M. malmoense* RGM *M. gordonae* *other SGM*	Number of MAC isolates (% of all NTM)	Distribution of MAC *M. avium complex* *M. avium* *M. intracellulare*
Europe	**43**	**6804**		**2500 (36.9)**	
North America	**4**	**4913**		**2553 (52)**	
South America	**3**	**393**		**123 (31.3)**	
Australia (Queensland)	**1**	**453**		**322 (71.1)**	
Asia	**3**	**1974**		**1062 (53.8)**	
South Africa	**2**	**5646**		**2849 (50.5)**	
TOTAL	**56**	**20183**		**9409 (46.6)**	

Table 1: Continental distribution of respiratory nontuberculous mycobacteria (NTM) isolates. MAC: *M. avium* complex; RGM: rapid growing mycobacteria; SGM: slow growing mycobacteria.

Species isolation worldwide

In this section, the isolation of different NTM is presented. Table 1 shows pie charts for *M. avium* complex and other common NTM per continent. Figure 1 presents the distribution of the most common NTM on a world map.

Mycobacterium avium complex

The *M. avium* complex (MAC) species accounted for 9421 (47%) of the 20182 isolates in the study. The highest relative contribution of MAC per continent was found in Australia (71%), the lowest in South America (31%). Per country, this figure differed from 79% in Japan to 16% in Hungary. Fifty-nine out of 62 laboratories were able to identify MAC isolates to species level; the relative frequency of *M. intracellulare* versus *M. avium* in different parts of the world is shown in Table 1. The most striking difference is the relative predominance, within the participating sites, of *M. avium* in North- and South America.

In contrast, *M. intracellulare* was most frequent in Australia-Queensland (57% of all mycobacteria cultured; 80% of the MAC), and South Africa (40% ; 77.5%). MAC isolates that were not identifiable to (sub)species level were common in participating laboratories in Asia and Europe (21% and 15% of all MAC isolates, respectively) but relatively rare in the participating laboratories in North America (8%).

Mycobacterium gordonae

M. gordonae is the second most isolated NTM worldwide in this study mainly due to a high isolation rate found in Europe where *M. gordonae* was the second most isolated NTM. On all other continents, *M. gordonae* ranked third (North America, South America, Africa), or fourth (Asia, Australia).

Mycobacterium xenopi

After MAC and *M. gordonae, M. xenopi* was the third most frequently isolated species in the survey, though its isolation was limited to distinct geographical regions, mainly in Europe and Ontario, eastern Canada (but not Alberta, western Canada). In Hungary, *M. xenopi* is the predominant NTM isolate comprising 49% of all the NTM in this country. In Croatia *M. xenopi* was the second most frequent isolated NTM after *M. gordonae*. Furthermore, *M. xenopi* was prevalent in the English Channel region, being the second most frequent NTM isolate in Belgium and south-east England and ranked third in France after MAC and *M. gordonae.*

Differences within countries were also observed: *M. xenopi* was the predominant NTM isolate in the Barcelona area but only ranked third after MAC and *M. fortuitum* in the Madrid area. *M. xenopi* was not isolated in the participating centers from Asia, Australia and South America.

Mycobacterium kansasii

Overall, *M. kansasii* was the sixth most frequently isolated NTM. In South America *M. kansasii* was the second most isolated NTM after MAC accounting for 19.8% of all NTM isolated. In Europe, Slovakia, Poland, and the UK had the highest *M. kansasii* isolation rates of 36%, 35%, and 11% respectively, compared to a mean isolation of 5% in Europe. In the Paris region of France, *M. kansasii* was the third most isolated NTM after MAC. In the participating laboratory in Japan, *M. kansasii* ranked fourth after MAC, *M. gordonae* and *M. abscessus*. In South Africa, *M. kansasii* ranked sixth overall. In a center with a large miners community in the Johannesburg region however, *M. kansasii* was the second most frequent NTM isolated.

Rapid growers

M. abscessus and *M. fortuitum* were the most frequently isolated rapidly growing mycobacteria (RGM) worldwide. Other RGM were only sporadically isolated.

Among the RGM, important geographical differences were observed. RGM were highly prevalent isolates in the participating centers in East Asia, where they make up 27% of all NTM isolates in comparison to isolation frequencies of 17.9%, 16%, and 14% in the participating centers in North America, South America and Europe, respectively. However, important differences in frequency of RGM isolation among countries within Asia were also noted. In Tokyo, Japan, rapid growers accounted for only 6.6% of all isolates, in contrast to the participating centers in Taiwan (50%) and South Korea (28.7%). Furthermore, in Taiwan, *M. fortuitum* and *M. abscessus* were the second and third most frequently isolated NTM species, while in South Korea, *M. abscessus* was the second most frequently isolated NTM after MAC.

Rare and geographically restricted NTM species

M. malmoense was found more often in Northern Europe (total of 80 of 3107 isolates; 2.6%) in comparison with Southern Europe (21 of 3696 isolates; 0.6%). In South Africa, 43 *M. malmoense* isolates were found (43 of 5646; 0.76%). Five *M. malmoense* isolates from the participating laboratories from North America were reported (5 of 4913 isolates; 0.1%). *M. malmoense* was not found in Queensland-Australia, Asia, and South America. *M. simiae* was found worldwide except Asia. A total of 97 patients were found in this study with an *M. simiae* isolate (0.5% of all 20182 NTM isolated). *M. scrofulaceum* was the second most isolated NTM in South Africa (383 isolates of 5646; 6.8%). *M. lentiflavum* was found in several labs worldwide (generally below 1% of all NTM isolated per lab) but was found more often in Portugal (6%) and Finland (5.8%). The majority of the 91 different NTM species in this study were infrequently isolated, e.g. *M. smegmatis* (n=5; 0.03%) and *M. interjectum* (n=36; 0.2%).

Focus on Europe

Because of a substantial number of participating countries in Europe, this continent was studied in more detail, with a focus on north-south differences. We collected data of 3107 isolates from Northern Europe and 3696 from Southern Europe. In Figure 2, the pie-charts of species diversity in all participating countries in Europe are shown.

MAC was isolated more frequently in Northern Europe (44% of all mycobacteria) than in Southern Europe (31%). In both north and south Europe, M. avium was the most frequent isolated subspecies of the MAC.

M. xenopi was more frequently isolated in Southern (778 of 3697 isolates; 21%) compared to Northern Europe (190 of 3107 isolates; 6%) partly due to substantial contribution of *M. xenopi* isolated in a single country (Hungary). On the other hand, *M. bohemicum* was especially found in Northern Europe, mainly Finland, in contrast to only 1 isolate from Southern Europe and no isolate from any participating laboratory in other regions of the world.

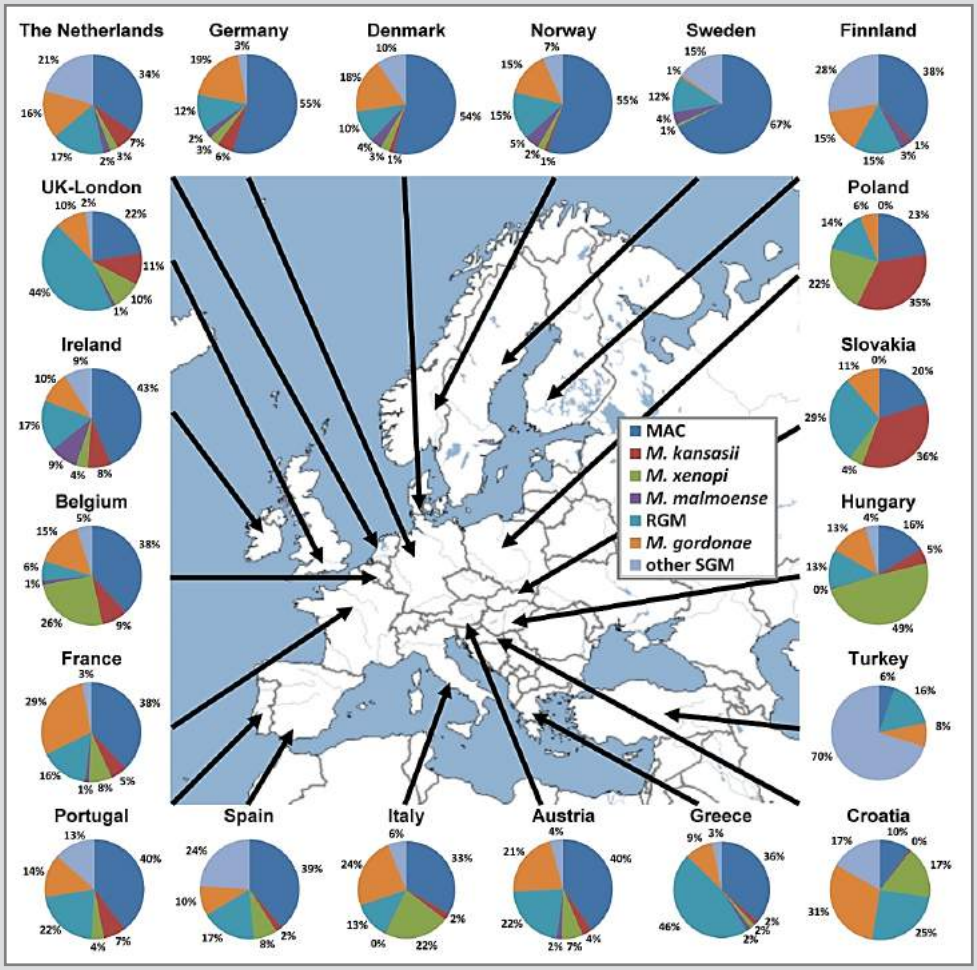


Figure 2: Distribution of different nontuberculous mycobacteria from pulmonary samples in 2008 in Europe. MAC: *M. avium* complex; RGM: rapid growing mycobacteria; SGM: slow growing mycobacteria.

Discussion

For the first time, we provide a snapshot of the worldwide distribution of NTM species isolated from pulmonary samples. This snapshot illustrates that the species distribution among NTM isolated from pulmonary specimens in the year 2008 differs by region and differs by country within these regions. For many regions or countries that participated in this study, these are the first data covering this topic. The NTM species distribution in a country or region may have profound impact on the frequencies and manifestations of pulmonary NTM disease. It is now generally accepted that NTM species differ in their ability to cause lung disease in

humans[3-6] and that the clinical relevance of a particular species can differ in different parts of the world.[7, 8] The species distribution presented in this study can help in identifying factors associated with human NTM infection like climate differences, population density, or host factors. In the past, it has been suggested that differences in NTM species distribution may also affect the efficacy of BCG vaccination.[9]

The two studies that approached this subject were published by Martin-Casabona and colleagues in 2004[2] and by Marras and Daley in 2002;[3] the former reported on NTM isolation over three decades, ending in 1996. The spread of molecular tools for identification in the years between 1996 and 2008 make the comparison of the two surveys difficult. The Martin-Casabona study only incorporated laboratories from Europe, Turkey, Iran and Brazil and sampling was not systematic as different laboratories produced data from different time periods. The review by Marras and Daley adds more historical published data and is not a survey. Still, both showed the predominance of MAC isolation worldwide together with the characteristic geographical distribution of *M. xenopi, M. kansasii,* and *M. malmoense*. The review by Marras and Daley also showed that differences in species distribution may occur over time.[3]

In this study, the members of the *M. avium* complex predominated in most regions, though *M. xenopi* predominated in Hungary and *M. kansasii* in Poland and Slovakia. The relative distribution of the various members of MAC again reveals geographic differences. While *M. avium* predominated in the participating centers in North and South America and Europe, *M. intracellulare* was most frequently isolated in South Africa and Australia. In Australia a significant increase in the isolation of this species has been reported before[5]. Few laboratories were able to reliably distinguish the novel MAC members, such as *M. colombiense* and *M. chimaera*.[10, 11]

Mycobacterium xenopi was particularly prevalent in the region covering Hungary, Croatia, Northern Italy, in Ontario-Canada and in the areas bordering the English Channel; the latter two are in line with earlier data.[1, 2] The frequently stated link to coastal areas[1, 2] does not hold true in the light of the observed predominance in Hungary and absence in coastal regions outside Europe. Nonetheless, presence of a specific environmental niche for *M. xenopi* is a likely explanation. Other factors to be considered are differences in the potential to colonize or infect human airways between *M. xenopi* strains and host factors.

The geographical distribution of *M. kansasii* has been the subject of previous studies. In our study, *M. kansasii* was frequently isolated in South America, Eastern Europe and metropolitan centers Paris, London and Tokyo and the Johannesburg region of South Africa. These findings match previously published data from those regions.[2, 8] Its isolation has been related to mining activities[12-14] as well as urbanization and may be related to working and living conditions.[15] In Northern Europe, isolation frequencies of *M. kansasii* have been in decline for the past three decades,[16] but the underlying causes are unknown. The high isolation frequency of *M. scrofulaceum* in South Africa is in accordance with the literature and probably related to mining activities.[14]

M. simiae was traditionally thought to be confined to the Southern USA, Cuba and Israel,[7] but in this study we demonstrate that this species has a global distribution (except Asia), making up 0.67% of all isolates in this study, with the highest isolation of 1.1% found in northern Europe. *M. malmoense* was found especially in Northern Europe, which is in accordance with the literature,[8] but also in South Africa. *M. malmoense* isolation from clinical specimens from this continent has never been reported before, although its presence in soil was.[17]

Rapid growers made up 10-20% of all NTM isolates worldwide, although they proved more prevalent in East Asia where they compose up to 50% (Taiwan) of all NTM isolated from pulmonary samples. The high isolation frequency of RGM, particularly *M. abscessus*, was also noted in previous studies, focusing on this region.[18-20] The reason for the high isolation rate of RGM in Asian countries remains unclear, though geographical or climatic factors, host and laboratory factors have been suggested.[19] An important reason to perform studies on geographical distribution of NTM is to help identify factors associated with differences in worldwide isolation patterns of specific species. Studies focusing on species-specific environmental niches and subsequent transmission to humans are not systematically performed. Yet, these can offer important clues that aid to prevent (re)infection of susceptible patients.

In the current study, a multitude of molecular identification techniques was used. It is well known that these techniques can produce discrepant results.[21, 22] Yet, this is likely to influence a minority of isolates and will not affect the snapshot of the geographic diversity in NTM species isolated from pulmonary samples, as presented in this study. The current study revealed the occasional presence of many rare species. For many of these species no data other than reports of single cases of disease were available. Often such case reports or novel species descriptions seem to suggest a very limited geographic spread of the bacterium and, as they mostly concern cases of true disease, overestimate the clinical relevance of these rare species. We hope that the current study also provides a reference to clinicians and microbiologists faced with rare NTM species.

An important shortcoming of this study is the low number of participating laboratories, especially outside Europe. Inclusion of more data from different laboratories will probably reveal other isolation patterns.

Follow-up studies covering different time periods together with more participating laboratories may reveal additional clues on changing NTM pattern over time and geographic differences of NTM distribution on the national scale.

We have chosen pulmonary samples specifically because these best reflect the distribution of species in local environments. Nevertheless, the presence and persistence within the human airways is also likely to represent a selection bias, as does the fact that we collected identification results of cultured isolates. The low isolation frequency of the majority of the 91 different NTM species may reflect an inability to persist in human airways or scarce environmental presence.

In summary, this snapshot illustrates that the species distribution among NTM isolated from pulmonary specimens in the year 2008 differs by region and differs by country within these regions. MAC predominates in most settings, but the frequency of isolation of rapid growers, or slow growers like *M. xenopi, M. kansasii* and *M. malmoense* differs per setting. These differences in species distribution may determine, in part, the frequency and manifestations of pulmonary NTM disease in each region. Future studies need to address species-specific environmental niches as a cause of differences in worldwide isolation patterns.

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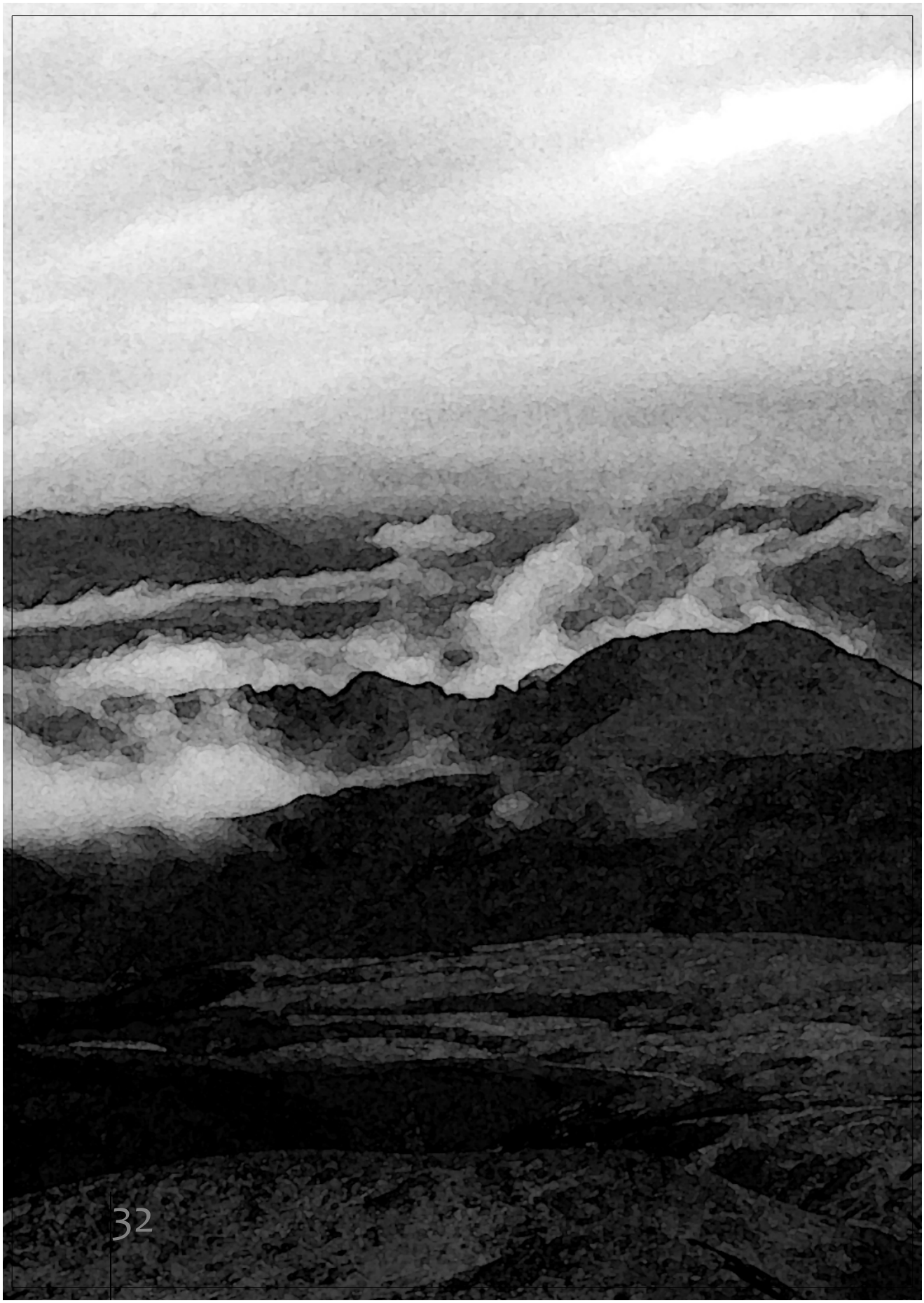

Chapter 3

The changing pattern of clinical *Mycobacterium avium* isolation in the Netherlands

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Int J Tuberc Lung Dis 2010; 14: 1176-1180

Abstract

Setting: National mycobacteria reference laboratory, the Netherlands.
Objective: To assess the role of factors other than laboratory improvements in the increasing isolation frequency of nontuberculous mycobacteria (NTM) in the Netherlands; laboratory improvements are often considered key factors in this increase.
Design: Laboratory database study. All clinically isolated NTM referred to the national reference laboratory between January 2000 and January 2007 were retrieved from the laboratory database and divided by species, patient age group and sample origin. Data were compared to national demographic data.
Results: Clinical *Mycobacterium avium* isolates accounted for most of the increase in referred NTM. The number of respiratory *M. avium* samples in patients >40 years of age increased over time. This age group increased in size during the study. In this age group, the prevalence of chronic obstructive pulmonary disease (COPD) increased during the study period. Mycobacterium avium isolation from lymph nodes in children remained stable, whereas extrapulmonary *M. avium* isolation in the middle age group, including HIV-associated bloodstream isolates, decreased.
Conclusions: The increasing NTM notification in the Netherlands is unlikely to have been a result of laboratory improvements alone: the ageing population with an increasing prevalence of COPD is likely as important. Environmental characteristics may specifically favour *M. avium.*

Introduction

Nontuberculous mycobacteria (NTM) are mostly opportunistic pathogens that are widely present in natural and man-made environments.[1] The frequency of isolation of NTM is increasing in many countries, mainly were the incidence of tuberculosis (TB) is in decline.[1-3] This increase in isolation frequency has previously been related to laboratory improvements for detection and identification of NTM, including automated liquid culture systems and molecular identification kits.[1-2] The pathogenic potential of these NTM, however, is increasingly recognized, especially in patients at risk due to local or systemic impaired immunity.[1]
At the Dutch national mycobacteria reference laboratory at the National Institute for Public Health and the Environment (RIVM), we also noted an increase in the number of referred NTM, most notably isolates belonging to the *Mycobacterium avium* complex (MAC).
In the present study, we explored the factors underlying this increase in NTM isolation.

Methods

Records of all clinical isolates of nontuberculous mycobacteria referred in between January 2000 and January 2007 were extracted from the reference laboratory database and divided by species, sample origin (respiratory, lymph node or other) and age group; one isolate per patient per year was analyzed, unless multiple species were identified. Age was defined as the age at the date of primary sampling. We chose the following categories: 1-12 years old to include mostly the children with lymphadenitis; 13-39 years to include mostly immunocompromised patients, including co-infection with the human immunodeficiency virus (HIV) and cystic fibrosis patients; >40 years to include mostly patients with pre-existent pulmonary disease, e.g., chronic obstructive pulmonary disease (COPD)[4]. The latter group was subdivided in the 40-60 and >60 years. We compared the laboratory data with publicly available demographic data of the Dutch Central Bureau for Statistics (CBS; http://www.cbs.nl); data on population size, age distribution and COPD prevalence were extracted.

The isolates of all patients were subjected to laboratory diagnosis by the RIVM, the national reference laboratory that performs identification, drug susceptibility testing and genotyping of *Mycobacterium* isolates for all hospitals in the Netherlands. An estimated 85% of all clinically isolated NTM is referred here. To identify mycobacteria, a GenoType MTBC reverse line probe assay (Hain Lifesciences, Nehren, Germany) is used after polymerase chain reaction based amplification to determine whether an isolate is a member of the *M. tuberculosis* complex. If the reaction is negative, an Inno-LiPA Mycobacteria v2 (Innogenetics, Gent, Belgium) reverse line blot assay is used to differentiate between the more common species of NTM. If no species-specific result is obtained, 16S rDNA gene sequencing is performed. Prior to 2004, 16S rDNA gene sequence analysis was performed on request of referring physicians, if AccuProbe *M. tuberculosis* complex, *M. avium* complex, *M. intracellulare* and *M. avium* DNA probes (GenProbe, San Diego, USA) yielded negative results.
Ethical approval was waived for this retrospective study.

Results

During the study period, the annual number of referred NTM rose in absolute terms from 480 to 747 per year, as well as relative to *M. tuberculosis* isolation (Table 1). Isolates belonging to the MAC, made up of *M. avium* and *M. intracellulare*, are the NTM most commonly isolated in the Netherlands, and their number rose sharply during the study period from 170 to 292 per year, comprising respectively 35% to 39% of annually referred NTM (Table 1).

During our study period, the increase in referred MAC isolates was caused mainly by an increase in *M. avium* isolates (Table 1) from 60 in 2000 to 210 in 2006, a 250% increase. This increasing annual trend, as compared to the general annual NTM count, proved statistically significant (Pearson's r 0.857; p=0.014). The number of *M. intracellulare* isolates was stable (Table 1). This rise in M. avium isolates has two important features. First, it is most pronounced in patients aged >40 years (Table 2). Second, it is mainly caused by a rise in M. avium isolates from pulmonary samples (Table 2). These pulmonary isolates are cultured almost exclusively from the patients aged >40 years ($p<0.001$); within these group, pulmonary isolates are significantly associated with age > 60 years ($p<0.001$). In general, the sample origin recorded was in agreement with the origins expected within the age group (Table 3).

During the study period, the demographic composition of the Netherlands changed. Between 2000 and 2008, the population increased by 3.3%, although this percentage was 12.2% for those aged 40 to 65-years and 10.9% among those aged >65 years, and by 2008, 50.1% of the Dutch population was aged >40 years (Table 4). The self-reported prevalence of COPD increased from 7.0% in 2000 to 7.6% in 2006; in persons aged 45-65 it increased from 5.2% to 7.7%, while in those aged >65 years it increased from 9.6% to 12.7%.

The number of extrapulmonary samples, including those from children with cervical lymphadenitis, remained relatively stable throughout the study period (Table 2).

The increase in the annual number of referred isolates is not confined to *M. avium*, although for this species it is most pronounced. *M. gordonae* isolates are also increasing in number, whereas isolation of other important species including *M. intracellulare, M. malmoense* and *M. kansasii* has been mostly stable (Table 1).

Table 1: Annual laboratory data for the study period*

Year	2000 *n* (%)	2001 *n* (%)	2002 *n* (%)	2003 *n* (%)	2004 *n* (%)	2005 *n* (%)	2006 *n* (%)
Total mycobacteria	1584	1604	1536	1480	1567	1561	1537
M. tuberculosis complex	1104	1111	1098	1009	933	893	790
Total NTM	480	493	438	471	634	668	747
MAC	170	175	161	191	267	278	292
-*M. avium*	60	61	56	81	191	215	210
-*M. intracellulare*	26	33	18	26	59	58	54
-Untyped MAC	84	81	93	84	17	5	28
M. malmoense	17	10	18	6	16	20	17
M. gordonae	57	45	40	49	112	99	152
M. marinum	5	8	7	7	13	12	20
M. kansasii	39	39	32	34	53	45	50

*Percentages are fractions of "Total NTM". The increase in the isolation frequency of *M. avium* is not mirrored in other NTM species, including *M. kansasii*, *M. malmoense* and *M. marinum*. Many NTM species were referred in low numbers and not included in the Table to increase clarity. NTM, nontuberculous mycobacteria; MAC, *Mycobacterium avium complex;*

Table 2: Isolation sources and age distribution of *Mycobacterium avium* isolates by year

	2000	2001	2002	2003	2004	2005	2006
Total	60	61	56	81	191	215	210
Pulmonary	38	42	42	59	125	155	156
Lymph node	12	7	4	12	42	39	32
Other	10	12	10	10	24	21	22
Age Group, years*							
1-12	11	10	5	11	43	40	26
13-39	8	9	9	9	33	24	18
40-60	18	12	14	27	32	47	53
>60	23	30	28	34	83	104	113

*The increase in *M. avium* isolation mainly results from pulmonary isolates and patients aged >40 years.

Table 3: The number of MAC isolates by sample origin and age group*

Age group	Lymph node	Other	Pulmonary	Total
0-12	159	16	10	185
13-39	7	70	112	189
40-60	10	52	326	388
>60	6	24	748	778
Total	182	162	1196	1540

*Pulmonary isolates are significantly associated with patients >40 years of age ($p<0.001$); within this group, they are significantly associated with patients >60 years old ($p<0.001$)

Table 4: Demographic data for the Netherlands, 2000-2008

Year	2000	2008	Increase	%
Population size	15863950	16405399	541449	3.3%
Age group, years*				
0-20	3873008	3940450	67442	1.7%
20-40	4761504	4267063	-494441	-10.4%
40-65	5076996	5783060	706064	12.2%
65-80	1652103	1799337	147234	8.2%
>65	2152442	2414826	262384	10.9%
>80	500339	615489	115150	18.7%

*The Dutch population is ageing; growth of the population is concentrated in the segment >40 years old.

Discussion

The rise in isolation frequency of NTM in the Netherlands is caused mostly by increasing numbers of *M. avium* isolates from respiratory samples in patients aged >40 years. These are probably patients suffering from COPD, the most common predisposing condition for NTM lung disease within this age group in the Netherlands and an argument for frequent culture of sputum samples.[5] A recent study in the Netherlands demonstrated that *M. avium* was more common than *M. intracellulare* in pulmonary samples. Moreover, pulmonary *M. avium* isolation represented true disease in 24 of 59 cases (41%) as opposed to 2/16 cases (13%) for *M. intracellulare*, based on the 2007 ATS criteria.[1,5]
Importantly, this study also demonstrated that among pulmonary MAC isolates, the percentage representing true MAC disease did not change significantly in the 1999-2004 period;[5] this suggests that the increase in isolation frequency is not a mere result of an increased sample volume. Moreover, clinical guidelines for COPD or other pulmonary diseases predisposing to NTM disease have not changed with respect to Mycobacterium culture during the study period. Reports from the USA have noted that the nodular-bronchiectatic type of pulmonary MAC disease is most frequent there and *M. intracellulare* its most common causative agent.[6] These differences remain largely unexplained. It is possible that *M. avium* bacteria isolated in the Netherlands differ in virulence from those isolated in the USA; previous studies have identified genetic differences in MAC isolates from different human and environmental sources.[7-8]

The improvements in culture and identification techniques often held responsible for rising NTM isolation frequencies[1-2] are also relevant in the Netherlands. Automated liquid culture systems were introduced in September 1997; their spread largely predates our study. Improvements in the identification methods at our reference lab in 2004 (see Methods) led to a decrease in the number of untyped MAC strains and an increase in the number of isolates identified as *M. intracellulare* or *M. avium* (Table 1). It is evident from Table 1 that this provides only a partial explanation of the increase in *M. avium* isolates in 2004. Tables 1 and 2 show that, although boosted by improved identification methods, the increase in *M. avium* isolation, especially among the oldest age groups, began before the improvements in laboratory techniques. Moreover, the annual number of isolates of other species, including the commonly isolated *M. kansasii, M. intracellulare* and *M. malmoense* did not benefit from these technical improvements (Table 1), nor did the number of extrapulmonary *M. avium* isolates (Table 2) or the percentage of TB cases that could not be confirmed by culture; this has been steady in the Netherlands throughout the study period, at 30%.[9] Skin sensitization to *M. intracellulare* has increased over the last decades in the US, along with the incidence of *M. intracellulare* infection.[10] Combined, this suggests that laboratory techniques have a role in the increase in NTM, thus *M. avium* isolation, but are not its sole explanation.
Host factors provide a second partial explanation for the rise in NTM isolation frequency. The Dutch population is ageing (Table 4) and reports an increasing prevalence of chronic respiratory disease, an important risk factor for NTM disease.[5] The increasing prevalence of COPD was also noted in a recent study of morbidity and interventions in general practice.[11] The prevalence of COPD may partially explain the increase in NTM notification in pulmonary samples (Tables 2 and 3). Patients in older age groups are more likely to have chronic pulmonary diseases accompanied by sputum production than younger patients. However, this offers

no explanation for the rise in isolation specifically of *M. avium* unless COPD predisposes to infection by *M. avium* rather than other NTM species. In the Netherlands, the fibrocavitary disease type is more prevalent, primarily affects COPD patients and is mostly caused by M. avium.[5] It is uncertain whether the predominance of cavitary MAC disease results from the strong presence of *M. avium* as opposed to *M. intracellulare*, or from host factors including COPD. Rather than rising COPD prevalence, the simultaneous decline in tuberculosis incidence (Table 1) may imply that exposure to tuberculosis infers cross-protection to NTM disease; this is supported by the fact that countries that halted bacille Calmette-Guerin (BCG) vaccination subsequently noticed an increase in the incidence of paediatric cervicofacial lymphadenitis caused by MAC.[12-13]

A third explanation for the increase in *M. avium* isolation in the Netherlands may be changes in the environment, either natural or man-made, that favour *M. avium* and impede or influence other NTM; these could result in increased exposure of humans specifically to *M. avium*. Similarly, in the 1970's *M. scrofulaceum* was replaced by MAC as the leading causative agent of cervical lymphadenitis in children. This phenomenon has been attributed to chlorination of tap water, which likely selects for the more chlorine resistant *M. avium*.[14] Studies reviewed by Collins et al., however, did not demonstrate important differences in chlorine susceptibility between NTM species and considered tap water chlorine levels too low to significantly reduce mycobacterial loads.[15]

The number of *M. avium* isolates from lymph node samples referred to our reference laboratory has remained relatively stable; the temporary elevation in 2004-2005 results from a clinical trial in that period.[16] The stable low numbers of other extrapulmonary *M. avium* isolates probably result, in part, from the availability of highly active antiretroviral therapy (HAART) and MAC prophylaxis for patients infected with HIV; one of the important limitations of the current study is the lack of clinical data, including HIV serostatus. A decline in the incidence of disseminated *M. avium* disease during and after HAART introduction has been recorded and reviewed before.[17] NTM infection usually affects patients with very low CD4 counts and their numbers have been reduced after the introduction of HAART.

In summary, the rise in notification of nontuberculous mycobacteria at the reference laboratory in the Netherlands was mainly caused by increasing isolation of *M. avium* from respiratory samples in patients aged >40 years. Changes in laboratory techniques are unlikely to be the sole explanation of this increase. The ageing population, with an increasing prevalence of chronic diseases including COPD, is likely as important. Decreasing cross-protection to NTM disease from exposure to TB may also play a role. Environmental changes may specifically favour *M. avium.*

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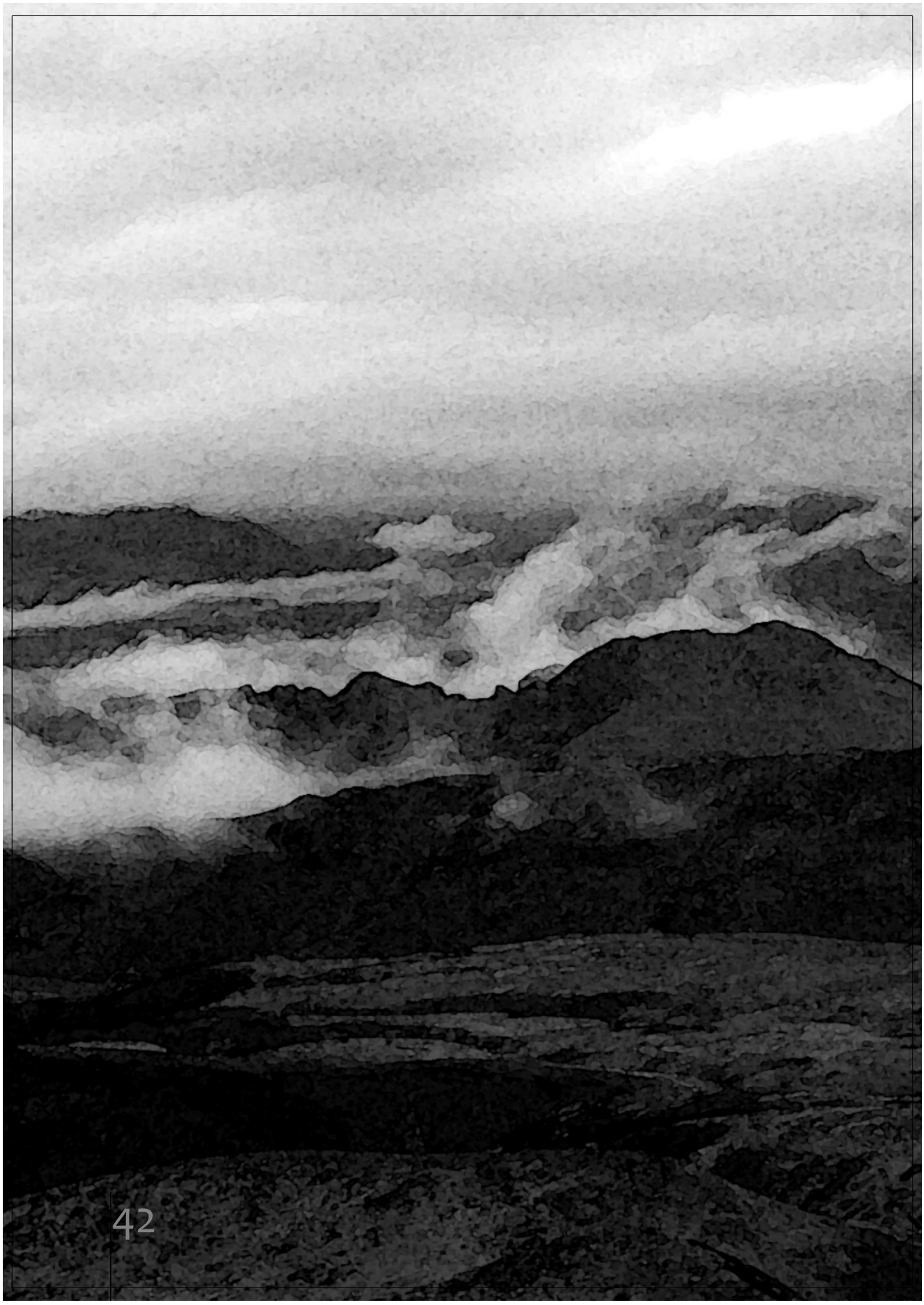

Prevalence of nontuberculous mycobacteria in COPD patients with exacerbations

Chapter 4

Wouter Hoefsloot, Jakko van Ingen, Cecile Magis-Escurra, Monique H. Reijers, Dick van Soolingen, Richard P.N. Dekhuijzen, Martin J. Boeree

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Abstract

Objective: The aim of the present study was to investigate the isolation prevalence of NTM among patients presenting with an acute exacerbation of chronic obstructive pulmonary disease (AECOPD).

Design: All patients with an AECOPD who were admitted to our pulmonary hospital or seen at the outpatient department between January 2010 and January 2011 were included in this prospective cohort study. At least 3 separate sputum samples were tested for the presence of NTM by culture on solid and liquid media.

Results: We included 73 patients with a mean age of 70.4 ± 10,7 years, of whom 44 were male (60%) with a mean FEV1 of 44.2% predicted. A total of 206 sputum samples were obtained; 21 samples (10.1%) of 16 patients (22%) yielded mycobacteria, 14 of 21 were *M. gordonae*. One patient was diagnosed with definite NTM lung disease. Patients with a positive mycobacterial culture were significantly older than patients without a positive mycobacterial culture.

Conclusion: In the current cohort of patients presenting with an AE COPD, 22% harbored NTM in their sputum. Further study is required to address the clinical importance of these NTM and their relation with COPD.

Introduction

Nontuberculous mycobacteria (NTM) are environmental micro-organisms omnipresent in soil, plants and natural and treated water. More than 130 different NTM species have been identified and still new species are described. Humans contract NTM infections mainly from environmental sources (aerosols, ingestions); person-to-person transmission has not been proven. The frequency of NTM isolation in clinical practice is increasing in many countries, including the Netherlands.[1-4] NTM are mostly opportunistic pathogens and capable of causing disease in patients with local or systemic impaired immunity.
Pulmonary NTM disease is most frequent and generally affects patients with pre-existing chronic pulmonary diseases, mainly chronic obstructive pulmonary disease (COPD).[1,3] COPD is the fourth leading cause of death in the world, and further increase in its prevalence and mortality can be anticipated in the coming decades. Pulmonary infections, including those by NTM, may contribute to the progression of COPD. There is little published information about the prevalence of NTM in the lung of COPD patients. Only when there are strong indications of a possible role of NTM in the disease progression, mycobacterial culture is requested. We performed a prospective cohort-study in COPD patients presenting with an exacerbation to determine the prevalence and clinical relevance of NTM isolation in COPD patients.

Materials & Methods

Study design and setting

We performed a prospective cohort study. The cohort includes all patients admitted to our pulmonary hospital and patients seen in the outpatient department between January 2010 and January 2011with an acute exacerbation of COPD (AECOPD). AECOPD was chosen as an inclusion parameter because of the potential ease of sputum collection in exacerbated patients. A COPD diagnosis made by spirometry was required before inclusion in the study. An AECOPD diagnosis was established by the responsible physician. AECOPD was defined, according to the Global Initiative for Chronic obstructive Lung Disease (www.goldcopd.com) as an event in the natural course of the disease characterized by a change in the patient's baseline dyspnoea, cough, and/or sputum that is beyond normal day-to-day variations, is acute in onset, and may warrant a change in regular medication. We excluded patients referred to our clinic for previously diagnosed pulmonary NTM disease. Written informed consent was obtained from all patients. The study was approved by the local ethics committee.

Study procedures

The main outcome in this study was the result of mycobacterial sputum culture. As part of routine clinical practice in our center, sputum samples and chest radiographs were obtained. We aimed to obtain three sputum samples per patient on separate days for mycobacterial culture. If one of these samples grew positive for mycobacteria, two successive samples were obtained on separate days in order to determine whether the American Thoracic Society

(ATS) microbiological criteria for NTM disease were met. In the ATS statement, true NTM lung disease is defined by at least two positive sputum cultures in combination with fitting clinical parameters (pulmonary symptoms and radiologic abnormalities associated with NTM disease).[1] Patients treated in the outpatient department were asked to mail successive sputum samples to the hospital. A systematical description of chest radiographs was not part of the study protocol. Patients were asked to complete a short written questionnaire about symptoms, and possible factors associated with NTM presence (smoking history, (inhaled) corticosteroid use, previous exacerbations, and hospital admissions). Level of dyspnoea was measured using the mMRC dyspnoea scale as part of routine clinical practice in our hospital.[5] No systematic follow-up was planned for this study.

Mycobacteriological examination

Concentrated sputum samples were decontaminated for 10 minutes using 1% N-acetyl-L-cysteine-sodium hydroxide and incubated on solid egg-based media (Lowenstein-Jensen, BD Biosciences, Erembodegem, Belgium) at 30 and 35°C as well as in liquid medium (BacTec MGIT960, BD Biosciences, Erembodegem, Belgium) at 35°C. We subjected all isolates to Ziehl-Neelsen staining and positives were subjected to molecular identification using the INNO-LiPA Mycobacteria v2 reverse line blot assay (Innogenetics, Ghent, Belgium); if a non-specific result was obtained, the hypervariable region A of the 16S rRNA gene was sequenced.[6]

Statistical analysis

Data are described as mean + standard deviation or median (quartile). Comparisons between groups were done with Student t test for normally distributed variables and with Mann-Whitney Rank Sum Test for those with non normal distribution. Chi-square and Fisher exact tests were used for categorical variables.

Results

Inclusion

Eighty-one patients were eligible for inclusion, of whom 73 were ultimately included during the study period. Eight patients were excluded. The reasons for exclusion were: pulmonary function tests showed no airflow obstruction (n=3) or pulmonary function tests not available (n=2), no informed consent given (n=1) or no sputum cultures available (n=2). The mean age of the 73 patients was 70.4 ± 10.7 years, of whom 44 were male (60%) with a mean FEV1 of 44.2% predicted. Sixty-four patients (88%) were admitted to the hospital at time of inclusion. Twenty-one patients (29%) showed dense airspace opacities on chest X-ray at inclusion. None of the patients were treated with a macrolide before inclusion.

Sputum cultures

A total of 206 sputum samples were collected during the study period (206/73=2.82 sputum samples/patient (SD ± 0.78)). Of these 206 sputum samples, 21 were positive

for mycobacteria (10.1%; 14 *M. gordonae,* 1 *M. avium*, 3 *M. chimaera*, 2 *M. simiae,* 1 *M. nonchromogenicum*). The total number of patients from whom a mycobacterium was cultured was 16 (22.2%); 11 *M. gordonae*, 1 *M. avium*, 2 *M. chimaera*, 1 *M. simiae*, 1 *M. gordonae* and *M. nonchromogenicum*). Four patients (25% of all the patients with a positive NTM culture and 5.4% of all the included patients) had two positive cultures yielding the same NTM species (*M. simiae* [n=1], *M. chimaera* [n=1] and *M. gordonae* [n=2]) (Table 1).

The patient with *M. simiae* isolated was diagnosed with definite NTM lung disease. The patient with two *M. chimaera* isolates showed dense airspace opacities on radiologic examination that recovered with standard antibiotics. He was known with fibrotic abnormalities of both upper lobes including bronchiectasis that were not changed on chest radiograph. Clinical data of the patients with one positive NTM culture (n=11) or two NTM cultures of different species (n=1) is presented in Table 2.

One patient, diagnosed with bronchiectasis, had 2 different mycobacteria cultured (*M. gordonae* and *M. nonchromogenicum*). Of the patients with a positive mycobacterial culture, six were previously diagnosed with bronchiectasis, in five patients bronchiectasis were excluded by a recent HRCT and in four patients the presence of bronchiectasis was unknown. Only the patient with *M. simiae* isolates had a *Ziehl-Neelsen* stain positive sputum smear.

The 16 patients with a positive mycobacterial culture were significantly older than patients without a positive mycobacterial culture (76.3 ± 8.4 years vs. 68.51 ± 10.7 respectively; p=0.009) (Table 3). When the patients with one positive *M. gordonae* culture (n=9, 12.3% of all the included patients) were excluded, the patients with positive mycobacterial isolates were still significantly older than patients without mycobacterial isolates (76,9 ± 7,6 years vs. 69.5 ± 10.5 respectively; p=0.009). Patients with a NTM isolated were all non-smokers at inclusion. Other patient characteristics were not associated with NTM isolation (Table 3).

Non-mycobacterial pathogens were cultured in 45 patients (62%). *Haemophilus influenzae* was the most frequently seen pathogen (cultured in 14 patients, 19.2%), followed by *Pseudomonas aeruginosa* (cultured in 12 patients, 16.4%), and *Streptococcus pneumoniae* (cultured in 5 patients, 6.8%). Positive sputum culture with non-NTM species was not associated with having a positive NTM culture (p=0.508).

Tabel 1: Clinical aspects and treatment of the patients with two mycobacteria cultured of the same species.

Patient	Sex/ Age	NTM species	Other pathogens	FEV1 (%pred)	Radiology	ATS criteria met?	Treatment	Routine follow-up
A	M/78	*M. simiae*	*S. aureus*, later: *E. cloacae*	81	HRCT: Emphysema and bronchiectasis. Nodular pattern left lower lobe.	Yes	R/E/Cla 1 year	Sputum conversion
B	M/81	*M. chimaera*	*S. pneumonia*	42	Radiograph: dense airspace opacities right lower lobe. Fibrotic lesions and bronchiectasis both upper lobes, unchanged.	No	None	*M. intracellulare* (1 x)
C	M/80	*M. gordonae*: sputum and BAL (8 weeks in between)		37	HRCT: bronchiectasis lower lobes	No	Co-trimoxazol for suspected pulmonary nocardiosis	No mycobacteria cultured
D	F/66	*M. gordonae* (isolated on same day)	None	85	Radiograph: Consolidation right upper lobe. Emphysema.	No	None	No mycobacteria cultured

ATS: American Thoracic Society; Cla: clarithromycin; E: ethambutol; E. cloacae: Enterobacter cloacae; FEV1: forced expiratory volume in 1s; HRCT: high resolution computed tomography; NTM: nontuberculous mycobacterium; %pred: % predicted; *S. aureus: Staphylococcus aureus; S. pneumonia: streptococcus pneumonia;* R: rifampicin.

Tabel 2: Clinical aspects of the patients with one positive mycobacterial culture.

Patient	Sex/Age	NTM species	Other pathogens	FEV1 (%pred)	Bronchiectasis
E	F/75	*M. gordonae*	*H. influenzae*	55	Unknown
F	M/82	*M. gordonae*	None	31	Unknown
G	M/89	*M. gordonae* *M. nonchromogenicum*	*P. aeruginosa*	53	Unknown
H	F/70	*M. gordonae*	*H. influenzae*	44	No
I	F/60	*M. gordonae*	*H. influenzae*	49	No
J	F/65	*M. gordonae*	*Serratia* species	36	Yes
K	F/73	*M. chimaera*	None	39	Yes
L	F/81	*M. gordonae*	*H. influenzae*	61	No
M	M/88	*M. gordonae*	*H. influenzae*	28	No
N	M/85	*M. gordonae*	HSB	59	Unknown
O	F/71	*M. avium*	None	41	Yes
P	M/88	*M. gordonae*	None	70	Unknown

F: female; FEV1: forced expiratory volume in 1s; *H. influenza: Haemophilus influenza*; HSB: *Haemolytic streptococcus* group B; M: male; NTM: nontuberculous mycobacterium;; *P. aeruginosa: Pseudomonas aeruginosa;* %pred: % predicted.

Table 3: Characteristics of patients with and without a nontuberculous mycobacteria cultured during the study period.

	No NTM (n=57)	NTM (n=16)	p-value
Age	68,51 (10,7)	76,3 (8,3)	0.009
Male	35 (61,4%)	8 (50%)	0.41
Post-bronchodilatation FEV1 %predicted FVC % predicted FEV1/FVC ratio	 43,1 82,5 0.40	 48,1 82,4 0,44	 0.31 0.99 0.3
Mean number of admissions in previous year	1,4	0,75	0.097
MRC dyspnea score at t=0	2.98 (1,06)	3,2 (0,86)	0.47
ICS use Beclomethason equivalent (mcg/day)	43/57=75.4% 1418 (200-4000)	13/16=81.2% 1346 (200-4000)	0.97 0.8
Oral steroid use Mean dosage (mg/day)	12/57=21.0% 6.8 (5-10)	4/16=25.0% 10 (5-15)	0.74 0.08
Smoking status Never Ex Current Unknown	 1 (1,8%) 40 (71,4%) 13 (18,1%) 1 (1,8%)	 2 (12,5%) 14 (87,5%) 0 2 (12,5%)	 * 0.19 * *
Alcohol consumption Yes No Unknown	 19 (34,5%) 32 (58,2%) 4	 2 (12,5%) 14 (87,5%) 0	 p=0.089

*: p-value not calculated because of low number of patients for that variable; FEV1: forced expiratory volume in 1s; FVC: force vital capacity; ICS: inhaled corticosteroid use; MRC: Medical Research Council; NTM: nontuberculous mycobacterium; OS: oral steroids.

Discussion

Among all the 73 patients with an acute exacerbation of COPD (AECOPD) included in this study, 22% of the patients had a positive mycobacterial culture. Such high isolation prevalence have not previously been reported in COPD patients. Ko et al.[7] performed a prospective 1-year study among AECOPD patients in Hong Kong, in which 1.8% of all the mycobacterial cultures were positive; 0.4% yielded *Mycobacterium tuberculosis* and 1.4% NTM. In the study by Ko et al. only solid media were used for mycobacterial culture which probably (partly) explains the higher NTM isolation in our study since we used both solid and liquid media. Other prospective studies concerning solely AECOPD patients have not included mycobacterial cultures in their analysis. A large retrospective analysis performed in the Netherlands showed that of all positive pulmonary mycobacterial cultures, 49% were from COPD patients, 22% of these COPD patients were found to have clinically relevant NTM disease.[3]

COPD seems to be a risk factor for pulmonary NTM presence and even NTM disease. In a study among asthma patients, those with NTM disease had a significantly worse FEV1 compared to asthmatic patients without NTM isolated.[8] This finding probably reflects chronic obstruction to be a risk factor for NTM presence or disease. Bronchiectasis is a known risk factor for NTM disease and can be found in up to 50% of patients with moderate to severe COPD.[9, 10] A prospective study among 98 patients with bronchiectasis, NTM isolation prevalence was found to be 10.2%.[11] Although no COPD diagnosis was reported for these patients, the mean FEV1- and FVC-predicted in this patient group were 63.5% and 85.8% respectively suggesting that a substantial number of COPD patients were enrolled. Severe airflow obstruction, one or more hospital admissions in the previous year, and a positive sputum culture are known factors associated with bronchiectasis in COPD patients.[9] Since these factors were commonly found among our study population, bronchiectasis may have played a central role in our observed NTM prevalence. A more definite association cannot be extracted from our data since high resolution computed tomography for bronchiectasis diagnosis was not part of the study protocol. In our study, one patient met the ATS diagnostic criteria for NTM lung disease (1.4%); this patient was previously diagnosed with bronchiectasis. In another patient with known bronchiectasis, two *M. chimaera* isolates were found but the clinical condition and dense airspace opacities improved on a short course of doxycycline. None of the patients with one positive NTM culture showed clinical abnormalities associated with possible NTM disease; this is in line with the landmark study by Tsukamura[12] and supports the microbiologic criteria of the ATS diagnostic criteria.[1]

Patients with a positive mycobacterial culture in our study were significant older than patients without a positive NTM culture. While no differences in airflow obstruction or corticosteroids use was seen between these groups, ageing may be associated with NTM presence in COPD patients. In the study by Fritscher et al.[8] asthmatic patients with NTM disease were also significantly older than asthmatic patients without NTM isolates or disease. Other risk factors to be considered are systemic and inhaled steroid use, a factor which was found to be associated with NTM disease in COPD[13] and asthmatic patients.[8] In the study by Andréjak[13] inhaled steroid use showed a dose dependent risk for NTM disease in their case-control study. Steroid use was not significantly different between patients with and without NTM isolation in our study but still, the high isolation rate of NTM in our study among patients

with an AECOPD may be associated with the frequent use of inhaled steroids found in 78% of patients in our study group. The exact role of NTM in the disease process of COPD as well as the mechanisms by which COPD predisposes to NTM lung disease warrant additional study.

M. gordonae contributed importantly to the observed high NTM prevalence (14/21 = 66.7%). *M. gordonae* is one of the most frequently isolated mycobacteria in the Netherlands,[14] and in general, pulmonary *M. gordonae* isolates in immunocompetent patients are considered clinically insignificant.[1] There was no clustering in time among the *M. gordonae* isolates which makes laboratory cross-contamination unlikely. In previous studies, *M. gordonae* isolates have been obtained from hospital tap water, and from sputum samples of patients collected after a tap water mouth rinse.[15-16] However, 11 of 95 patients in the retrospective analysis of Arnow et al. had multiple *M. gordonae* positive cultures and were classified as having *M. gordonae* colonization.[15] Still, the local immune suppression in COPD patients may lead to *M. gordonae* infection and disease and this needs to be actively ruled out.

Our data are not definite applicable to other settings since our study is a single center study. NTM are environmental mycobacteria and exposure to NTM and therefore NTM presence or disease in humans can differ per environmental setting. Multi-center studies are therefore needed to confirm our results.

In conclusion, NTM were isolated from 22% of the patients presenting with an AECOPD. NTM isolation was more frequent in elderly patients. No other factors were shown to be associated with NTM isolation in our study although bronchiectasis and steroid use can be important predisposing factors. The most frequently isolated species was *M. gordonae* and these isolates had no clinical significance. Further study is necessary to confirm our findings and to explore the association between COPD, bronchiectasis and pulmonary NTM isolation, including follow-up of patients to find out more about NTM isolation and progression to NTM disease and accelerated lung function decline in COPD.

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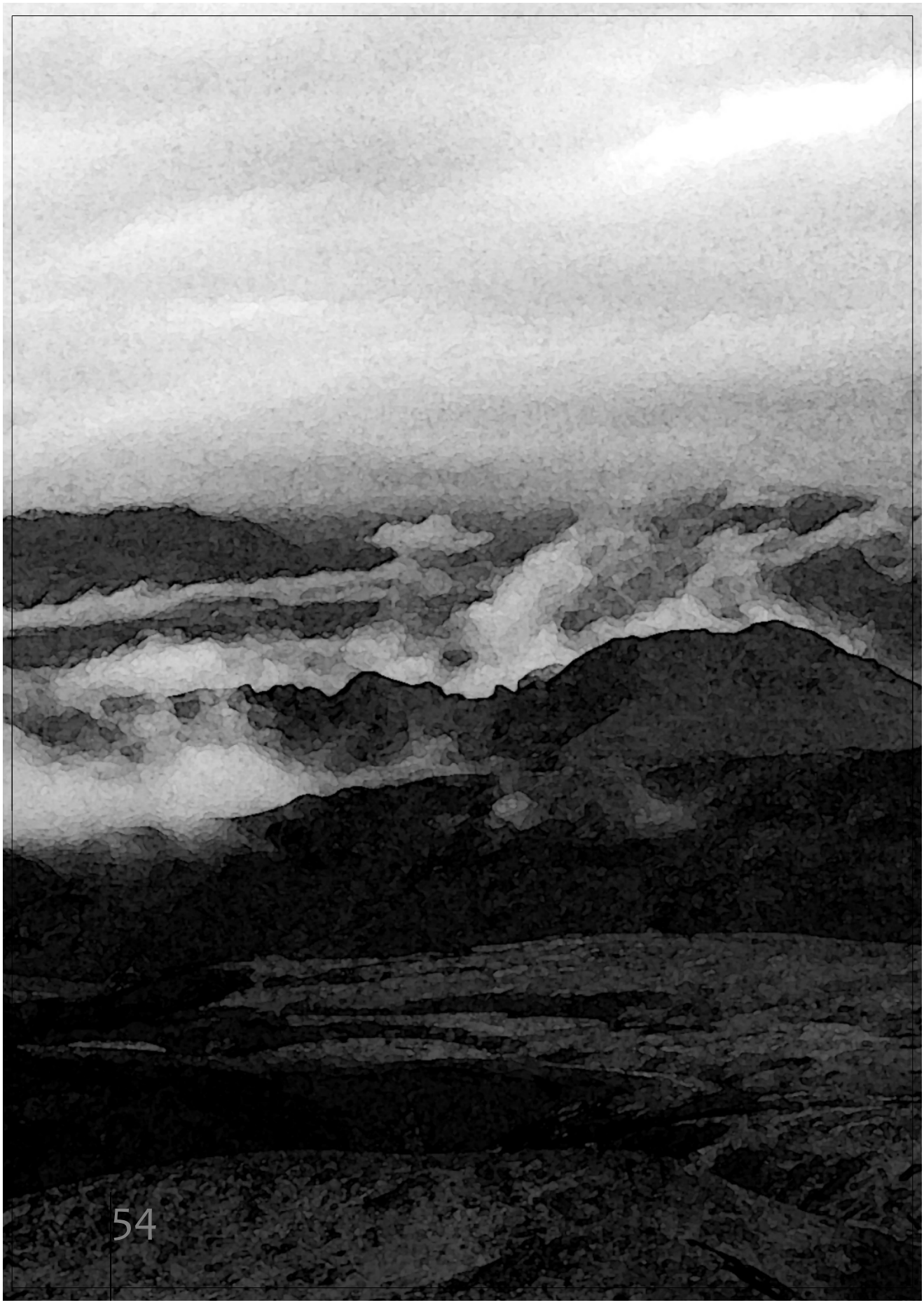
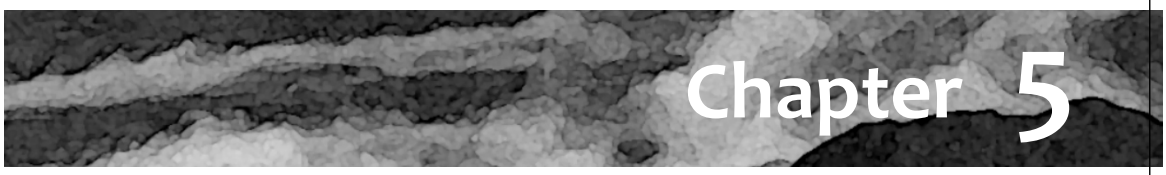


The rising incidence and clinical relevance of *Mycobacterium malmoense*

A review of the literature



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Int J Tuberc Lung Dis 2008; 12: 987-993

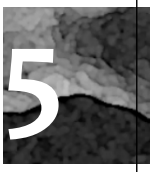
Abstract

The incidence of *Mycobacterium malmoense* infections, compared to other nontuberculous mycobacteria (NTM) has increased since 1980, especially in northern Europe. Based on various epidemiological and clinical reports outside northern Europe, there is a wide distribution of these infections. Infections with *M. malmoense* cause pulmonary disease comparable with tuberculosis (TB). The main extra-pulmonary disease type is paediatric cervical lymphadenitis. *M. malmoense* isolates are clinically significant in about 70-80% of patients. Like other NTM infections, *M. malmoense* is often found in patients with chronic obstructive pulmonary disease (COPD) and may cause serious morbidity and mortality when inadequately treated. The best treatment consists of a 2-year regimen with rifampicin and ethambutol. The literature on infections with *M. malmoense* is reviewed with respect to epidemiology, clinical presentation, treatment and outcome.

Introduction

Since Koch discovered *Mycobacterium tuberculosis* in 1882, a range of mycobacterial species have been identified. They can be divided into *M. tuberculosis* complex, *M. leprae*, and atypical mycobacteria or nontuberculous mycobacteria (NTM). NTM are also known as environmental mycobacteria due to their presence in natural water and soil.[1-2] NTM are transmitted to humans mainly from environmental sources (aerosols, ingestion). Person-to-person transmission, or transmission from animal sources has not been proven.[2-3] NTM can be subdivided in at least 120 different species with different rates of evolutionary divergence, varying biochemical characteristics, clinical presentation, clinical relevance in human beings and susceptibility to anti-mycobacterial agents. Many NTM have proven to be clinically relevant, stimulating interest for these NTM in recent years.[7]

The diagnostic criteria set in a Statement by the American Thoracic Society (ATS)[8] and those published by the British Thoracic Society (BTS)[12] are generally used to differentiate between true NTM infection, pseudo-infection and contamination. This differentiation is made using clinical, radiological and microbiological features (see Box). A diagnosis of infection with an NTM – correct or incorrect- leads to a long period of treatment with anti-mycobacterial drugs.[8,12-13] The ATS and BTS also provide evidence-based guidance on treatment of NTM infections. Increasing numbers of clinical NTM isolates have been observed in recent years.[14-15] *M. malmoense*, first isolated in 1954 from patients close to the Swedish city of Malmö, is one of the NTM that, according to the ATS and/or BTS criteria, is frequently considered clinically relevant. First described as a respiratory tract pathogen in 1977 by Schroder & Juhlin,[16] *M. malmoense* is now considered the second most serious pathogen after *M. avium* complex in northern Europe.[8,12,14] However, few references on *M. malmoense* are found in NTM treatment guidelines.[8]



The present paper reviews the current knowledge of infections with the NTM *M. malmoense* with respect to epidemiology, clinical presentation, laboratory diagnosis, treatment and outcome.

Box: Summary of the 2007 American Thoracic Society diagnostic criteria

American Thoracic Society Diagnostic Criteria of Nontuberculous Mycobacterial Lung Disease
Clinical criteria
1. Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or an HRCT scan that shows multifocal bronchiectasis with multiple small nodules.
and
2. Appropriate exclusion of other diagnoses.
Microbiological criteria
1. Positive culture results from at least two separate expectorated sputum samples. (If the results from the initial sputum samples are nondiagnostic, consider repeat sputum AFB smears and cultures.)
or
2. Positive culture results from at least one bronchial wash or lavage.
or
3. Transbronchial or other lung biopsy with mycobacterial histopathologic features (granulomatous inflammation or AFB) and positive culture for NTM or biopsy showing mycobacterial histopathologic features (granulomatous inflammation or AFB) and one or more sputum or bronchial washings that are culture positive for NTM.

Abbreviations: CT = Computed tomography, HRCT = High resolution computed tomography

Methods

We performed a literature search using Pubmed (National Centre for Biotechnology Information [NCBI]; http:// www.pubmed.gov). Medical Subject Headings (MeSH) terms "atypical mycobacteria" and "atypical mycobacterium infection" combined with *"Mycobacterium malmoense"* were used. The literature was searched from 1954 (when *M. malmoense* was firstly isolated) to 2007. Articles that used data derived from a mycobacterial reference laboratory, with a known and well defined catchment area[9] were used for describing epidemiology. The term notification is used to describe the number of isolates, irrespective of its clinical significance. Randomised clinical trials, retrospective medical file studies, and reports describing four or more patient cases were included for summarising clinical symptoms and signs, treatment and outcome.

Results

Epidemiology

Ten articles were identified that report on a well defined region and are therefore included to describe the epidemiology. Several studies described a rise in the notification of *M. malmoense* infections between 1980 and 1994 in northern Europe (table 1). The increase observed in England and Wales was more obvious than for *M. kansasii* and *M. xenopi*, making *M. malmoense* the secondmost frequently occuring NTM after *M. avium* in this region.[17] Moreover, *M. malmoense* was the secondmost frequently occurring NTM infection after *M. avium* complex in 117 non-HIV patients between 1995 and 1999 in an urban community in the UK.[18] A specific geographic clustering of *M. malmoense* infections was observed in the specific region of Lothian (Scotland), while the number of *M. malmoense* isolates as part of the total NTM isolates was not different for Lothian in comparison with the rest of Scotland.[9] Except for some case reports of (extra) pulmonary infections,[19-22] studies concerning *M. malmoense* were rare outside northern Europe until the mid-1990s.

The first retrospective study from a reference laboratory showed an increase in notification between 1993 and 1995 from 11 to 27 in the USA.[10] In the mid-1990s, a specialised laboratory in Italy received 15 *M. malmoense* isolates in 25 months.[23] An increase in the number of *M. malmoense* isolates was described in a recent multi-country retrospective survey in Europe. In the period 1985-1990, 102 *M. malmoense* isolations were reported (1.7% of all the isolated NTM), and within the period 1991-1996 this number was 463 (2.2%). This increase was mainly attributable to a rise of *M. malmoense* isolation outside the UK, especially Switzerland and Germany.[15] In the Netherlands, an average of 13 patients with *M. malmoense* isolates were notified annually in the period 2000 to 2006 (D. van Soolingen, Personal Communication, National Mycobacteria Reference Laboratory).

Despite some anecdotal reports from Scotland,[24] man-to-man transmission of *M. malmoense* is not reported . A molecular epidemiology study performed in Scotland, found no evidence for transmission between patients.[25] Environmental sources are not well defined, although the isolation of *M. malmoense* from water and soil is described.[1, 26]

Clinical Presentation

The clinical presentation of pulmonary *M. malmoense* disease sometimes mimicks pulmonary disease caused by *M. tuberculosis* with productive cough, dyspnoea, fever, haemoptysis, and weight loss.[4, 10] Radiological features of pulmonary *M. malmoense* infections are mostly impressive cavitation and/or infiltration (Figure 1 and 2).[4, 6, 11, 27] Most patients with pulmonary infection are males, with a mean age of 60 years.[5-6, 11] Extra-pulmonary *M. malmoense* infection is rare, except for lymphadenitis in children and tenosynovitis.[28-30] In England and Wales, in the study period 1982 to 1994, 83% of the *M. malmoense* infections presented in the lung, 7.7% in lymph nodes, 4% in the skin or wounds and another 3.4% as abscesses.[28]
In Sweden, 221 cases of *M. malmoense* infection were described , identified between 1968 and 1989, of which 21% were of extra-pulmonary origin.[6] A detailed review of the extra-pulmonary cases has been presented by Zaugg et al. who concluded that extra-pulmonary infection with *M. malmoense* is rare and that dissemination is only observed in patients with severely impaired immunity.[29]

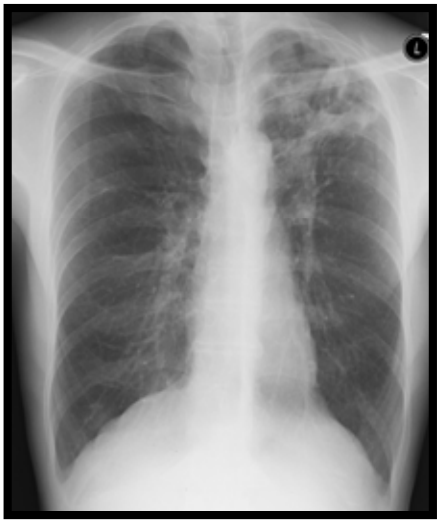


Figure 1 Chest radiograph showing a cavity in the left upper lobe in a patient with positive sputum smears with a cultured *M. malmoense* in a patient suffering COPD. (source: Hospital Drachten, the Netherlands. With permission).

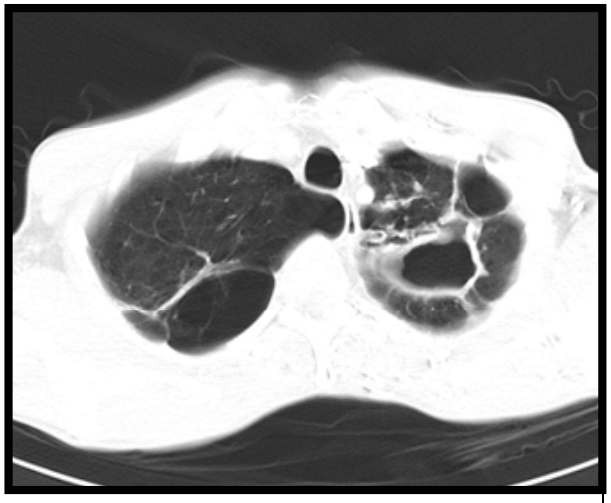


Figure 2 Computed Tomography scan of the thorax of the same patient as in figure 1 showing widespread cavitation in the left upper lobe bullous changes in the right lung as well around the cavity. (source: Hospital Drachten, the Netherlands).

In a Danish survey, *M. malmoense* was found to be clinically relevant in 83% of patients with *M. malmoense* isolates.[11] The clinical significance of *M. malmoense* in the USA was remarkably lower,[10] than in studies performed in northern Europe. In Italy, 10 of 15 patients from whom *M. malmoense* isolates were obtained had a clinically significant infection.[23] The clinical relevance of *M. malmoense* isolation in different studies is mentioned in Table 2.
The prevalence of underlying diseases in *M. malmoense* infections is summarized in Table 3. Infections with *M. malmoense* are prevalent among patients with pre-existing pulmonary disease, especially chronic obstructive pulmonary disease (COPD) (26-45%).[11, 19] In the BTS trial 55% of the studied patients with a clinically significant *M. malmoense* infection had a history of chronic bronchitis, emphysema or asthma and old, healed TB. In the urban community of Leeds, UK, infection by *M. malmoense* was especially prevalent among COPD patients, more so than other NTM, including *M. avium* and *M. kansasii*.[19] Two patients with clinical symptoms due to HIV infection in a group of seven patients with clinically relevant infection were found in the USA.[10] Bollert et al.[9] reported one patient with AIDS and 19 HIV-negative patients with clinically relevant *M. malmoense* infections. In Italy, two of 10 patients with clinically significant *M. malmoense* infection had AIDS; both were suffering from cavitary pulmonary disease.[23] Case reports describing *M. malmoense* infections in HIV-positive patients are uncommon, but dissemination has been reported.[29, 31, 32] Immune suppression due to chronic steroid use has been described in 3.5-13% of patients with *M. malmoense* infection (Table 3).

Table 1 Epidemiology: notification of *M. malmoense* (total number of positive isolates irrespective of clinical significance) in different geographical areas.

Author (reference)	Study area	Study period	Notification (year)
Banks, 1985[4]	Wales	1978-1983	Total: 46
Jenkins, 1985[28]	England and Wales	1954-1984	every year n=1-4 (1954-1980) 10 (1981) 31 (1984)
France, 1987[24]	Scotland	1982-1984	Total: 20
Katila, 1991[5]	Finland	1971-1987	Total: 66
Henriques, 1993[6]	Sweden	1968-1989	19 (1968-1977) 22 (1978-1980) 39 (1981-1983) 70 (1984-1986) 71 (1987-1989)
Bollert, 1995[9]	Scotland	1990-1993	Total: 95
Lamden, 1996[17]	England and Wales	1982-1994	10 (1982) 80 (1993)
Buchholz, 1998[10]	USA	1993-1995	11 (1993) 35 (1994) 27 (1995)
Thomsen, 2002[11]	Danmark	1995-1996	Total: 20
Henry, 2004[18]	Leeds	1995-1999	3 (1995) 5 (1996) 6 (1997) 2 (1998) 5 (1999)

Table 2 Clinical significance of *M. malmoense* in different studies. The criteria for determining clinical significance used in the different studies are presented in column 3. ATS= criteria as determined by the American Thoracic Society (6). Modified ATS: bacteriological criteria were modified to criteria used in Denmark (not further specified).

Author (reference)	Patients (n)	Criteria	Clinical significance
Banks, 1985[4]	34	Repeated isolates compatible symptoms & signs	84%
Katila, 1991[5]	66	Unclear	81%
Henriques, 1993[6]	221	ATS	70%
Bollert, 1995[9]	41	ATS	75%
Buchholz, 1998[10]	60	ATS	10%
Thomsen, 2002[11]	20	Modified ATS	Bacteriological criteria: 83% Radiographic criteria: 100% Clinical criteria: 88%

Table 3 Age, HIV, immune suppression and co-morbidity among *M. malmoense* infected patients. PTB= pulmonary tuberculosis, PD = pulmonary disease, PF = pulmonary fibrosis. * = Co-morbidity in patients with pulmonary *M. malmoense* infection (n=131).

Authors	Patients (n)	% Male	HIV	Immune suppression	COPD	Other co-morbidity
Banks, 1985[4]	34	60	unknown	12% (chronic steroids n=3; immunosupp. n=1)	47%	Bronchiectasis: 6% Coal workers pneumoconiosis: 9% History PTB: 9% Peptic ulcers: 36%
France, 1987[24]	20	65	unknown	10% (chronic steroids)	45%	Pneumothorax: 10% TB, PF, aspergillosis, pneumoconiosis: all n=1 Malignancy: 20% Peptic ulcers: 40%
Henriques, 1993[6]	221	53	unknown	13% (chronic steroids)	unknown	History PTB: 33%* (12% also PD) PD alone: 28%* Malignancy: 11%* Leukemia: 1.5%*
Lamden, 1996[17]	470	unknown	Unknown	3.5%	unknown	unknown
Bollert, 1995[9]	41	unknown	Positive: n=1	unknown	unknown	unknown
Buchholz, 1998[10]	73[6]	62	Positive: n=2 (both active AIDS)	No	n=2 (33%)	History of PTB n=2 (33%)
Thomsen, 2002[11]	13	69	None	None	n=6	Cancer n=1
BTS, 2003[33]	106	62	Excluded	13% (condition?)	CB/ Emph/A: 26%	old/cured PTB: 21% Bronchiectasis: 1%
Henry, 2004[18]	18	unknown	Excluded	unknown	44%	Bronchiectasis: 5%; History PTB: 10%;

Labaratory diagnosis

Although acid-fast bacilli (AFB) smear microscopy is still the starting point, the laboratory diagnosis of NTM disease has changed dramatically over the past two decades. The recent introduction of liquid culture systems has substantially shortened the time to detection for NTM, as well as increasing sensitivity. The disadvantage of these liquid culture systems is that they do not allow quantification and assessment of colony morphology.[8]

Identification of NTM has long been based on growth rate, colony morphology and a series of biochemical tests performed on live cultures, combined with, or substituted for, cell wall fatty acid composition analysis.[7, 8] The advent of molecular tools, especially 16S rDNA gene sequence analysis, has improved and accelerated this identification process. In addition, these techniques have shed new light on mycobacterial taxonomy.[7, 8, 32]

The differences (two basepairs [bp]) between *M. malmoense* and *M. szulgai* have long been considered one of the drawbacks of 16S sequencing. However, one bp difference can result in important phylogenetic differences. Furthermore, sequencing is still expensive and technically demanding, and its use is therefore limited to large or reference laboratories. Nevertheless, 16S sequencing and high-performance lipid chromatography, both widely used for mycobacterial identification, can be successfully applied to identify *M. malmoense*.[8]

To provide molecular identification suitable for use in a clinical setting, line probe assays have been developed that use DNA probes rather than sequencing for identification of mycobacteria. A multitude of commercial line probe asays is now available, most of which include M. malmoense-specific probes.

The in vitro drug susceptibility testing (DST) of *M. malmoense* has not been found to be associated with response to treatment.[6, 33-35] The only published correlation linked ethambutol (EMB) resistance to treatment failure of a 2-year rifampicin (RMP) and EMB based regimen.[33] However, in vitro resistance to EMB and RMP, tested separately, has been observed in strains susceptible to a combination of the two drugs.[36]

Management

Current guidelines recommend that treatment for pulmonary *M. malmoense* infections consists of a 18-24 months regimen with EMB and RMP.[12] The ATS guidelines published in 2007 suggest a three drug regimen with RMP, isoniazid (INH) and EMB[8]. Banks et al.[4] and France et al.[24] concluded that adding EMB to other first-line drugs improved outcome, supported by a synergistic effect with EMB observed in in vitro tests. Patients receiving second-line drugs had poorer outcome related to drug toxicity and poor patient adherence.[6, 33] According to a special research committee of the BTS, a 2-year regimen of RMP and EMB is better tolerated than RMP and EMB combined with INH. This recommendation is based on a randomised clinical trial in 106 non-HIV patients with pulmonary *M. malmoense* infection.[33, 35] Ten per cent of the patients remained culture-positive after 2 years of treatment with RMP and EMB, with or without INH. Henry et al.[18] found a significant association between appropriate treatment and a successful outcome in non-HIV patients with clinically relevant pulmonary and extra-pulmonary NTM infections, including infections by *M. malmoense* (n=18). Appropriate therapy was defined as adherence to the BTS criteria and treatment for 2 years with RMP + EMB for *M. malmoense*. Forty-four per cent received appropriate therapy. In this group, 25 %

died, in contrast to 80% deaths in the group treated for less than 12 months.
Furthermore, the involvement of two or more lung zones is found to be an independent predictor of death.[33] A recently published in vivo clinical trial studying the combination of RMP + EMB with quinolones (ciprofloxacin) or macrolides (clarithromycine [CLM]) has not shown improved outcome of these adjunctive treatment modalities in *M. malmoense* infections. Because of a higher observed "all-cause death rate" among patients treated with RMP + EMB and ciprofloxacin and the observation of more significant side-effects among RMP + EMB and CLM treated patients, first line treatment for pulmonary *M. malmoense* disease is still RMP + EMB.[37]

Discussion

An increase in the number of clinical *M. malmoense* isolates has been observed, especially in northern Europe, since the 1980s. This increase is more pronounced for *M. malmoense* than in other clinically significant NTM.[14, 28] Possible explanations for this increase are: 1) changes in host defence mechanisms or a larger number of patients with impaired immunity; 2) increased awareness or focus of physicians on requesting mycobacterial sputum cultures; 3) selection within NTM due to environmental factors, for example differences in susceptibility to desinfectants used in tap water; 4) improved culture methods, e.g. liquid media; and 5) improved identification techniques for NTM.
In the early 1980s, reports of *M. malmoense* isolation were mostly from northern Europe, some suggesting regional clustering.[38] The prevalence of *M. malmoense* outside northern Europe remains unclear with an increase in notification in the USA[10] and the presence of *M. malmoense* in the environment of the Democratic Republic of Congo (former Zaire)[22] and Japan[26] but absence of isolation in Ontario, Canada.[39] Some geographically determined environmental niches (water reservoirs) or characteristics (temperature) may favour survival of the mycobacterium and thus transmission to humans.[1, 2] Further molecular epidemiology studies are needed to search for environmental resources and transmission vectors for human infections.
Infections with *M. malmoense* cause considerable morbidity and mortality. Studies performed in northern Europe found clinically relevant infection (defined by fulfilment of the ATS diagnostic criteria or a modification of them) in over 70% of cases. Clinical relevance of pulmonary *M. malmoense* in the Netherlands is found to be over 80% as determined by a retrospective study and, in comparison with other NTM, the most relevant.[40] In the USA a retrospective reference laboratory study found only 10% of clinical isolates to be clinically significant between 1993 and 1995, according to the ATS criteria.[10] Confounding factors include differences between countries regarding indications for mycobacterial strains and cultures and variation in the assessment of clinical relevance. Differences in pathogenicity of *M. malmoense* between continents have not yet been studied.

COPD, and especially emphysema, is frequently observed as an underlying condition in *M. malmoense* infections. The pathological conditions of COPD patients (impaired local immunity) probably favour infection with *M. malmoense*. It is not known if infection with M. malmoense occurs more frequently in severe COPD. The male predominance may be partly explained because, at least in the past, males more often suffer COPD and therefore

have a higher probability to be evaluated for, or to be infected by, NTM. While few cases of pulmonary *M. malmoense* infection have been described in HIV positive patients, in some epidemiological studies a considerable number of patients are found to be HIV positive,[10, 23] together with case reports describing *M. malmoense* dissemination in patients with HIV with advanced disease (CD4 count<100 cells/µl).[31-32] Recent data suggest an underestimation of clinically relevant NTM infections among HIV-positive and HIV–negative patients in Africa.[41] Further study is therefore needed.

The BTS guidelines supply treatment guidance based on a solid randomized clinical trial that was designed also for *M. malmoense* infections, and they can therefore be recommended. Inadequate treatment of *M. malmoense* infections may cause significant mortality in COPD patients. In vitro evidence suggests increased susceptibility for a combination of RMP, EMB and CLM when compared to the standard treatment regimen of RMP and EMB[33]. There is no evidence that DST contributes to the choice of treatment regimen. However, in vitro testing may be helpful in choosing alternative drug regimens in treatment failure cases. To date, there is no evidence for the additional value of INH or CLM, for example in patients with treatment failure or relapse.[37]

In conclusion, rising notifications of *M. malmoense* observed in different countries have been more obvious than those of other clinical relevant NTM, and it is responsible for clinical relevant disease, especially in COPD patients. Epidemiological studies remain important to assess a possible further increase in incidence. The incidence of clinically significant infections should be investigated in different countries. Genotyping of *M. malmoense* is of great value as a tool for studying epidemiology, as well as for identifying suspected sources of infection from the environment. While the only evidence based treatment is 18 months RMP and EMB, more studies are needed, including studies of other drug combinations.

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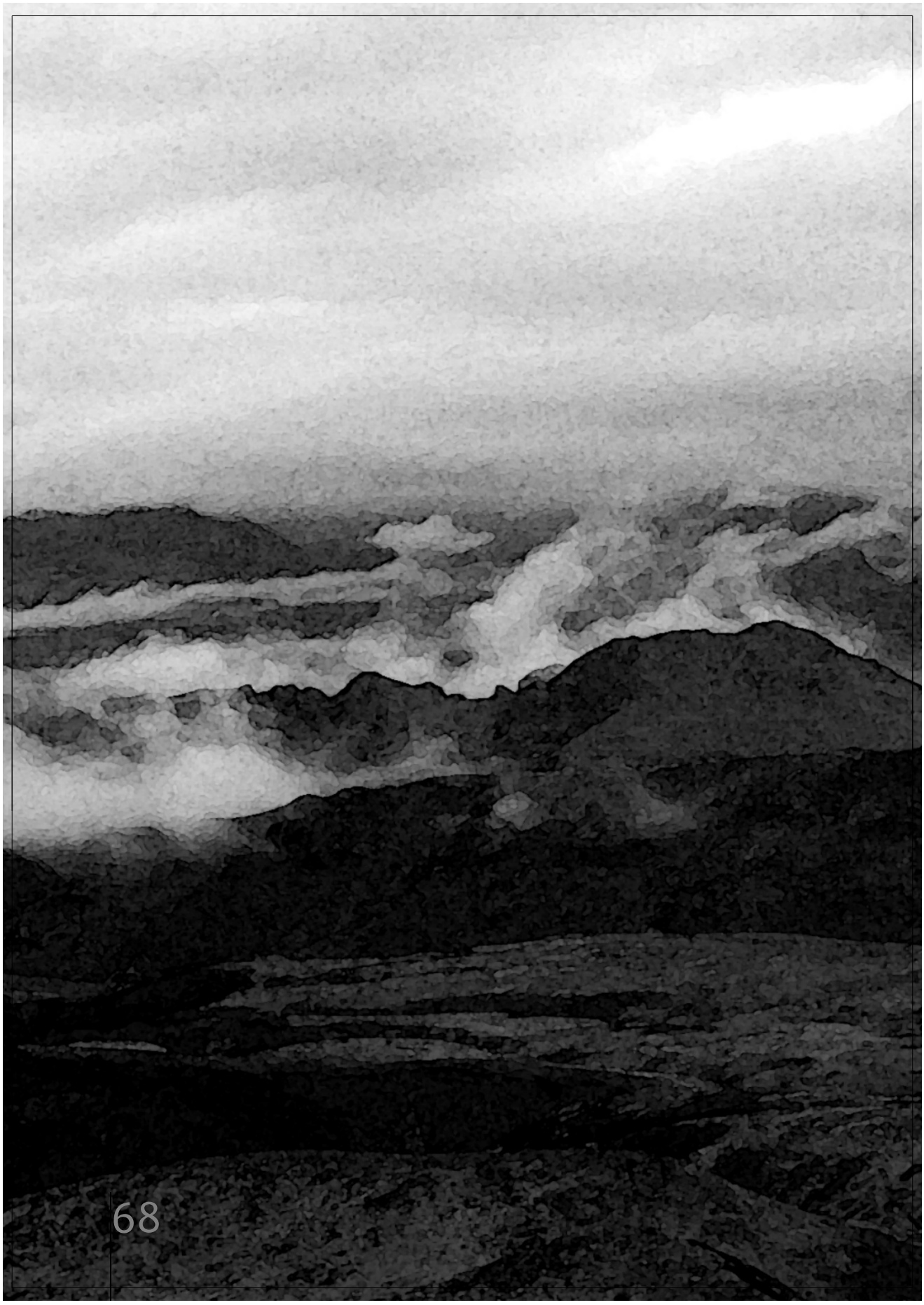
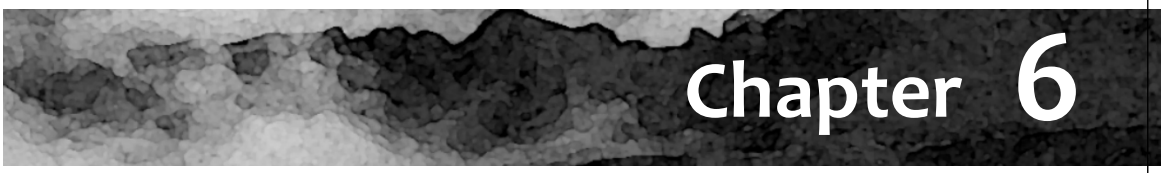


Clinical relevance of *Mycobacterium malmoense* isolation in the Netherlands



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Abstract

Uncertainty exists about the clinical relevance of *Mycobacterium malmoense* isolation, especially in pulmonary samples. We therefore determined clinical relevance, treatment and outcome of *M. malmoense* isolation in the Netherlands.

A retrospective medical file study was conducted for all patients in the Netherlands from whom *Mycobacterium malmoense* had been isolated between January 2002 and January 2006. Diagnostic criteria for nontuberculous mycobacterial (NTM) disease published by the American Thoracic Society (ATS) were used to determine clinical relevance. Treatment was compared with guidelines published by the British Thoracic Society.

In total, 51 patients were found from whom *M. malmoense* was isolated. Of these, 40 (78%) patients had pulmonary isolates and 32 (80%) of them met the ATS diagnostic criteria. Cavitary disease was most common (n=28; 88%). Patients with pulmonary disease were mostly males, with an average age of 56 years and pre-existing chronic obstructive pulmonary disease. Cervical lymphadenitis was the most common extrapulmonary disease type. Adherence to treatment guidelines was poor. A good clinical response to treatment was observed in 70% and 73% of patients treated for pulmonary and extrapulmonary disease, respectively.

In conclusion, *M. malmoense* is a clinically highly relevant NTM in the Netherlands causing serious pulmonary morbidity. Adherence to treatment guidelines is not satisfactory.

Introduction

First described as a respiratory tract pathogen in 1977 by Schröder and Juhlin,[1] *Mycobacterium malmoense* is among the most frequently isolated and clinically relevant nontuberculous mycobacteria (NTM) in northern Europe.[2-4] The environment is the suspected source of transmission of NTM to humans through aerosols and ingestion. Person-to-person transmission or transmission from animal sources has not been proven[3, 5-6]. The presence of NTM in the environment implies that a NTM cultured from a non-sterile body site, such as the respiratory tract, may result from contamination or occasional presence of the NTM in a sample. Hence, it is important to distinguish contamination from true NTM disease. The American Thoracic Society (ATS) has published guidelines to assist in this distinction.[3] The clinical relevance of a NTM species can be quantified by assessing the percentage of patients with positive cultures of the respective NTM who meet the ATS diagnostic criteria.
In the current study, we quantified the clinical relevance of *M. malmoense* isolation in the Netherlands between 2002 and 2006 by applying the ATS diagnostic criteria, and evaluated treatment and outcome.

Materials & Methods

Study subjects

Patients were identified by reviewing the database of the Dutch National Institute for Public Health and the Environment (RIVM; Bilthoven, the Netherlands) for *M. malmoense* positive cultures. The RIVM is the national mycobacteria reference laboratory that provides identification and drug susceptibility testing for all hospitals in the Netherlands. We reviewed medical records of the identified patients from whom *Mycobacterium malmoense* was cultured between January 2002 and January 2006.

Study design and setting

The present study was a retrospective observational study concerning all patients with *M. malmoense* positive cultures identified at the RIVM. The Central Committee on Research involving Human Subjects, Arnhem-Nijmegen regional office (Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands) approved the study. We recorded demographic data, clinical data, drug susceptibility, treatment and outcome. The 2007 ATS diagnostic criteria were used to determine clinical relevant infections (Box 1).[3]
Treatment was compared with guidelines published by the British Thoracic Society (BTS): a NTM based treatment regimen was defined as consisting of rifampicin or rifabutin and ethambutol.[7] An adequate response to treatment was defined as symptomatic improvement and reversion to at least three subsequent negative cultures.

The RIVM subjected isolates of most patients to laboratory diagnosis. All patients' isolates were subcultured in both liquid and solid media and identified using the Inno-Lipa Mycobacteria v2 (Innogenetics, Gent, Belgium) reverse line blot assay which has specific probes for *M. malmoense*. Prior to 2004, 16S rDNA gene sequencing (151bp hypervariable region A) was

performed, after ruling out membership of the *M. tuberculosis* or *M. avium* complex using the AccuProbe assays (GenProbe, San Diego, USA). Remaining isolates were identified at local hospitals, by 16S sequencing.
Drug susceptibility was tested using the agar dilution method.[8] Drugs included in the test panel were isoniazid, rifampicin, ethambutol, streptomycin, cycloserine, prothionamide, amikacin, ciprofloxacin, clofazimine, clarithromycin, and rifabutin.

Data analysis

Pearson Chi-squared and Fisher exact tests were used for statistical correlations.

BOX 1: SUMMARY OF THE 2007 AMERICAN THORACIC SOCIETY DIAGNOSTIC CRITERIA

American Thoracic Society Diagnostic Criteria of Nontuberculous Mycobacterial Lung Disease
Clinical criteria
1. Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or an HRCT scan that shows multifocal bronchiectasis with multiple small nodules.
and
2. Appropriate exclusion of other diagnoses.
Microbiological criteria
1. Positive culture results from at least two separate expectorated sputum samples. (If the results from the initial sputum samples are nondiagnostic, consider repeat sputum AFB smears and cultures.)
or
2. Positive culture results from at least one bronchial wash or lavage.
or
3. Transbronchial or other lung biopsy with mycobacterial histopathologic features (granulomatous inflammation or AFB) and positive culture for NTM or biopsy showing mycobacterial histopathologic features (granulomatous inflammation or AFB) and one or more sputum or bronchial washings that are culture positive for NTM.

Results

M. malmoense was isolated from 51 patients in the study period. In total, 40 (78%) patients had pulmonary isolates; in 11 (22%) cases these were of extra-pulmonary origin. No patients in the study group were HIV-infected.
During the study period, no increase in notification of *M. malmoense* isolation was observed each year.

Pulmonary isolates

Of all 40 patients with pulmonary *M. malmoense* isolates, 32 (80%) met the ATS diagnostic criteria and were likely to suffer *M. malmoense* lung disease. The baseline patient characteristics are detailed in Table 1.

Table 1: Baseline characteristics of patients with a *Mycobacterium malmoense isolates*

	ATS criteria met	ATS criteria not met	Total	P value
N	32	8	40	
Males	21 (66)	7 (88)	28	0.67
Mean age (range)	56 (28-81yr)	57 (33-83yr)	56 (28-83yr)	0.84
Pre-existing pulmonary disease	26 (81)	7 (88)	33	0.57
COPD	21 (66)	5 (63)	26	0.59
Prior TB	2 (6)	2 (25)	4	0.18
AFB smear positive	27 (84)	2 (25)	29	0.03
Cavitary lesion	28 (88)	1 (13)	29	< 0.001
Nodular lesion(s)	4 (13)	1 (13)	5	0.74

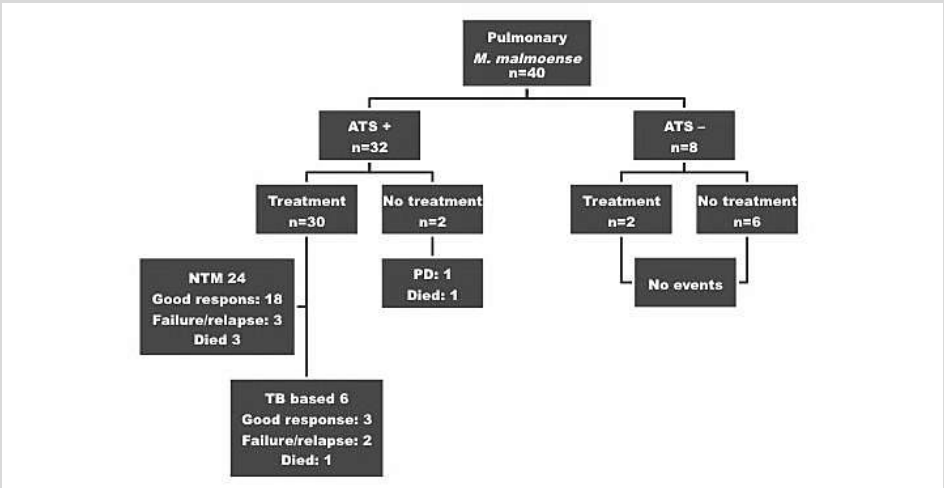
Data are presented as n or n (%). ATS: American Thoracic Society; COPD: Chronic obstructive pulmonary disease; TB: tuberculosis; AFB: acid fast baccilli

The predominant patient profile is a male with pre-existing pulmonary disease, mainly chronic obstructive pulmonary disease (COPD). The seven patients without a previous diagnosis of pre-existing pulmonary disease were mostly smokers, with radiographic features suggestive of pulmonary disease. Most patients reported productive cough (n=37; 93%), weight loss (n=24; 60%), and fatigue (23; 58%). Night sweats (n=10; 25%), hemoptysis (n=7; 8%) or fever (n=11; 28%) were infrequently reported. Only patients who reported weight loss were more likely to meet the ATS diagnostic criteria (p=0.048; odds ratio 7.333; 95%CI 1.072-50.145). In the group of patients that did not meet the ATS diagnostic criteria, four failed to meet the bacteriological criteria (three because only one sputum sample was collected) and four failed to meet the bacteriological and radiological criteria. Overall, 75% (n=24) of the 32 patients that met the ATS criteria for pulmonary NTM disease presented with cavitary lesions visible on chest radiographs. Additional computed tomography scanning revealed 4 extra cases of cavitary disease (total n=28; 88%), not identified as such using plain chest radiographs. Two patients presented with multiple nodular opacities on chest radiograph; two had a single pulmonary mass.

The 30 patients who met the ATS diagnostic criteria for pulmonary NTM disease started treatment. Figure 1 summarizes treatment and outcome in the study group. The mean duration of antimycobacterial treatment was 12 months (range 1–26 months). Macrolides were added in 22 patients (18 clarithromycin, 4 azithromycine; 92%), fluoroquinolones in six patients (four ciprofloxacin, two moxifloxacin; 25%). Nine patients received therapy for presumed tuberculosis, prior to the diagnosis of NTM disease, for a mean duration of 48 days (range 2-123 days), and completed a NTM based regimen afterwards. Six patients with *M. malmoense* pulmonary disease only received a complete first-line tuberculosis treatment.

Of the 30 patients treated, 21 (70%) patients showed an adequate response, five (17%) suffered a failure or relapse (mean time to relapse 13 months, range 5-24 months) and four (13%) died (Figure 1). Although the percentage of patients with an adequate response was lower in those receiving macrolide containing regimens (43 versus 63%), this difference was not statistically significant (p=0.344). The mean duration of treatment among patients who later relapsed was shorter than for patients with an adequate response, although not significantly (320 versus 358 days; p=0.709). The frequency of adequate response was not significantly different between patients treated with a tuberculosis-based regimen and those treated with a NTM regimen (p=0.260). Two patients who met the ATS criteria refused treatment: one patient died, the other showed progressive disease. Follow-up of patients not meeting ATS diagnostic criteria was uneventful; no more positive cultures were recorded. Symptoms regressed in absence of antimycobacterial treatment.
Six (20%) patients received the 24 months of rifampicin and ethambutol regimen based on the BTS trials. This did not affect the percentage of patients with an adequate response (83% for the BTS regimen versus 71% for other regimens; p= 0.426).
Contact-tracing studies were initiated for two patients with pulmonary *M. malmoense* isolates; both were presumed to have pulmonary tuberculosis. Some contacts received 6 months of isoniazid, based on a tuberculin skin test conversion.
In the current study, one case of disseminated *M. malmoense* disease was noted in a patient who received immunosuppressive treatment after kidney transplantation. He presented with pulmonary M. malmoense disease, which extended to histologically and bacteriologically proven lymphadenitis and mediastino-esophageal fistula, with blood cultures yielding *M. malmoense*. Interestingly, this patient had strong epidemiological links to a patient diagnosed with smear positive pulmonary *M. malmoense* disease one year before.

Figure 1: Frequency, treatment and outcome of pulmonary *M. malmoense* disease in the Netherlands between 2002 and 2006.



NTM based: treatment given to treat nontuberculous mycobacteria; TB based: group of patients treated only for a presumed tuberculosis infection; ATS+: fulfilment of the criteria of the American Thoracic Society; PD: progressive disease.

Extra-pulmonary isolates

Eleven patients had extra-pulmonary *M. malmoense* isolates; we noted 10 cases of cervicofacial lymphadenitis, including two in elderly patients. One case of tenosynovitis of the second and third digit of the right hand was observed in a plant handler with a history of multiple wounds to the right wrist. He had an adequate response after surgical debridement followed by a macrolide based regimen of 13 months' duration. The eight pediatric cases of lymphadenitis were three boys and five girls without predisposing conditions, with a mean age of 36 months (range 22-46 months). All presented with painless cervical or submandibular swelling, without fever or other symptoms. Surgical excision was the most frequent treatment and resulted in an adequate response in all patients. One of the elderly patients had a relapse after surgery; the other had an adequate response. Overall, eight (73%) patients with extra-pulmonary isolates had an adequate response after the initial therapy.

In vitro drug susceptibility testing was performed on the primary isolates from 46 patients. Isolates were resistant to isoniazid (all), streptomycin (70%), amikacin (70%), ciprofloxacin (61%); intermediately susceptible (39%) or resistant (46%) to ethambutol; and susceptible to rifampicin (72%), rifabutin (96%), clarithromycin (all), cycloserine (98%), prothionamide (96%) and clofazimine (all). Relapse or treatment failure among patients with pulmonary *M. malmoense* disease was not associated with in vitro rifampicin or ethambutol resistance (p=0.327 and p=0.405 respectively).

Discussion

M. malmoense is one of the most clinically relevant NTM in the Netherlands. Overall, 80% of patients with pulmonary isolates met the ATS diagnostic criteria, compared to 21 (47%) relevant infections among 45 patients with pulmonary *M. xenopi* isolates, and 11 (73%) among 15 patients with pulmonary *M. szulgai* isolates.[9, 10] We observed *M. malmoense* disease exclusively in HIV-negative patients, which is in contrast to *M. avium, M. kansasii, M. xenopi* and *M. szulgai*.[3, 9-10] Patients were mainly males with pre-existing pulmonary disease. The ATS diagnostic criteria were designed for infections with *M. avium, M. kansasii* and *M. abscessus*; they may be less applicable to *M. malmoense*. Because of the high degree of true pulmonary *M. malmoense* infections observed, judgment on the clinical relevance of pulmonary *M. malmoense* isolates could probably be based on less strict criteria, as is advocated for *M. kansasii*,[3] to prevent a prolonged period of inadequate treatment. The high degree of clinical relevance is in accordance with previous observations from northern Europe, varying from 70% to 84% using either ATS criteria or a modification of these criteria.[11-14]

Interestingly, a dramatically lower clinical relevance of 10% was found in a retrospective case study of 73 patients in the USA.[15] There is no explanation for this difference; however it suggests less pathogenic strains of *M. malmoense* in northern America compared with northern Europe. There are no known bacterial virulence factors for *M. malmoense*. Phylogenetically related *M. szulgai* and *M. kansasii*, both of which are suggested to be among the most pathogenic NTM [3, 9] are known to harbor a region of difference 1-like genetic element (including esat-6 and cfp-10 genes) which is a well-known virulence factor for *M.tuberculosis*.[16] *M. malmoense*,

however, lacks this element.[17] To date, immunological studies have focused on *M. avium* and *M. abscessus*,[18-19] rather than *M. malmoense*. Studies of *M. malmoense* pathogenesis and virulence in murine models are warranted, as are studies on the role of host genetic factors in *M. malmoense* disease.
In a recently published retrospective study of the prevalence of all NTM in Ontario, Canada, between 1997-2003, *M. malmoense* was not isolated.[20] This observation is in contrast with the increase in *M. malmoense* notification in Europe since 1980, including increasing numbers of countries reporting isolation of *M. malmoense*.[21] This contrast suggests environmental niches favouring transmission to humans in Europe. Whether this observation can be linked with the lower clinical relevance observed in the United Sates needs to be studied.

Human transmission has never been proven, even in a setting of geographic clustering of cases. In a study published by Doig et al., small differences observed using pulsed-field electrophoresis were sufficient to show a lack of correlation between strain type and epidemiological or patient characteristics, making person-to-person spread unlikely.[22]
The BTS treatment guidelines for pulmonary *M. malmoense* disease were not well adhered to, including choice of antimycobacterial therapy and duration of therapy. Treatment of *M. malmoense* disease was often preceded by or consisted only of tuberculosis treatment. This observation reflects the similar clinical presentation of pulmonary *M. malmoense* and *M. tuberculosis* complex infection and is a cause of concern. Increasing the use of PCR to rule out *M. tuberculosis*, providing a quick and definite NTM diagnosis, will probably decrease morbidity and mortality and prevent initiation of unnecessary contact tracing studies. Although hampered by our limited study group size and duration of follow-up, successful clinical response (83%) in the current study in the optimally treated group is comparable with a 75% successful outcome found by Henry et al.[23] The failure and relapse rates found in the recently published BTS trial (12% in the group treated with R and E, 5% in the group treated with R and E combined with clarithromycine or ciprofloxacin) are lower compared to the rate found in our study (17%).[24] The shorter mean duration of antimycobacterial therapy probably negatively influenced treatment outcome in our population.

The mortality rate (13%) found among adequately treated patients in the present study is comparable to that found by Banks et al (15%),[11] and Henry et al (11%).[23] The NTM disease-related mortality after 5 years of follow-up was found to be low (3,6%).[24] Mortality has been related to the length of delay between diagnosis and start of the treatment, while the occurrence of relapse has previously been associated with total time span of treatment.[11] Other factors suggested to independently affect mortality are in vitro resistance to ethambutol and the involvement of more than one lung zone.[15] In our study population, there was no significant association between ethambutol resistance and treatment failure.
The recently published BTS trial showed no additional benefit of adjunctive clarithromycin or ciprofloxacin over the 24-month regimen of rifampicin and ethambutol for pulmonary M. malmoense disease. The addition of clarithromycin even led to more side-effects.[24] These data are clinically important considering the extent of adjunctive macrolide and/or fluorquinolone use in our study group.
The frequency and types of extrapulmonary disease in our study are similar to those found in a survey in Sweden, in which 21% out of 221 patients had extra-pulmonary isolates, mainly lymphadenitis.[14] Pediatric cases of cervicofacial lymphadenitis are most frequent and tend

to affect children in a limited age range, which may be related to environmental exposures specific to this age category,[25] or the state of development of the immune system in children. Contrary to pulmonary disease, *M. malmoense* lymphadenitis is a relatively benign condition. Surgery is considered to be the optimal treatment and yields good results [3, 26].[3, 26]
Tenosynovitis due to *M. malmoense* is rare, although case reports are available in the international literature.[27] Extra-pulmonary infection with *M. malmoense* is rare and dissemination is only observed in patients with severely impaired immunity, although rarely in HIV/AIDS.

In conclusion, pulmonary *Mycobacterium malmoense* isolation is clinically relevant in 80% of all patients in the Netherlands, reflecting a level of virulence unmatched by other NTM species. Pulmonary disease resembling tuberculosis, and pediatric lymphadenitis are the most common types of *M. malmoense* disease. Some patients are incorrectly treated for tuberculosis for a lengthy period. We recommend the use of molecular diagnostic tools for every sample positive for mycobacteria to enable quick initiation of adequate therapy. Treatment outcome is relatively favourable when compared to other NTM infections. Future studies are necessary to optimize treatment regimens and to discern host and pathogen factors determining virulence and transmission to humans.

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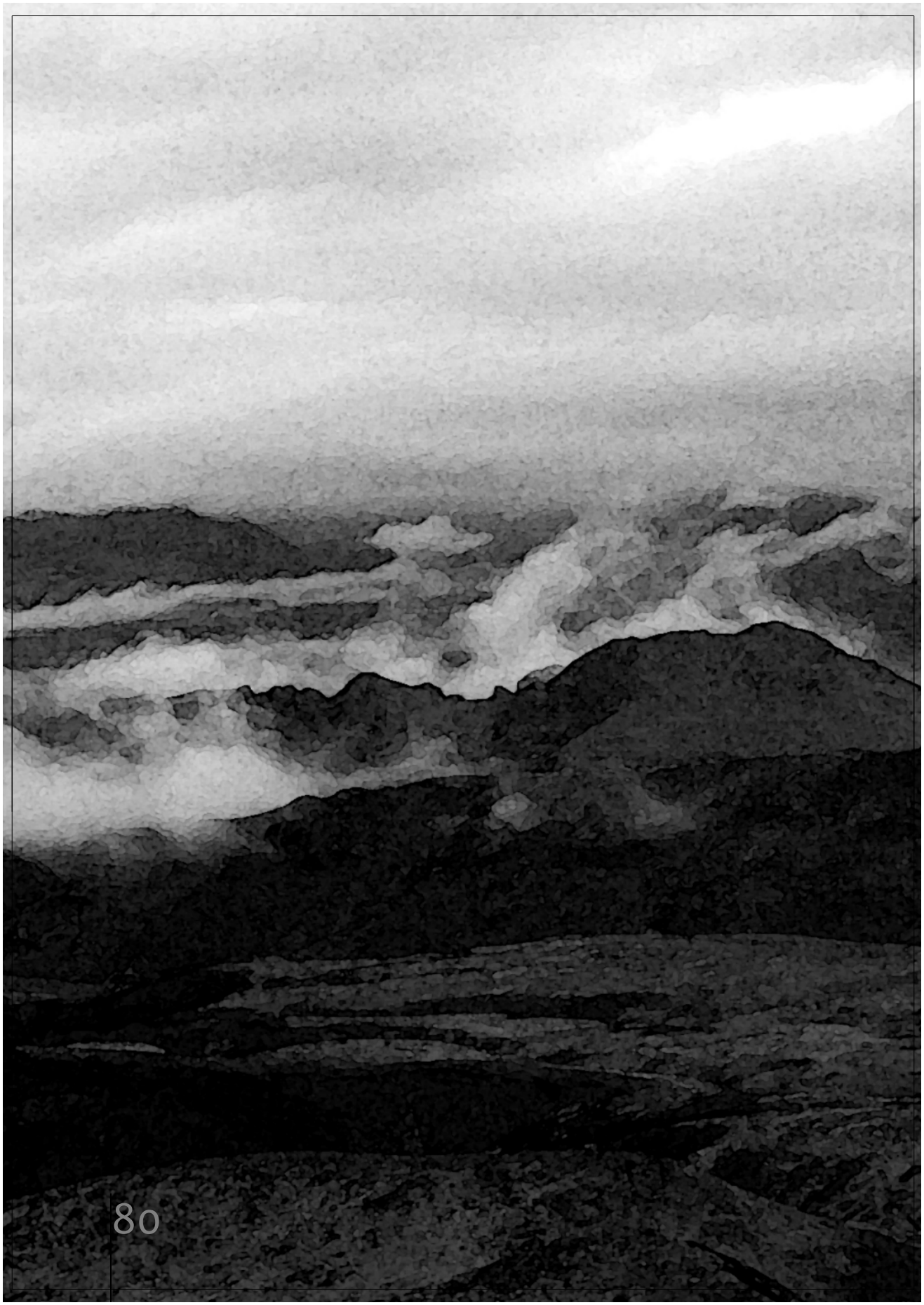

No human transmission of *M. malmoense* in a perfect storm setting

Chapter 7

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Abstract

Nontuberculous mycobacteria (NTM) are opportunistic pathogens that arewidely present in our environment. Human transmission of NTM infections has never been proven.
We report a perfect storm scenario for NTM human transmission in which an acid fast bacilli (AFB) positive, coughing patient, diagnosed with cavitary *Mycobacterium malmoense* lung disease, is in close contact with an immunocompromised patient who was 11 months after the diagnosis of the AFB positive patient, found to have also a *M. malmoense* lung disease. However, multi-locus sequence typing and genotyping results including sequencing of the hsp65 gene made human transmission in this case highly unlikely.

Introduction

Nontuberculous mycobacteria (NTM) are opportunistic pathogens that are widely present in our environment, i.e. in the soil and in natural and processed water. This group of organisms, with varying biological properties and clinical relevance, has gained notoriety over the past two decades due to their capability to cause severe disease in patients with immunodeficiency and/or chronic lung disease.[1] Despite their rising isolation frequency and growing clinical importance, NTM infections receive little scientific or public health attention. This partly results from the dogma that these NTM infections are not transmitted from male-to-male.[1]

During our previous research of *Mycobacterium malmoense* infection in the Netherlands,[2] we studied two patients with *M. malmoense* pulmonary infection with a strong epidemiological link. Human transmission was suspected because an acid-fast bacilli (AFB) smear positive patient was in close contact with an immunocompromised patient. Herein, we present details of the cases and the results of the molecular diagnostics that were performed. The patients gave informed consent; ethical approval was not required for this retrospective study.

The cases

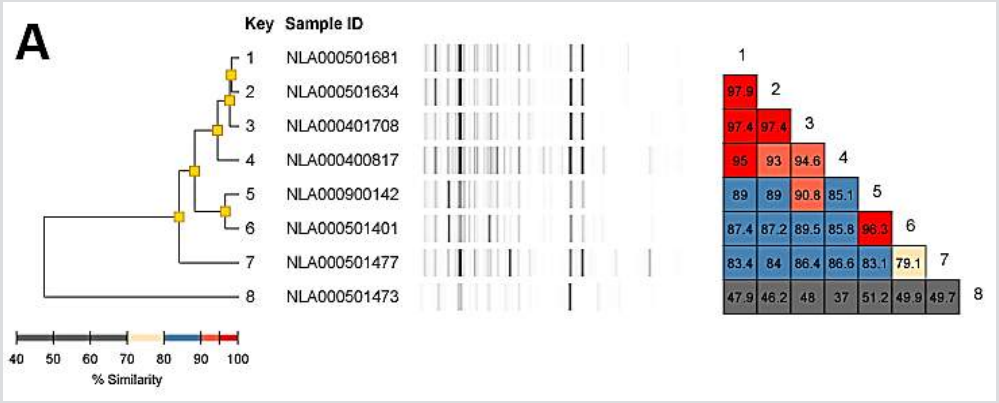
Case 1. A 38- year old male with a medical history of chronic obstructive pulmonary disease and spontaneous pneumothorax reported to the respiratory physician with progressive dyspnoea, productive cough, fever, night sweats, malaise and weight loss. A chest radiograph revealed infiltrates with cavities in the upper lobes. Laboratory diagnostics showed a raised erythrocyte sedimentation rate (90mm/h) and leukocytosis (white blood cell count 22.4 cells/mL); serological tests for HIV were negative. Three sputum smears were negative for AFB on direct microscopy. Bronchoalveolar lavage was performed, which was positive for AFB on direct microscopy. A regimen of isoniazid, rifampicin, ethambutol and pyrazinamide was initiated, based on a presumptive diagnosis of pulmonary tuberculosis. Later, sputum appeared to be positive for AFB. All corresponding cultures yielded *M. malmoense*, identified by Inno-LiPA Mycobacteria v2 reverse line blot assay. Since the patient met the ATS diagnostic criteria for clinical disease[1] the regimen was changed to 24 months rifampicin, ethambutol and clarithromycin and the patient slowly improved.

Case 2. 11 months after the diagnosis was made in patient one, a second patient was diagnosed with *M. malmoense* disease in the same hospital. This patient, a 59-year old male, had a history of kidney transplantation and subsequent immunosuppressive treatment with cyclosporine and prednisone. He presented with productive cough, malaise, fever and weight loss. Erythrocyte sedimentation rate was 110mm/hr and white blood cell count was 8.5 cells/mL. A chest computed tomography scan revealed an infiltrate with cavitation in the left upper lobe and mediastinal lymphadenopathy. A transbronchial biopsy showed granulomatous inflammation with central necrosis and visible AFB on histological examination. Sputum samples revealed AFB on direct microscopy and corresponding cultures, and two simultaneously obtained blood cultures yielded *M. malmoense*. A regimen of rifampicin,

ethambutol and clarithromycin was initiated; owing to clarithromycin intolerance, this drug was changed to ciprofloxacin. After initial progression of disease, including sepsis, progressive pulmonary infiltrates and spontaneous rupture of a nectrotizing mediastinal lymph node to the esophagus, the patient recovered slowly. Both patients completed 24 months of drug therapy and have remained culture negative during 2 years of follow-up. Patient 2 died after the 2 year follow-up period due to a Grawitz tumor.

Epidemiological investigation revealed that the two patients were acquainted and had intensive contact because they very regularly met in the same bar. The first patient worked in a flower bulb processing factory. Isolates of both patients, as well as unrelated patients from the same region and bordering regions, were subjected to genotyping by repetitive element (rep)-PCR typing. The results were confirmed using amplified fragment length polymorphism (AFLP) typing as described previously.[3-4] Rep-PCR typing revealed 97.4% similarity between isolates of both patients, suggesting they were possibly the same single strain. However, this same (>97% similarity) pattern was also observed from the *M. malmoense* isolate of an elderly woman with pulmonary *M. malmoense* disease living in a nearby city; this patient was not epidemiologically related. Other strains from nearby and more distant regions, as well as from the same region though a later period, revealed distinct patterns by rep-PCR typing (Figure 1A). However, AFLP typing showed that the isolates of both patients had only 90% similarity. AFLP included the strain of a patient with pulmonary *M. malmoense* disease from the neighbouring region (05-1477) in the cluster with isolates of both our patients (Figure 1B). We subsequently sequenced the 16S rDNA gene, 16S-23S internal transcribed spacer (ITS) as well as partial hsp65 and rpoB genes, as previously described.[5] The isolates of both patients had identical 16S, ITS and rpoB sequences; the 441bp Telenti-fragment of the hsp65 gene showed 2 base pairs difference between both isolates.[6]

Figure 1: Gene sequencing results of the isolates of the patients described including related strains isolated from other patients.



1A: Typing results obtained by rep-PCR (DiversiLab, BioMerieux).

Figure 1: Gene sequencing results of the isolates of the patients described including related strains isolated from other patients.

1B: Amplified Fragment Length Polymorphism typing results.

Note: 04-1708 is the strain isolated from patient 1, 05-1634 was isolated from patient 2; 97.4% similarity is calculated between the two using rep-PCR (figure 1A). 90% similarity is calculated between the two using AFLP (figure 1B). Strain 05-1681 was isolated from an elderly woman with pulmonary *M. malmoense* disease in a nearby city, 04-817 was isolated from a child with lymphadenitis in the same region; 05-1477 was isolated from a patient with pulmonary *M. malmoense* disease in a neighboring district. More distantly related 09-142 and 05-1401 were isolated in the same region, but four years later, and in a region in another part of the country, respectively. Strain 05-1473 is an *M. fortuitum* isolate used as an outgroup; *M. smegmatis* mc2-155 and *M. marinum* 2000-01053 are control strains.

Discussion

Although there is a strong epidemiological link between both patients with a perfect scenario for human transmission (an AFB positive and coughing source in close contact with an immunocompromised receiver), the results of AFLP and the hsp65 gene sequences, showing two base-pairs difference between the strains of both patients, render human transmission in this case unlikely. Cases of NTM human transmission have been suggested previously, as was reviewed by Wolinsky.[7] One setting showed some similarities to ours with two brothers who developed pulmonary *Mycobacterium kansasii* disease, with an interval of 1 year.[8] Still, human transmission of NTM has, however, never been proven.

The interpretation of genotyping results in order to make definite conclusions about human NTM transmission is complicated: even if both strains would have been fully identical by the methods described, this cannot rule out the possibility of infection from an identical environmental reservoir. The similarity with other strains from the same region (Figure 1) suggests presence of a local ecotype. If local ecotypes exist, as our typing data suggests, the environmental source need not even have been identical; they might have just been infected in the same region. In conclusion, we couldn't establish human transmission in this case of an AFB positive and coughing source in close contact with an immunocompromised receiver.

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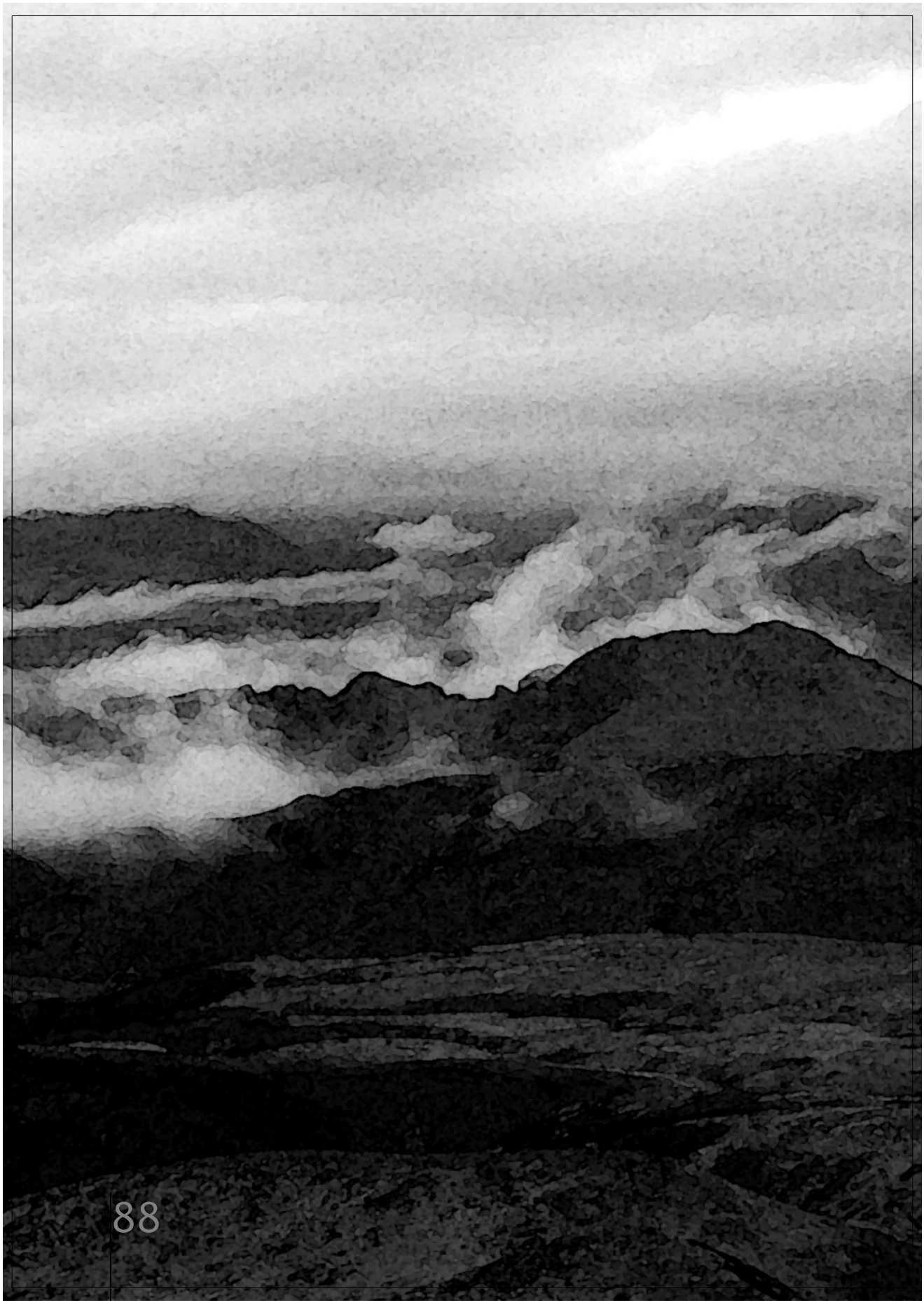

Mycobacterium genavense in the Netherlands: an opportunistic pathogen in HIV and non-HIV immunocompromised patients.

An observational study in 14 cases.

Chapter 8

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Abstract

Mycobacterium genavense is an opportunistic nontuberculous mycobacterium previously mostly associated with HIV-infected patients with CD4 counts below 100/µl. In this retrospective observational study of medical charts we studied all Dutch patients in whom *M. genavense* was detected between January 2002 and January 2010. Of the 14 patients identified, 13 (93%) showed clinical relevant *M. genavense* disease. All patients with *M. genavense* disease were severely immunocompromised, including HIV-infected patients, solid organ transplant recipients, those with chronic steroid use in combination with other immune modulating drugs, recipients of chemotherapy for non Hodgkin lymphoma, and immunodeficiency syndromes. Two patients had non-disseminated pulmonary *M. genavense* disease. Of the 12 patients treated, eight (75%) showed a favourable outcome. Four patients died in this study, three despite treatment for *M. genavense* disease. We conclude that *M. genavense* is a clinically relevant pathogen in severely immunocompromised patients that causes predominantly disseminated disease with serious morbidity and mortality. *M. genavense* is increasingly seen among non-HIV immunocompromised patients.

Introduction

Among the nontuberculous mycobacteria (NTM), *Mycobacterium genavense* has been described as a pathogen causing disease with significant morbidity and mortality, especially in HIV-infected patients with CD4 counts below 100/µl.[1, 2] It was first described by Böttger et al. in 1993,[3] and retrospectively first isolated in 1987 from an HIV patient with a disseminated infection.[4]

In the period after the introduction of antiretroviral therapy (cART) in the late 1990s, the epidemiology of *M. genavense* changed. *M. genavense* was now acknowledged as an opportunistic pathogen in patients with non-HIV immunodeficiencies such as solid organ transplant recipients,[5-7] patients with both innate and acquired impairment of cell-mediated immunity, as in some lymphoproliferative malignancies,[8] after allogeneic stem cell transplantation,[9] and patients on immunosuppressive therapy.[10-13] The available literature focuses only on case reports. Little is known about clinical aspects, treatment and outcome in this category of patients.

M. genavense can be isolated from tap water, healthy birds, and the gastrointestinal tract of healthy individuals. Hence, it is important to distinguish contamination and colonization from true infection if *M. genavense* is isolated from a non-sterile source (e.g. the digestive or respiratory tract).[14-15] The American Thoracic Society (ATS) has published guidelines to assist in this distinction.[16] The clinical relevance of an NTM species may be quantified by assessing the percentage of patients with positive cultures of the respective NTM who meet the ATS diagnostic criteria.

We studied the frequency and clinical relevance of *M. genavense* isolation in the Netherlands, using the ATS diagnostic criteria. We focus on clinical aspects, treatment and outcome of *M. genavense* disease.

Methods

The present study is a retrospective observational study of all patients in the Netherlands who had *M. genavense* detected in a clinical sample during the 2002 to 2010 period. Patients were identified by reviewing the database of the Dutch National Institute for Public Health and the Environment (RIVM) for clinical samples in which *M. genavense* was detected. The RIVM is the national mycobacteria reference laboratory that provides identification and drug susceptibility testing for all hospitals in the Netherlands. In addition, all hospitals that perform identification of NTM without referral to the RIVM were contacted to include their patients with clinical samples in which *M. genavense* was detected. We reviewed medical records of all identified patients in whom *M. genavense* was detected in the study period. We recorded demographic data, clinical data, treatment and outcome. The 2007 ATS diagnostic criteria were used to determine clinical relevant infections;[16] because *M. genavense* is extremely difficult to culture on routine media, we considered molecular detection of *M. genavense* to equal culture, as the ATS criteria are based on cultures. The ATS diagnostic criteria are designed specifically for pulmonary NTM disease. For non-pulmonary infections in this study,

clinically relevant infection was defined by positive culture or molecular detection of *M. genavense* with clinical signs in accordance with the isolation site and, if applicable, fitting radiologic findings.
To identify NTM, the RIVM used the INNO-LiPA Mycobacteria v2 (Innogenetics, Ghent, Belgium) reverse line blot assay was used, which has specific probes for *M. genavense.* Prior to 2004, 16S rDNA gene sequencing was performed by previously published methods,[17] after ruling out membership of the *M. tuberculosis* or *M. avium* complex using the AccuProbe assays (GenProbe, San Diego, USA). Similarly, 16S sequencing or INNO-LiPA assays were mostly used by the local hospitals. Drug susceptibility testing was not performed.

The local ethics committee approved the study.

Results

We identified 14 patients in whom *M. genavense* was detected in a clinical sample. Thirteen (93%) patients met the ATS diagnostic criteria. For 11 patients, *M. genavense* was detected by molecular methods only, in clinical specimens. In three, scanty growth was observed on Middlebrook 7H10 solid (n=1) or 7H9 liquid medium (n=2) supplemented with sheep blood and Mycobactin J and identified by the INNO-LiPA Mycobacteria v2 assay as *M. genavense*; no growth was observed upon subculture. Seven (54%) patients had *M. genavense* detected in pulmonary samples of whom two had localized *M. genavense* disease (no other site from which *M. genavense* was detected). In 11 (85%) patients extra-pulmonary samples including bone marrow (n=6), blood (2), lymph node (4), liver (2) and stool (3) yielded *M. genavense*. The mean age of the patients at the time of isolation was 54.7 years (range 33-68 years). Of the patients with a clinically relevant *M. genavense* infection, four patients were HIV positive and nine patients were immunocompromised due to causes other than HIV infection.

HIV-infected patients

All four HIV-infected patients presented with disseminated *M. genavense* disease. The mean CD4 count at time of *M. genavense* isolation was 35/µl (range 10-83). The patients mostly reported fever (n=3), malaise (2) and gastro-intestinal symptoms (2). In three patients enlarged abdominal lymph nodes were observed. A pulmonary localization of *M. genavense* was proven in one patient who presented with pleural fluid and multifocal nodular lesions on chest computed tomography (CT). Treatment was given in all cases and included ethambutol and clarithromycin in all patients. In one patient rifampicin was added to the regimen. Two patients were cured of *M. genavense* infection based on negative culture results and normalization of radiology results (Table 1). One patient died after 7 months treatment. Postmortem examination revealed the cause of death in this patient to be the combination of a disseminated *M. genavense* disease and a cerebral malignancy. One patient is currently still on treatment and showed complete resolution of *M. genavense* related symptoms. As a result, a favourable response to treatment was observed in three HIV positive patients.

Table 1: Individual clinical characteristics, treatment and outcome of the HIV positive patients with *M. genavense* disease.

Patient	Age / Sex	Year of diagnosis	CD4 count per µl	Detection site	Disseminated disease	Treatment	Outcome
1	33 / M	2002	83	Abdominal lymph nodes.	Yes	E/Cla 7.5 years	Cured
2	53 / M	2007	52	Liver	Yes	E/Cla	Chronic treated
3	49 / M	2002	10	BM, faeces, pleural fluid, ascites, lung biopsy	Yes	R/E/Cla 26 months	Cured
4	64 / M	2005	10	Faeces. Postmortem: AFB+ material in: BM, spleen, liver and lung	Yes	E/Cla several months	Died Cause of death: MG disseminated disease and intracerebral tumor

AFB: acid fast bacilli; BM: bone marrow; Cla: clarithromycin; E: ethambutol; Hb: hemoglobin; MG: *Mycobacterium genavense*; R: rifampicine;

Non-HIV immunocompromised patients

M. genavense was detected in ten non-HIV patients. Nine patients had a clinically relevant *M. genavense* isolation, all of whom were diagnosed with an innate or acquired immunodeficiency. Details of the non-HIV patients with a clinically relevant *M. genavense* isolation are summarized in Table 2.

Table 2: Individual clinical characteristics, treatment and outcome of the non-HIV immunosuppressed patients who presented with *M. genavense* disease.

Patient	Age/ Sex	Underlying condition	Immune suppression	Detection site	Disseminated disease	Treatment	Outcome
5	55 / M	Possible RA	Prednisone, azathioprine, leflunomide	Sputum, lung biopsy	No	R/E/Cla 18 months	Cured
6	57 / M	Sarcoidosis, diagnosed 10 years before first isolation	Several years prednisone; stopped several months before first isolation.	Sputum	No	R/Cla/ Moxifloxacine	Resolvement of complaints. AFB negative. Still on treatment
7	63 / M	NHL	Chemotherapy (including retuximab) 3 months before first isolation	BM	Yes	R/E/Cla	Resolvement pancytopenia after 3 weeks treatment. Chronic treated
8	72/ M	Sarcoidosis	Prednisone, azathioprine,	BM	Yes	(R)/E/Cla/ Ciprofloxacin R stopped after 1 month treatment because of hepatoxicity	Died, within 8 weeks after MG isolation because of multi-organ failure
9	73 / F	Renal transplantation 18 years before first isolation	Prednisone, mycophebolate mofetil	Abdominal LN	Yes	R/E/H/Z	Died, within 4 weeks after MG isolation
10	54 / F	Liver transplantation 19 years before first MG isolation	Prednisone, azathioprine	Blood, feces, BM	Yes	R/E/Cla 12 months	Resolvement pancytopenia after 2 weeks treatment Chronic treated R / Cla
11	43 / M	Innate IL-12 receptor deficiency	IL-12 receptor deficiency	Sputum, cervical LN, BM	Yes	R/E/Cla	Chronic treated
12	57 / M	Interstitial nephritis with granuloma	Prednisone, cyclophosphamide	Sputum	Yes	R/E/Cla 14 months.	Died
13	42 / M	Idiopatic CD4+ lymphocytopenia	Idiopatic CD4+ lymphocytopenia	BM	Yes	R/E/Cla.	Chronic treated

AFB: acid fast bacilli;BM: bone marrow; Cla: clarithromycin; E: ethambutol; F: female; H: isoniazide; LN: lymph node; M: male; MG: *Mycobacterium genavense*; NHL: Non-Hodgkin lymphoma; R: rifampicine; Z: pyrazinamide

Disseminated *M. genavense* disease

Seven of nine patients showed disseminated *M. genavense* disease. Two patients were solid organ transplant recipients, the other five patients were immunocompromised due to an immunological disorder, immunosuppressive drugs or a haematologic malignancy (Table 2). Patient number 13, diagnosed with an idiopathic CD4+ T cell lymphocytopenia, also had *Mycobacterium xenopi* and *Mycobacterium simiae* isolated from a bronchoalveolar lavage. Fever was the most common clinical sign (Table 3). Pancytopenia was seen at the time of M. genavense isolation in four cases, all of whom had *M. genavense* isolated from bone marrow. Enlarged para-aortic and mesenteric lymph nodes were the most common abnormalities on radiologic examination (n=4), followed by pulmonary dense airspace opacities (2).
Six patients were treated for *M. genavense* disease of whom two died; the other patients showed a good clinical response and are still, or chronically treated. One patient received a treatment for a presumed *M. tuberculosis* infection and died several weeks after start of this treatment.

Table 3: Frequency (%) of symptoms and signs in non-HIV immune suppressed Dutch and previously published cases presenting with disseminated *M. genavense* disease.[5-13]

Symptom or sign	Dutch patients (n = 7)	Literature (n = 9)
Fever	86	67
Weight loss	57	22
Malaise/Fatique	57	22
Productive cough	43	22
Night sweats	29	11
Diarrhea	29	44
Dyspnea	29	0
Abdominal pain	14	44
Vomiting	14	0

Pulmonary *M. genavense* disease

Two patients had *M. genavense* detected repeatedly, but in pulmonary samples only. They had no clinical suspicion for other organ involvement of *M. genavense* (patients 5 and 6 in Table 2). Patient 6 was free of any immune suppression at time of first *M. genavense* detection. Fever was reported by both patients. Other complaints were dyspnoea, productive cough and chest pain. Radiologic examination showed cavitations together with a reticulonodular pattern in both patients (Figure 1). Treatment was initiated in both, one was cured and the other is still being treated.

Clinically irrelevant detection of *M. genavense* occured in bronchoalveolar lavage fluid obtained in a patient with a community-acquired pneumonia who improved on a course of amoxicillin and clavulanic acid. The patient was known to have type 2 diabetes; he had not been using immunosuppressive drugs before. No clinical signs or radiologic abnormalities suggestive of mycobacterial disease were found during 2 years of follow-up.

Among the 12 patients treated for *M. genavense* disease (i.e. with regimens including a macrolide, ethambutol and often rifampicin) we recorded a favourable outcome in 75% (9/12) of patients (cured, 25% (n=3); chronically treated but with resolution of *M. genavense* related complaints: 50% (n=6)); three died.
Overall, four patients (4/13, 30%) died in this study because of (disseminated) mycobacterial disease: the three treated for *M. genavense* disease and one who received treatment for presumed *M. tuberculosis* infection (Tables 1 and 2).

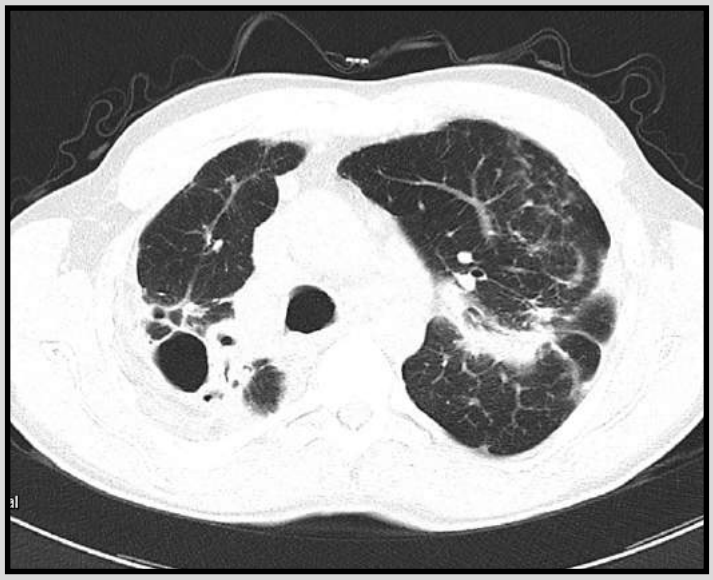

Figure 1: Computed tomography of patient 6. Cavitations are seen in the right upper lobe together with a reticulonodular pattern, especially seen in the left lung.

Discussion

Mycobacterium genavense detection proved to be indicative of clinically relevant disease in 93% (13/14) of the patients in this study. Disseminated disease was seen in all HIV positive patients and in 78% of the non-HIV immunosuppressed patients. To the best of our knowledge, we have reported the first two patients with a non-disseminated pulmonary *M. genavense* disease. One of these patients used prednisolone and azathioprine, a drug combination that was also used by one of the patients with disseminated disease. The other patient had been on systemic steroids and had a pre-existing pulmonary fibrosis with bullae which probably predisposed to a pulmonary *M. genavense* infection. Although little is known about the pathogenesis of *M. genavense* infection, Ehlers and Richter[18] showed that immunocompetent mice cleared intravenous-injected M. genavense, in contrast to syngeneic gamma-interferon-gene deficient mice. This observation is in agreement with our study, in which *M. genavense* disseminated disease was only seen in severely immunocompromised patients including HIV-infected patients, solid organ transplant recipients, chronic steroid use in combination with other immune modulating drugs, recipients of chemotherapy for non Hodgkin lymphoma and immunodeficiency syndromes.

Prior to the cART era, *M. genavense* disease was predominantly seen among HIV-infected patients. Tortoli *et al*. retrospectively reviewed all patients in Italy with *M. genavense* isolation between 1992 and 1996. All 24 patients appeared to be HIV positive with a mean CD4 count of 23/µl.[1] The epidemiology seems to have changed since the introduction of cART; 71% of the patients in this study were HIV negative. This finding is in line with the replacement of HIV related *M. genavense* case reports by non-HIV related *M. genavense* case reports identified in the literature since the publication of Tortoli.[5-13] In the Netherlands this epidemiologic shift has also been observed since the last literature report of HIV-related *M. genavense* infection was published in this country in 1998.[19]
This epidemiologic transition probably reflects the rising number of indications for the use of immunosuppressive drugs, and increasing number of solid-organ transplant recipients, together with a better life expectancy of this group of patients. Anti-TNF therapy is increasingly seen as one of the important immunosuppressive drugs that is giving a higher risk for mycobacterial disease.[20 21] However, we have not found any patient using anti-TNF therapy. No case reports have been described in literature either regarding *M. genavense* disease related to anti-TNF therapy. TNF inhibition alone might not be enough for *M. genavense* to become pathogenic in humans.

Whether the number of HIV patients presenting with a *M. genavense* infection is decreasing cannot be concluded from our data. However, because *M. genavense* infection is especially observed at CD4 counts below 100/µl, the early initiation of cART in the disease course of an HIV patient has certainly influenced its epidemiology; a similar phenomenon has been noted for *M. avium* infections in this group.[22] The exact environmental source of *M. genavense* remains unknown. *M. genavense* has been found in hospital tap water,[23] and has been isolated with high bacterial load from intestines of apparently healthy birds.[24] Furthermore, Dumonceau et al.[14] reported DNA isolation of *M. genavense* from macroscopically healthy intestinal biopsy samples in five out of nine HIV negative patients.

All these patients were free of any clinical sign of infection. This finding supports the hypothesis that *M. genavense* may colonize the gut and disseminate from it when patients become immunocompromised.[25] If colonization can be found in a high number of healthy individuals, as suggested by Dumonceau, then the risk of dissemination from the gut is low since *M. genavense* isolation frequency in the Netherlands (2-4/year) is only a small part of the total number of NTM isolated (800/year).[26]
Considering the difficulty in culturing *M. genavense*, underestimation of *M. genavense* disease incidence is likely. Further study is warranted to explore the frequency of *M. genavense* presence in intestines in healthy individuals and factors associated with dissemination. Probably, HIV infection with low CD4 counts is an important risk factor for dissemination from a colonized gut since the Swiss HIV cohort study found *M. genavense* in 12.8% of the disseminated NTM infections diagnosed between 1990 and 1992.[2]

Diagnosis of *M. genavense* infection remains a challenge for clinicians because of the absence of specific symptoms and the difficulties with culturing the organism using standard mycobacterial culture methods. In an immunocompromised host, especially with signs of disseminated disease (pancytopenia, abdominal lymph nodes), mycobacterial disease including M. genavense dissemination has to be considered.
Nucleic acid amplification techniques are useful to establish a fast and reliable diagnosis. Attempts to culture *M. genavense* are probably best done on solid media (Middlebrook 7H11) supplemented with blood and charcoal and acidified to pH 6.2 ± 0.2.[27]

Before cART became available, survival from disseminated *M. genavense* infection was poor and comparable with disseminated *Mycobacterium avium* complex (MAC) disease with a mean survival time of 8,5 months.[1, 2, 22, 28] After cART was introduced, no studies were published concerning treatment outcome and survival for *M. genavense* infected HIV patients. In our study, one patient died and one patient is still treated. Probably, as also observed in MAC disease, survival for *M. genavense* improved dramatically because of the combination of cART and anti-mycobacterial drugs. However, this has to be confirmed in future (international) studies. Little is known about factors associated with mortality in the *M. genavense* infected non-HIV population. Delay in diagnosis and thus treatment, ongoing immune suppression and treatment duration are factors to be considered.[8, 13]

The optimal treatment regimen for *M. genavense* disease remains unclear. In vitro susceptibility data are limited because of the fastidiousness of the organism. Previous studies, reviewed in the ATS Statement, have recorded in vitro susceptibility to rifamycins, streptomycin, macrolides, fluoroquinolones and amikacin.[16] In our study, all initiated treatments for M. genavense consisted of clarithromycin. Ethambutol was added in nine patients, rifampicin in eight and moxifloxacin in one patient.

Current ATS guidelines only state that macrolide based regimens are preferable over other regimens; the rifampicin, ethambutol and macrolide regimen advised for *M. avium* complex disease is likely preferable for *M. genavense* disease, too. Lifelong treatment was given in a high percentage of patients and may be indicated when the immunosuppressive condition persists; otherwise, therapy may be continued until the patient has been culture-negative for 12 consecutive months.[16]

In conclusion, *M. genavense* is an opportunistic pathogen in HIV and non-HIV immunocompromised patients, causing mostly disseminated disease with serious morbidity and mortality. After the introduction of cART, the epidemiology has changed, with a switch to non-HIV immunocompromised patients. The optimal treatment regimen and duration is unclear. Since the organism can colonize the gut of healthy individuals, more research is needed to determine factors associated with dissemination and disease.

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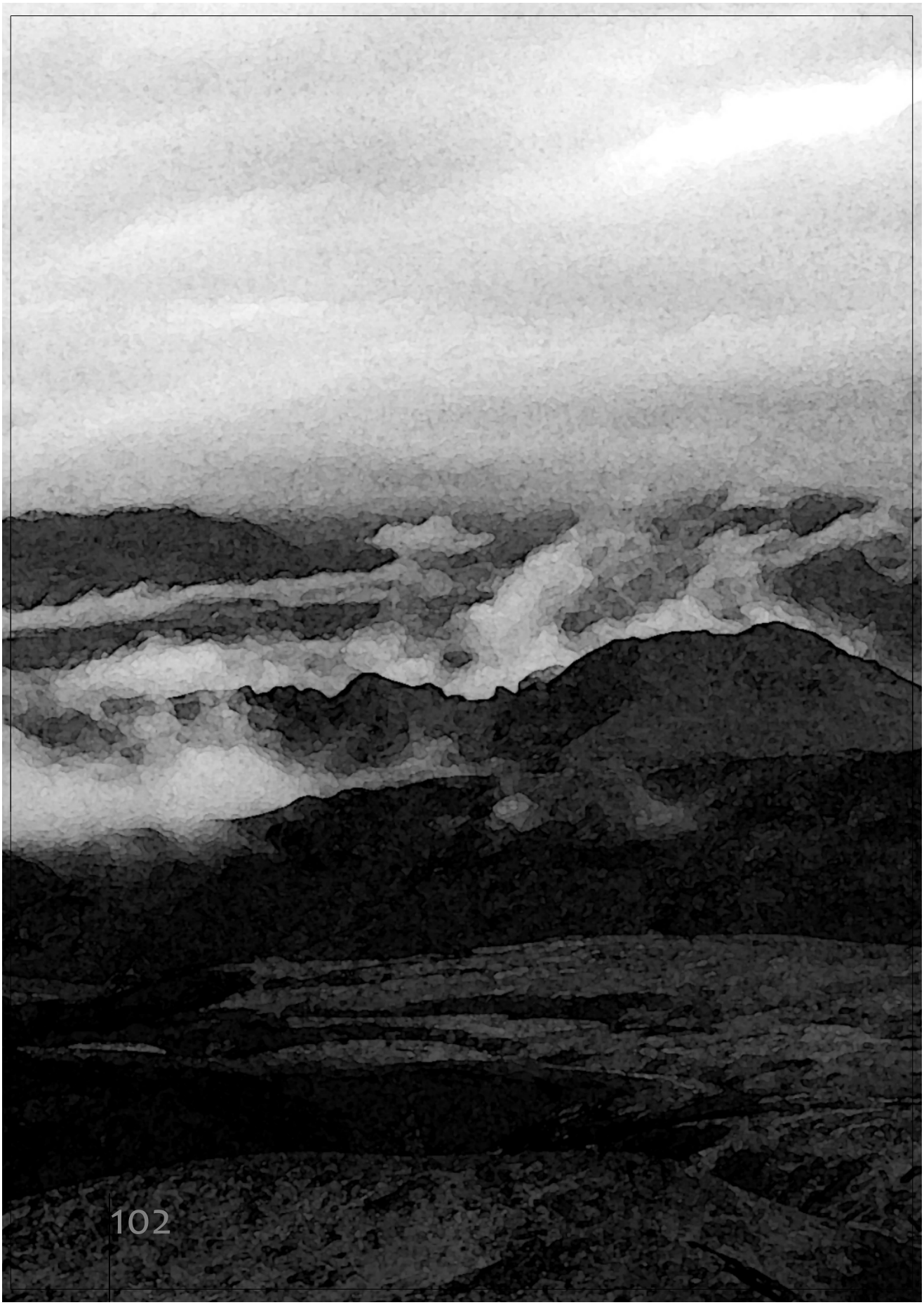

General discussion

Chapter 9

General discussion

Disease caused by nontuberculous mycobacteria (NTM) is a growing health problem. There is an observed increase in isolation frequency and disease incidence and prevalence, mainly in countries with a low incidence of tuberculosis.[1-2] However, in contrast to infections with *M. tuberculosis* complex little is known about NTM including epidemiology, pathogenesis, transmission to humans, virulence factors and treatment.

In this thesis, the focus is on epidemiology and clinical relevance of NTM isolation. The geographical distribution of NTM is studied from a single time period in chapter 2. The majority of the observations made in this study were expected. Still, some other observations may inspire future research on environmental or host factors related to the observed differences. Not only isolation rates of NTM species differ between continents, also clinical relevance may vary as exampled in the literature review on *M. malmoense* (chapter 5). In chapter 3, the epidemiology of NTM in the Netherlands is studied. An increase in NTM isolation was observed, mainly due to *M. avium* isolation in subjects of older age. COPD is an important risk factor for NTM pulmonary disease. We have prospectively studied the prevalence of NTM in COPD patients with exacerbations not suspected of NTM disease (chapter 4). A surprisingly high isolation rate was observed, though only 6% was clinically relevant. Again, higher age was significantly related to NTM isolation. This study confirms the importance of the ATS diagnostic criteria for NTM disease, and the need for further study on the association between COPD and NTM pulmonary disease. Finally, two important species of NTM were studied in more detail, both known to show high clinical relevance in patients with local depressed immunity (*M. malmoense* pulmonary disease seen especially in patients with COPD), and systemically depressed immunity (*M. genavense* disseminated disease).

Epidemiology

Worldwide species distribution

In chapter 2, we describe the worldwide species distribution among NTM from pulmonary specimens. There is a difference by region and by country within the regions. *M. avium* complex (MAC) is the most prevalent NTM worldwide; the frequency of isolation of the other NTM differs by setting. *M. avium* predominates in the participating centres in North and South America and Europe; *M. intracellulare* was most frequently isolated in South Africa and Australia. In Australia, a significant increase in the isolation and predominance of *M. intracellulare* has been reported before.[3] In Japan, *M. intracellulare* appeared to be the most prevalent in the South, *M. avium* was more prevalent in the North, including Tokyo.[4] In our study, the data available from Tokyo, Japan, only provided MAC isolation figures without subspecies identification. *M. intracellulare* was the most frequent isolated NTM in a study performed in Texas, southern US,[5] although this study was not a population-based study. Isolation prevalence of the different subspecies within the MAC seems to differ between countries and probably even within countries. *M. xenopi* is an important respiratory tract pathogen (ranked third on isolation frequency after MAC and *M. gordonae*) and increasing in different parts of the world as also seen by Martin-Casabona and co-workers.[2] On the other hand, *M. kansasii* ranked sixth in our study, which is in agreement with the observed decline in isolation frequency in recent years.

Several arguments may explain the observed differences in worldwide species distribution:
First, the species distribution may be a reflection of the environmental distribution of NTM. Thomson suggested the source of *M. intracellulare* to be drinking water supplies. This has never been systematically studied as a factor in the observed geographical differences in MAC subspecies isolation, neither for other NTM species. In a study by Brown-Elliot et al.[6] a hospital and community outbreak of *M. porcinum* was identified and related to hospital- and household drinking supplies. In a 5 year period, *M. porcinum* isolation from clinical specimens was clinical relevant in 46% of the cases. In Australia, *M. lentiflavum* showed a dominant environmental clone. This pathogen, isolated from tap water, was closely related to clinical strains based on automated repetitive sequence-based PCR genotyping.[7] Wether environmental presence (partly) explains the patterns of isolation observed in chapter 2 remains to be studied.

Second, geographic differences in NTM isolation can be the result of differences in host factors between continents. Among HIV patients, extra-pulmonary MAC disease is the predominant NTM disease presentation.[8] We studied only pulmonary isolates, so HIV is not a likely explanation for the observed geographical differences in MAC isolation. The high isolation rate of RGM in Asia can be related to the high tuberculosis incidence in Asian countries. Host genetic differences may also account for geographical NTM isolation patterns. Several studies from Asian countries report on gene polymorphisms of certain candidate genes for mycobacterial infection susceptibility. For example, Koh et al. found an association between polymorphisms in the natural resistance-associated macrophage protein 1 gene (*Nramp1*, synonym: *slc11A1*) and susceptibility to pulmonary NTM disease in Korea. This protein is involved in ion transport into the lysosome during phagocytosis of mycobacteria.[9] Although

especially patients from whom the RGM *M. abscessus* was isolated were studied by Koh, it is unclear if this Nramp1 polymorphism is associated with the frequently found RGM isolation in Asia. In a population-based study from the US among elderly subjects, pulmonary NTM disease was mainly prevalent among Asians/Pacific islanders in contrast to whites.[10] No information was given regarding the NTM species isolated in this study, i.e. whether these patient had frequent RGM isolates. Overall, it remains to be studied whether among Asian people a certain genetic predisposition accounts for the relatively high RGM isolation.

Third, the observed geographical differences in species isolation may be explained by differences in virulence within a NTM species. As discussed in chapter 5, *M. malmoense* showed high clinical relevance in Northern Europe in contrast to the US. Differences in clinical relevance within a NTM species is known for *M. kansasii* but turned out to be the result of different subtypes of *M. kansasii* with differences in clinical relevance.[11]
For *M. malmoense*, different subtypes have been identified, but without differences in clinical relevance. Whole genome sequencing studies will probably reveal intra-species variation in mycobacterial virulence factors associated with the observed difference in geographical isolation patterns.[12-13]

Isolation prevalence

We have shown in chapter 3 that in the Netherlands there is an increase in the total number of NTM isolated and a decline in isolation of *M. tuberculosis* in recent years. The first is mainly due to a rise in *M. avium* isolation in elderly subjects. The increase seems a real increase, since it began before improved laboratory techniques were introduced and isolation frequencies of the other NTM remained relatively stable during the study period. The same increase in NTM isolation, especially MAC, has been observed in other regions and countries like Massachusetts, USA,[14] Ontario, Canada[15] and Australia.[3] For many other regions worldwide, epidemiological figures of MAC isolation are lacking. Several studies have found an increase in overall NTM isolation prevalence[16] and disease over time.[3, 10, 17, 18] The increase in NTM disease incidence in the study by Thomson was mainly due to an increase in *M. intracellulare* disease among elderly subjects, especially females with nodular-bronchiectatic disease.[3] A remarkable increase in pulmonary NTM disease prevalence was observed among elderly subjects (age > 65 years) in the US among both men and women.[10] In Denmark, a population-based study showed no increase in NTM incidence rates among the general population between 1997 and 2007, except in the elderly.[19] Parallel to increases in NTM isolation from clinical specimens, increases in skin sensitization to NTM antigens have been observed; Khan et al. performed a study in which population-representative cohorts (1971-1972 vs. 1999-2000) were compared regarding skin sensitization to *M. intracellulare*.[20] Sensitization increased from 11.2% to 16.6% between the two time periods; age between 20-39 years, sex, race, birth place, education and occupation were associated with positive skin sensitization. This increase is in line with the observed increase in NTM isolation prevalence and disease, but it is not clear whether skin sensitization has an association with past or future NTM infection. Overall, NTM isolation and disease is increasing in developed countries and may not be fully attributed to improved laboratory techniques.

There are several explanations for the observed increase in isolation prevalence and disease rates . As discussed in chapter 3 and shown in other studies, improved awareness among clinicians of NTM disease, ageing of the population in combination with growing number of COPD patients, increasing numbers of patients with immunosuppressive diseases or therapies, and environmental changes are factors to be considered.[15, 18, 21-22]
Population-based studies are needed in each continent and country to monitor the change in NTM epidemiology over time to reach a better pathophysiological understanding of NTM disease and to identify country specific risk groups.

COPD and nontuberculous mycobacteria

The relationship between chronic pulmonary diseases and mycobacterial lung disease was known from experiments performed in the 1970s. Silicotic guinea pigs showed progressive lung disease when infected with some species of NTM.[23] Also from case series in these years, reviewed by Emanuel Wolinsky in 1979,[24] it became clear that occupational exposure to dust (silica), that may cause chronic lung disease, predisposes to NTM lung disease. Nowadays, NTM pulmonary disease exists among patients with pre-existing pulmonary disease, especially bronchiectasis and COPD.[18-19, 21, 25-26]
COPD is the fourth leading cause of death in the world, and further increase in its prevalence and mortality can be predicted for the coming decades. Investigation of risk factors related to the development of COPD, as well as investigation of cellular and molecular mechanisms involved in COPD deterioration, remain an important focus for research as advocated by the Global Initiative for COPD.[27] Infections (viral and bacterial) may contribute to the pathogenesis and disease progression of COPD. In chapter 4, we described the first performed prospective study focusing on NTM isolation in COPD patients not suspected of NTM disease. Among all the 73 patients included with an acute exacerbation of COPD (AECOPD), 22% of the patients had a positive mycobacterial culture. Only higher age was associated with NTM isolation. The high NTM isolation rate among the COPD patients in chapter 4 re-emphasizes the need for further study on COPD in relation to NTM isolation and disease. The question has to be answered whether isolation of a NTM from a patient without clinical signs of NTM pulmonary disease impose an increased risk of NTM disease and/or lung function decline during follow-up. If NTM are involved in accelerated deterioration of lung function or an increased exacerbation frequency in COPD patients, a more vigilant stance or even screening may be worthwhile. Furthermore, certain COPD phenotypes or risk factors can probably be identified for NTM pulmonary disease.

Retrospective evidence of lung function decline in COPD patients with NTM isolation has been found recently.[28] In cystic fibrosis (CF), significant more lung function decline have been observed in patients from whom mycobacteria are isolated in contrast to patients with no mycobacteria isolated.[29] Also deteriorated lung function tests prior to NTM isolation have been observed in CF patients.[30] Huang et al.,[28] found a relationship between NTM isolation and more frequent exacerbations of COPD. Still, one may speculate whether the NTM is responsible for the increased exacerbation rate and lung function decline in COPD, or that the presence of NTM is just a marker of a “bad” COPD phenotype as this maybe also the

case in CF related NTM infections. One may similarly speculate about the observation that use of (inhaled) corticosteroids in COPD patients is related to NTM pulmonary disease in COPD patients.[21] The prescription of inhaled corticosteroids can be related to a specific COPD phenotype (for example severe bronchial hyper responsiveness or more pronounced bronchiectasis with frequent exacerbations) that again predisposes to NTM presence, instead of the hypothesis that the effects of steroids are directly related to NTM presence and/or disease in the airways.

In our study, age was significantly related to NTM isolation in COPD patients. As is discussed in chapter 4, higher frequency of co-morbid conditions and use of immunomodulatory drugs in the elderly can play a role in this association. This was not addressed in our study. Since NTM disease and even colonization are related to increased mortality in the elderly,[10] more research on etiologic factors in this patient group is needed. For the clinician it is important whether screening for NTM in COPD patients is recommendable. In chapter 4 we suggest to perform mycobacterial cultures in all patients with bronchiectasis and in COPD patients with deteriorating clinical condition despite standard treatment measures. Important questions are: is treatment of a single potentially pathogenic NTM isolation necessary in case of an increased exacerbation rate? This in the background of increased mortality in patients of older age with a single NTM sputum culture. Or is optimization of COPD treatment sufficient?
Another argument for screening for NTM disease is the increased use of macrolides in clinical practice. Macrolides are prescribed more frequently since it was found that its use prevents exacerbations and lung function decline among non-CF bronchiectasis and COPD patients.[31, 32] However, the risk of inducing NTM macrolide resistance as a result of macrolide mono therapy with resulting worse treatment outcome in these patients is of concern,[33 34] a risk that applies especially for MAC, the most common NTM.[35]
Our research and that performed by others, shows that COPD predisposes to NTM isolation and disease,[18, 21] NTM can be detected in sputum of AECOPD patients who are not suspected of having NTM disease (chapter 4), higher age is associated with NTM isolation (chapter 4) and elder people who have NTM cultured from their sputum have increased mortality.[19] Inhaled corticosteroid use is probably associated with NTM disease,[21] and NTM isolation in COPD patient is possibly related to increased lung function decline.[28]

In focus: *Mycobacterium malmoense*

In this thesis, several aspects of *M. malmoense* are discussed. First, *M. malmoense* is a clinically relevant NTM in the Netherlands, causing serious pulmonary morbidity with cavitary disease as the most common disease presentation. Second, *M. malmoense* pulmonary disease is especially seen among patients with pre-existing COPD. Last, from a literature review we observed a rising incidence of *M. malmoense* isolation especially in Northern Europe together with a high clinical relevance in this region. However, in the USA, *M. malmoense* is rarely isolated (chapter 2) and clinical relevance of *M. malmoense* was observed to be much lower. Since pulmonary *M. malmoense* disease is mostly of the cavitary disease type, treatment for a presumed tuberculosis infection is frequently initiated. Thirty per cent of the patients in

the study presented in chapter 6 completed a standard 6 months anti-tuberculosis regimen for pulmonary *M. malmoense* disease. Contact tracing studies have been initiated based on cavitary disease in a sputum-smear positive patient that turned out to have a pulmonary *M. malmoense* disease; some contacts received 6 months of preventive therapy (chapter 6). In a setting where NTM prevalence is higher than tuberculosis, especially in a patient with COPD without tuberculosis risk factors, it is important to perform molecular identification techniques in order to prevent suboptimal treatment and unnecessary public health measures.

M. malmoense is an important NTM pathogen in Northern Europe, especially in the United Kingdom. In Lothian Scotland, it is the most frequently isolated NTM.[36] In our World Map study we have confirmed the geographic distribution of *M. malmoense* (chapter 2). However, *M. malmoense* was in this study also isolated from patients in South Africa, a country where *M. malmoense* isolation from clinical specimens had not been previously reported. It has been isolated from the Congo river in the Democratic Republic of Congo.[37] Interestingly, *M. malmoense* shows a very low isolation frequency in North America. In a study form Ontario, Canada, *M. malmoense* was not isolated between 1997 and 2003.[16] In our World Map study we have found five *M. malmoense* isolates from Canada (compared to a total of 3,443 NTM isolates from this country) and no isolates from the participating laboratories in the US (Portland and Denver). In a previous study from the Unites States, *M. malmoense* was isolated from 73 patients between 1992 and 1994. Most of the isolates in this study were from Florida (42/73) and two other southern states.[38] In chapter 5 we described that the isolation frequency of *M. malmoense* is on the rise in certain parts of Northern Europe. In the Netherlands, however, isolation frequency of *M. malmoense* remained relatively stable between 2002 and 2006 (chapter 6).

The increase in NTM isolation in the Netherlands, as discussed in chapter 3, was mainly due to increase in *M. avium* isolates. To date, we do not have a clear explanation for the observed geographic distribution of *M. malmoense*, nor do we have regarding differences in clinical relevance between Europe and the USA for this organism. Whole genome sequencing of the European and US strains can be helpful to identify genetic virulence factors responsible for the observed differences in clinical relevance of *M. malmoense*. Differences in the presence of other microbes in the environmental niches can be related to the observed differences in virulence within *M. malmoense*. For example, growth of *M. avium* within amoeba was shown to enhance virulence in a mouse model.[39] Host factors will certainly also be important in the observed differences in clinical relevance within NTM species (genetic differences, differences in habits like smoking, alcohol consumption, dietary factors).[25] Interestingly, for *M. malmoense,* polymorphism in the vitamin D receptor have been linked to the susceptibility to *M. malmoense* infection.[40]

In chapter 7, two patients with a *M. malmoense* pulmonary infection with a strong epidemiological link were studied. Human transmission of NTM disease was strongly suspected because a smear positive patient was in close contact with an immunocompromised patient. However, the results of genetic testing made human transmission even in this case highly unlikely. A case of probable NTM transmission was described from a cystic fibrosis clinic in the US. A highly drug-resistant *M. abscessus*, subspecies *M. bolletii* (former *M. massiliense*)

strain was isolated from an index case and 4 other clinic patients within 8 months.[41] It was hypothesized that the high mycobacterial load in the sputum of the index case may have contaminated the clinical environment and facilitated patient-to-patient transmission. High mortality was observed in the patients: three of the 5 infected patients died, the index case after lung transplantation. Very recently, a retrospective cohort study from a cystic fibrosis centre strongly suggested NTM transmission between cystic fibrosis patients. More study is necessary to prove that NTM human transmission may occur, especially in cystic fibrosis patients. Retrospective analysis of genotypic results are needed to further study probable human transmission of NTM, especially among cystic fibrosis patients, but also in COPD patients. The important question then is how this (indirect?) transmission can be prevented.

In focus: *Mycobacterium genavense*

In chapter 8, a retrospective medical file study is described on infections by *M. genavense*. All Dutch patients were studied from whom *M. genavense* was isolated between 2002 and 2010. *M. genavense* were clinical relevant in 93% of the patients. All patients with *M. genavense* disease were severely immunocompromised, including HIV-infected patients (CD4 count generally below 100/ul), solid organ transplant recipients, patients treated with immune modulating drugs, and patients with immunodeficiency syndromes. *M. genavense* disseminated disease is the most common disease presentation. The clinical presentation of disseminated disease is comparable between HIV patients and patients with an impaired immunity of other cause,[42] and includes fever, weight loss, abdominal pain, diarrhoea, and pulmonary symptoms. Abdominal lymph nodes are frequently found in disseminated *M. genavense* disease. Due to difficulty in culturing *M. genavense*, the diagnosis of *M. genavense* disease is a challenge for clinicians; in an immunocompromised patient with signs of disseminated disease, mycobacterial disease including *M. genavense* dissemination has to be considered. Molecular detection techniques have to be used to establish a fast and reliable diagnosis.

M. genavense was first described as an opportunistic pathogen in HIV infected patients with low CD4 counts. Among HIV patients with disseminated NTM disease, *M. genavense* is isolated in 4-13% of the cases.[43-46] As discussed in chapter 8, the introduction of anti-retroviral therapy has changed the epidemiology of *M. genavense* dramatically. Patients on drugs to prevent rejection after a solid organ transplant are now among the most frequently found case-reports in the literature. Interestingly, disseminated *M. genavense* in this patient group can be found years after transplantation: 18 and 19 years after renal and liver transplantation respectively in the patients presented in chapter 8, a mean duration of 33 months in 3 patients in case series from France,[42] and in other case-reports.[47-49] NTM infections that specifically occur months after starting immunosuppressive drugs are also seen among patients who use anti-tumor necrosis factor-α (anti-TNF-α) therapy.[50-52] This long interval between the start of immunosuppressive drug and development of active infection is in contrast to tuberculosis for which a median interval of 12 weeks is observed.[53] Probably the absence of a latent disease state for NTM, in contrast to tuberculosis is an explanation for this

observation. Although patients are on generally three different immunosuppressive drugs and thus severely immunocompromised, *M. genavense* disease is also reported in patients with sarcoidosis treated only with prednisone as the patient 8 in chapter 8, and in other case reports.[54-55] It remains unclear whether the immune reaction in sarcoidosis in combination with steroids predisposes to *M. genavense* disease, or the *M. genavense* infection was actually the cause of the observed granulomateous reaction in at least some patients.[56] Furthermore, the *M. genavense* infection can just be the result of another coexistent (innate) immune disorder in these patient. In chapter 8, we identified a patient with an IL-12 receptor deficiency and a patient with an idiopatic CD4 lymphcytopenia as the underlying disorder in the disseminated *M. genavense* infection. Some reports describe *M. genavense* patients with inherited disorders in the IL-12 / IFNy pathway (also called the Mendelian susceptibility to mycobacterial disease; MSMD) directly responsible for the disseminated M. genavense disease,[57] but in some immunesuppressive drugs still seem to be necessary to trigger *M. genavense* disease in this category of patients.[58] Anti-TNF-α therapy is not found in case series and case reports as a possible cause of *M. genavense* disease.[22] Probably, TNF- α inhibition is not enough to promote *M. genavense* disease but defects in IL-12/IFN-γ pathway that also result in diminished TNF- α production together with inhibition of various cytokines and chemokines do.[59]

Althought the treatment of *M. genavense* typically includes a macrolide, rifampicin and ethambutol (chapter 8),[42] the optimal treatment has still to be established. Besides anti-mycobacterial therapy, reducing the dosage of immunosuppressive drugs and/or substitution of cytokines/chemokines like IFN-γ have to be considered given the important role of the immune depressed state favouring *M. genavense* disease.

Clinical relevance of NTM

M. malmoense and *M. genavense* isolation in the Netherlands is clinically relevant in the majority of patients (chapter 6 and 8). In line with other studies from Northern Europe, clinical relevance of *M. malmoense* in the Netherlands was over 80% (chapter 6). It is one of the most clinical relevant NTM in the Netherlands, causing serious morbidity and mortality, although not frequently isolated. The same is true for *M. genavense*, an even higher clinical relevance was found of 93% in the Netherlands for this NTM, also infrequently isolated (chapter 8). Besides work presented in this thesis (chapter 5, 6, and 8), van Ingen et al.[18] published one of the key articles in the field of clinical relevance of NTM isolation. In this retrospective medical file review, all patients with a positive NTM isolated were studied to assess clinical relevance. Overall, clinical relevance was found to be 25%, but differed significantly by species: for some species, clinical relevance rose far over 50% (*M. malmoense*, *M. kansasii, M. szulgai*), in contrast to some species that showed almost no clinical relevance (*M. fortuitum, M. intracellulare, M. gordonae*). Based on these findings, as suggested by van Ingen, the ATS diagnostic criteria should differentiate between species of low and high clinical relevance, with less strict criteria to diagnose disease by highly relevant species and stricter criteria to be used for species with low clinical relevance. If criteria are applied less strict it may imply for example that one positive *M. malmoense* culture from a patient without clinical symptoms and with radiographic abnormalities that fits NTM disease, justify a much closer

observation of the patient or an earlier initiation of treatment after ruling out alternative diagnoses. Support for this approach was found by a follow-up study in patients with a single positive sputum sample with a potentially pathogenic NTM.[60] In the median follow-up time of 16 months, a NTM pulmonary disease diagnosis was made in 14% of cases. Only patients with MAC, *M. abscessus*, and *M. kansasii* isolates were included in this study, probably other NTM including *M. malmoense* will show comparable or even higher disease incidence after a single NTM sputum culture. The effects of early initiation of treatment versus close observation for these patients with a single NTM sputum sample can be addressed in future studies. In clinical practice, it is of greater importance to relate a positive NTM culture to co-morbid conditions. *M. genavense* isolation from a sterile body site, in a patient with an alleged normal immunity must trigger further investigation to detect an immunologic disorder. At the other side of the spectrum, a single pulmonary *M. gordonae* isolation in a patient without clinical signs and symptoms, and without asthma or COPD will likely be mere contamination.

Besides NTM virulence and host factors, clinical relevance of a NTM may be hard to assess if the organism is highly fastidious, as in the case of *M. genavense*. This very difficult to culture NTM species can be found in a high percentage of patients in intestine of healthy individuals.[61] Considering this *M. genavense* colonization of the human gut, clinical relevance figures would be much lower if culturing the pathogen was less complicated. In that case *M. genavense* will likely also more frequently be found in the respiratory system as a clinical irrelevant species, since *M. genavense* is acquired via the oral route. Furthermore, the presence of certain mycobacteria in niches close to humans will influence clinical relevance; lower clinical relevance figures will be found for NTM present in a high amount in tap water because of a higher change of culturing the NTM from the oral cavity after, for example a mouth rinse. Differences in clinical relevance between the species of NTM is not a matter of differences in pathogenicity between the species but a matter of differences in host factors, bacteriological factors and environmental factors combined. Because of the observed rise in NTM prevalence as discussed before together with geographical differences in species isolation (chapter 2) and differences in clinical relevance between continents within the same species (chapter 5), future studies remain important to monitor NTM epidemiology and clinical relevance.

Future research

Although the available literature on NTM has increased, NTM are poorly studied. The quality of NTM research issues will be greatly improve if NTM isolation becomes legally notifiable, or if a laboratory registration system is nationally introduced. The increase in prevalence of NTM disease in an ageing population that is increasingly at risk and the high mortality favour such measures. Although treatment outcome remains poor, national surveillance projects on NTM including central registration of demographic variables and for example postal codes will enhance insight into epidemiological factors and NTM transmission from the environment and can thus prioritize research. For example, NTM species that show increase in isolation in certain areas will trigger research on transmission vectors from the environment in that area.

Studies on NTM outbreaks are a possible source of knowledge regarding transmission vectors. Most of the studies available report on outbreaks of RGM infections. The majority describe RGM skin infections related to infected materials or fluids needed for example for acupuncture and tattoos.[62] Tap water supplies are an important source of NTM transmission to humans. Given the high mycobacterial load that can be found in tap water samples and shower heads,[63-65] transmission from these supplies with frequent exposure is perhaps more likely than transmission from a natural environmental source. Future studies on geographical distribution of NTM species have to address water supplies as a source of NTM transmission and differences between these supplies as probable cause of the observed geographical differences, an explanation that has been addressed before.[66] Important differences in isolation of mycobacteria from piped water supplies have been observed and was in accordance with the epidemiology of NTM disease in the studied countries.[67] To enhance insight in this matter, future reports on NTM isolation from environmental sources as well as from human populations should identify all isolates to the species level since the majority of population based studies only provide total numbers of NTM isolation.

We still need a better understanding of the important interplay between mycobacterial virulence and host factors. Why does NTM cause disease in some, but not all COPD patients after exposure to NTM from the (man-made) environment (showering, use of tap water). It has been hypothesized that disease is the result of a multifactorial process, in which both acquired local immunodeficiency (e.g. COPD), together with variation in for example dosage of inhaled steroid use, and deficits of cell mediated immunity have a role. The immunity to mycobacteria in humans is largely determined by the interferon- γ / interleukin-12 pathway, by which macrophages that capture the mycobacteria activate T lymphocytes to enable successful eradication of the invading mycobacteria. Analyses of host genetics may probably reveal polymorphisms in the genes that encode the essential proteins of the interferon-γ / interleukin-12 pathway. Among patients presenting with pulmonary NTM disease it was recently found that mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene were seen in 36.5% of the patients, significantly different from the 15.6% in an age-/sex-matched group.[68] Furthermore, these patients were taller and leaner than control subjects, with high rates of scoliosis, pectus excavatum and mitral valve prolapse.
Nodular-bronchiectatic disease was the most prevalent NTM disease type and the patients in this study are thus different from the COPD patients presenting more often with cavitating NTM disease. Whether this latter group of patients is also harboring a higher incidence of CFTR mutations remains to be studied. Other interesting genes that may play a role in NTM pulmonary disease susceptibility are natural resistance-associated macrophage protein 1 gene (Nramp1),[9] the major histocompatibility complex class I chain-related A (MICA) gene,[69] mutations in the vitamin D receptor[40] and toll-like receptor 2 (TLR-2) mutations[70] and have to be further studied for a possible role in COPD related NTM disease.

The important association between COPD and NTM deserve multi-center prospective case-control studies in order to find out more about NTM isolation and progression to NTM disease and accelerated lung function decline in COPD, especially in older subjects. Knowledge about risk factors derived from such studies will give insight into preventive strategies.
There is still a gap in knowledge on drug treatment of NTM pulmonary disease. A combination of rifampicine (RMP) and ethambutol (EMB) is the only treatment tested for slow growing

mycobacteria in a randomized clinical trial, in which addition of clarithromycin (CLA) showed no clear outcome benefit.[71] This is in contrast to studies from the United States, suggesting benefit of the addition of clarithromycin.[72] Regimens without RMP have been tested but received little attention in current guidelines. Clofazimin, EMB and CLA, however, showed sputum conversion rates above 80% and this regimen was in general well tolerated.[73] The clinical effect of the addition of CLA to the standard regime of RMP and EMB in MAC lung disease or lung disease caused by other slow growing NTM needs to be studied in more trials. Furthermore, potentially less toxic regimens (clofazimin, EMB and CLA) may be tested in multi-center clinical trials. Since treatment duration generally exceeds 12 months with toxic drugs, patients and NTM disease characteristics that most likely benefit from treatment have to be identified.

Regarding the RGM, *M. abscessus* is the most frequent encountered NTM; treatment results of pulmonary *M. abscessus* are very poor and have to be improved by further studies including clinical trials in CF related *M. abscessus* lung disease. Potential drug combinations that show synergistic results in vitro may be tested in vivo. The combination of amikacin and clofazimine showed synergistic effects across some clinical relevant NTM.[74] A multi-center clinical trial comparing the standard regimen of RMP/EMB with RMP/EMB combined with clofazimine and amikacin is needed. These trials have to include analysis of serum drug concentrations which can be related to the MICs and patient outcome.

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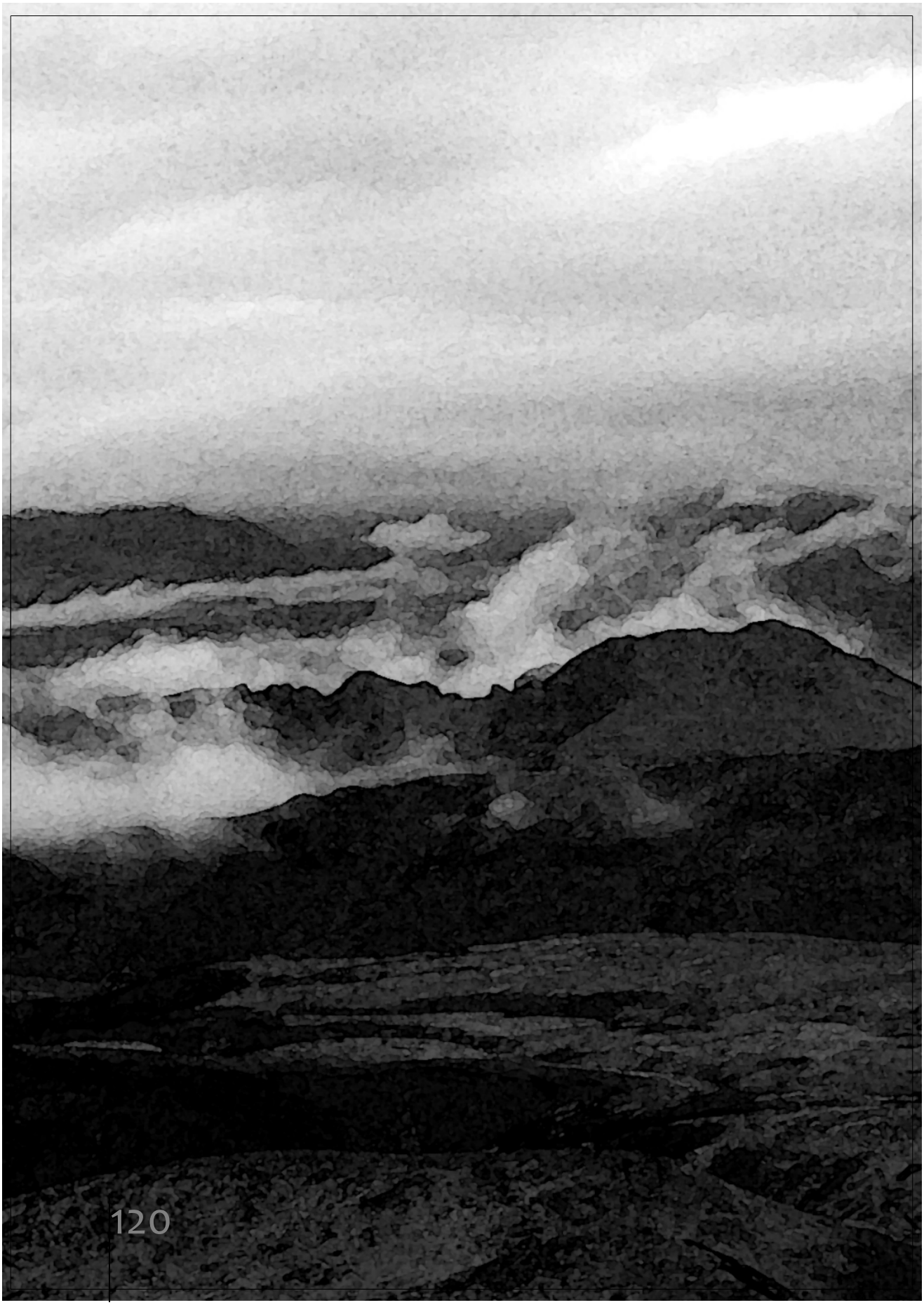

Summary

Summary

In this thesis studies on nontuberculous mycobacteria (NTM) performed by University Center of Chronic Diseases (UCCZ) Dekkerswald (part of the Radboud University Nijmegen Medical Center) in close conjunction with the National Institute for Public Health and the Environment (RIVM) are presented. The focus of the research is epidemiology and clinical relevance of NTM.

In **chapter 1** a general introduction on NTM is given. NTM are part of the genus *Mycobacterium*. *Mycobacterium tuberculosis* complex and *Mycobacterium leprae* are the other two groups that are part of the genus *Mycobacterium*. More than 120 different NTM species have been described. In contrast to *M. tuberculosis* complex and *M. leprae*, NTM are opportunistic pathogens. NTM are not transmitted from man-to-man. NTM are also called environmental mycobacteria due to their widespread environmental presence. Because of this environmental presence, isolation of mycobacteria from human samples can be related to true infection, pseudo-infection and contamination. The American Thoracic Society (ATS) diagnostic criteria are used to determine clinical relevance of the isolation. In the 20th century, the first case series of pulmonary NTM disease were published, a disease that showed important similarities with pulmonary tuberculosis. Several studies have shown a rise in the isolation prevalence of-and disease caused by- NTM in recent years. Because of serious morbidity and mortality related to NTM disease, together with poor treatment outcome, research on NTM must have a high priority.

In **chapter 2** the focus is on the NTM species distribution in pulmonary samples worldwide from a single time period. This snapshot illustrates that the species distribution among NTM isolated from pulmonary specimens in the year 2008 differs by region and differs by country within this region. For many regions or countries that participated in this study, these are the first data covering this topic. Furthermore, this study confirms previously suggested isolation patterns of certain mycobacteria. *M. malmoense* for example is a well known pathogen in Northern Europe (see also chapter 4 and 5 of this thesis) which is also shown in this study. Also within *Mycobacterium avium* complex, important differences are observed in worldwide isolation. While *M. avium* is mainly isolated in northern America, *M. intracellulare* is especially isolated in Australia. However, also some previously stated worldwide isolation patterns of certain mycobacteria do not hold true based on our study results. The study presented in chapter 2 will hopefully guide future research on transmission of NTM from the environment to humans.

In **chapter 3** the epidemiology of NTM isolation in the Netherlands is discussed. In many countries, including the Netherlands, an increase in NTM isolation has been observed. In this study we showed that the total number of NTM rose in absolute terms and relative to the number of *M. tuberculosis* isolates. The rise in NTM isolation was mainly caused by a sharp increase in *M. avium* isolation among patients aged >40 years. Although no review of clinical charts was performed in this study, based on previous studies, COPD seems to be an important factor for this observation. Since our research group showed COPD to be a possible risk factor for NTM disease, we studied this in more detail by a prospective cohort study in **chapter 4** in which the goal was to study the NTM isolation rate among patients presenting with an acute exacerbation of COPD. Seventy-three patients were included, 16 patients turned out to have one or more positive sputum culture yielding a NTM. In our study, only higher age was found to be associated with NTM isolation although bronchiectasis and steroid use may have predisposed as well. Although 4 patients were found to have two or more NTM of the same species isolated, only one patient turned out to have definite NTM disease according to the ATS diagnostic criteria. Further study is necessary to confirm our findings and to explore the association between COPD, bronchiectasis and pulmonary NTM isolation, including follow-up of patients to find more about NTM isolation and progression to NTM disease and accelerated lung function decline.

In **chapter 5-7** *M. malmoense* is studied in more detail. **Chapter 5** is a literature review concerning epidemiology, clinical relevance, and treatment of *M. malmoense.* Until the mid-1990s, *M. malmoense* isolation showed an increase in many countries in Northern Europe; outside Northern Europe *M. malmoense* was hardly described. An interesting difference in clinical relevance of *M. malmoense* isolation was observed in this review between isolates from northern Europe and North America: clinical relevance in North America was 10% in contrast to over 70% in Northern Europe. Clinically relevant isolation of *M. malmoense* was mainly observed in COPD patients in the studies included. Available data on treatment for *M. malmoense* is discussed.
In **chapter 6** the clinical relevance, treatment and outcome of *M. malmoense* in the Netherlands is studied. We reviewed medical records of all patients with a *M. malmoense* isolate between 2002 and 2006, identified via the national mycobacteria reference laboratory

RIVM. Fifty-one patients were identified in this retrospective analysis of whom 40 patients had a pulmonary *M. malmoense* isolate. Thirty-two patients (80%) met the ATS diagnostic criteria for pulmonary NTM disease. Of all patients with definite pulmonary *M. malmoense* disease, 81% had pre-existing pulmonary diseases. Cavitary lesions were seen in 88% of the patients. Eleven patients had an extra-pulmonary isolate of whom 10 had cervicofacial lymphadenitis including two elderly patients. None of the patients in this study were found to have an underlying immune deficiency like HIV. Adherence to treatment guidelines was relatively poor. Some patients were treated for a presumed pulmonary tuberculosis infection. A good clinical response to treatment was observed in 70% and 73% of patients treated for pulmonary and extrapulmonary disease, respectively. Two pulmonary *M. malmoense* patients with a strong epidemiological link identified in this study are described in more detail in **chapter 7**. Human transmission was suspected because an acid-fast bacilli (AFB) smear positive patient who was in close contact with an immunocompromised patient. However, using two molecular typing techniques and *hsp65* gene sequencing, strains of both patients proved unique and thus human transmission was highly unlikely.

Chapter 8 focuses on a *M. genavense*, a pathogen that has been described especially in patients with HIV with CD4 counts below 100/µl. Reviewing the NTM database of the RIVM, the infrequently isolated NTM *M. genavense* was observed to be isolated especially from sterile body sites like bone marrow and liver. In the era of successful HIV treatment, we expected a change in its epidemiology and etiology which has not been studied in detail before. Thirteen out of the 14 patients retrospectively identified had definite *M. genavense* disease. Four patients were HIV-positive, the other patients had mostly disseminated disease because of severe immunosuppression. Four patients died in this study, all because of *M. genavense* disseminated disease. The conclusion is made that *M. genavense* is a clinically relevant pathogen in severely immunocompromised patients that causes predominantly disseminated disease. *M. genavense* is increasingly seen among non-HIV immunocompromised patients.

In **chapter 9** our studies are discussed in a broader scientific context. Besides epidemiology and clinical relevance (the main focus of this thesis) also NTM in relation to COPD is discussed in more detail in this chapter. It is stated that research on NTM isolation and disease will be greatly improved if a national surveillance on NTM will be introduced. Furthermore, future research topics are discussed and include transmission vectors, mycobacterial virulence factors and host factors, NTM pathogenesis among COPD patients and treatment.

Samenvating II

Samenvatting

In dit proefschrift worden studies gepresenteerd op het gebied van nontuberculeuze mycobacteriën (NTM), uitgevoerd door het Universitair Centrum voor Chronische Ziekten (UCCZ) Dekkerswald (onderdeel van Radboud Universitair Medisch Centrum) in nauwe samenwerking met het Rijksinstituut voor Volksgezondheid en Milieu (RIVM). De focus van dit onderzoek is epidemiologie en klinische relevantie van NTM.

Hoofdstuk 1 geeft een algemene inleiding op NTM. NTM maken onderdeel uit van het genus Mycobacteriën. Naast NTM worden mycobacteriën onderscheiden die vallen onder het *Mycobacterium tuberculosis* complex en *Mycobacterium leprae*. Er zijn meer dan 130 verschillende soorten NTM bekend. In tegenstelling tot *M. tuberculosis* complex en *M. leprae* zijn NTM opportunistische pathogenen. NTM worden voor zover bekend niet van mens op mens overgedragen. NTM zijn milieubacteriën, ze komen wijd verspreid in onder andere water en grond voor. Omdat NTM in het milieu voorkomen moet een van een patiënt verkregen positieve NTM kweek altijd gerelateerd worden aan de kliniek: het verkregen isolaat kan een echte infectie betekenen, pseudo-infectie of contaminatie uit het milieu. De door Amerikaanse longartsen gepubliceerde diagnostische criteria (*American Thoracic Society criteria on NTM disease*) kunnen worden gebruikt om de klinische relevantie van een verkregen isolaat te bepalen. Halverwege de 20e eeuw zijn de eerste patiënten series beschreven waar pulmonale NTM ziekte werd vastgesteld. Pulmonale NTM ziekte heeft een vergelijkbare presentatie als

pulmonale tuberculose. Verschillende onderzoeken hebben inmiddels een stijging laten zien van het aantal gevonden NTM isolaten en het aantal bewezen infecties met NTM. Onderzoek op het gebied van NTM is gerechtvaardigd op basis van de morbiditeit en mortaliteit die gepaard gaat met infecties in combinatie met slechte uitkomsten van behandeling.

Hoofdstuk 2 beschrijft een onderzoek naar het wereldwijd voorkomen van de verschillende NTM species in het jaar 2008. Deze *snapshot* laat zien dat de wereldwijde NTM species verdeling in het jaar 2008 verschilt per continent en verschilt per land binnen een continent. Voor een aantal landen die data hebben aangeleverd voor deze studie zijn er niet eerder epidemiologische data over NTM gepubliceerd. Daarnaast worden eerder waargenomen wereldwijde isolatie patronen middels deze studie bevestigd, bijvoorbeeld het specifiek frequent voorkomen van *M. malmoense* in Noord Europa in tegenstelling tot andere werelddelen (zie ook hoofdstuk 4 en 5 van dit proefschrift). Ook binnen het *Mycobacterium avium* complex worden verschillen in wereldwijde isolatie waargenomen. *M. avium* wordt voornamelijk geïsoleerd in Noord Amerika, *M. intracellulare* met name in Australië. Sommige uit de literatuur bekende patronen van isolatie van enkele NTM species worden in deze studie niet bevestigd. Het onderzoek wat in dit hoofdstuk wordt gepresenteerd zal richting kunnen geven aan toekomstig onderzoek naar transmissie vectoren van NTM naar de mens.

In **hoofdstuk 3** wordt de epidemiologie van NTM in Nederland bestudeerd. In veel landen, inclusief Nederland, wordt de laatste jaren een stijging van het aantal NTM isolaten gevonden. In deze studie laten we zien dat het aantal gevonden NTM isolaten in Nederland stijgt in verhouding tot het aantal *M. tuberculosis* isolaten. Deze stijging in isolatiefrequentie werd met name bepaald door een scherpe stijging van het aantal gevonden *M. avium* isolaten onder patiënten ouder dan 40 jaar. Hoewel er in deze studie geen klinische gegevens zijn bestudeerd lijkt COPD een belangrijke factor hiervoor te zijn. Aangezien onze onderzoeksgroep eerder heeft aangetoond dat COPD een mogelijke risicofactor voor NTM pulmonale ziekte is, hebben we dit in meer detail bestudeerd in **hoofdstuk 4** middels een prospectieve cohort studie. Het doel van deze studie was het bestuderen van de prevalentie van NTM isolatie bij patiënten die zich presenteren met een acute exacerbatie van COPD. 73 patiënten werden geïncludeerd, 16 patiënten bleken één of meerdere positieve NTM sputumkweken te hebben. Hoge leeftijd was geassocieerd met een positieve NTM kweek. Er werden vier patiënten gevonden die twee of meer positieve NTM kweken hadden van hetzelfde species, een daarvan bleek klinisch relevant op basis van de ATS diagnostische criteria. Nader onderzoek is geïndiceerd om onze bevindingen te bevestigen en om de associatie tussen COPD, bronchiectasiën en pulmonale NTM isolatie verder te bestuderen, inclusief de follow-up van patiënten gericht op progressie naar pulmonale NTM ziekte en versneld longfunctieverlies.

In **hoofdstuk 5-7** wordt *M. malmoense* nader bestudeerd. **Hoofdstuk 5** is een overzicht van de literatuur voor wat betreft epidemiologie, klinische relevantie en behandeling van *M. malmoense.* Tot halverwege de jaren 90 werd er een stijging gezien van het aantal gevonden *M. malmoense* isolaten in Noord Europa; buiten Noord Europa werd *M. malmoense* nauwelijks gerapporteerd. Uit studies geïncludeerd in dit overzicht blijkt verder dat isolaten afkomstig uit Noord Europa veel vaker klinisch relevant zijn dan isolaten gevonden in Noord Amerika. Klinisch relevante isolatie van *M. malmoense* werd verder met name gevonden in patiënten met onderliggend COPD. Beschikbare literatuur over behandeling van *M. malmoense* wordt besproken.

In **hoofdstuk 6** wordt de klinische relevantie, behandeling en uitkomst van *M. malmoense* in Nederland bestudeerd. De medische dossiers van alle patiënten met een positieve *M. malmoense* isolaat die werden geïdentificeerd via het mycobacterieel referentie laboratorium van het RIVM tussen 2002 en 2006 werden ingezien. De klinische relevantie, behandeling en uitkomst werden geregistreerd. 51 patiënten werden geïdentificeerd in deze retrospectieve analyse, 40 patiënten hadden een pulmonale *M. malmoense* isolaat. 32 patiënten (80%) voldeden aan de ATS diagnostische criteria voor NTM pulmonale ziekte. Van deze patiënten had 81% een pre-existente longziekte. Caviterende afwijkingen werden gezien in 88% van de patiënten. 11 patiënten hadden een extra-pulmonaal isolaat waarvan 10 een cervicale lymfadenitis bleken te hebben, waaronder 2 oudere patiënten. Geen van de patiënten in deze studie bleek een onderliggende immuundeficiëntie te hebben zoals HIV. Behandelrichtlijnen werden slecht gevolgd. Sommige patiënten werden behandeld voor een pulmonale tuberculose infectie. Een goede klinische respons op behandeling werd gevonden in 70% en 73% van de patiënten behandeld voor respectievelijk pulmonale en een extra-pulmonale ziekte. Twee patiënten met een sterke epidemiologische link in deze studie werden nader bestudeerd in **hoofdstuk 7**. Humane transmissie werd vermoed op basis van sputum Ziehl-Neelsen (ZN) positieve patiënt die veelvuldig contact had met een immuungecompromiteerde patiënt. Humane transmissie kon echter niet worden bevestigd middels moleculaire technieken waaronder de hsp65 gen sequentie techniek.

Hoofdstuk 8 bestudeerd *M. genavense*, een pathogeen die met name beschreven is in HIV patiënten met een CD4-getal beneden de 100/µl. In de database van het RIVM werd gezien dat deze weinig frequent gevonden NTM met name geïsoleerd werd uit steriele organen zoals lever en beenmerg. In het tijdperk van succesvolle behandeling van HIV verwachtten wij een verandering in epidemiologie en etiologie welke niet eerder is bestudeerd. 13 van de 14 retrospectief geïdentificeerde patiënten met een *M. genavense* isolaat bleken relevante *M. genavense* ziekte te hebben. Vier patiënten waren HIV-positief, de andere patiënten bleken voornamelijk gedissimineerd *M. genavense* ziekte te hebben op basis van een ernstige immuundeficiëntie. Vier patiënten overleden in deze studie op basis van gedissimineerde *M. genavense* ziekte. Geconcludeerd wordt dat *M. genavense* een klinisch relevant pathogeen is in ernstig immuungecompromiteerde patiënten die voornamelijk gedissimineerde ziekte veroorzaakt. *M. genavense* wordt steeds vaker gezien in de non-HIV immuungecompromiteerde patiënt.

In hoofdstuk 9 worden onze studies in een breder wetenschappelijk perspectief besproken. Naast epidemiologie en klinische relevantie (de belangrijkste focus van dit proefschrift), wordt ook NTM in relatie met COPD bediscussieerd in dit hoofdstuk. Er wordt gesteld dat onderzoek op het gebied van NTM ziekte verbeterd zal worden als NTM isolatie meldingsplichtig gemaakt wordt. Daarnaast worden in dit hoofdstuk onderwerpen voor toekomstig onderzoek besproken zoals transmissie vectoren, mycobacteriële virulentie- en gastheer factoren, NTM pathogenese in COPD patiënten en behandeling.

Dankwoord III

Dankwoord

Tijdens mijn promotietraject zijn er veel mensen geweest die het werk zowel leuker hebben gemaakt als ook makkelijker. Mijn dank is daarvoor groot.

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Veel verpleegkundigen en secretaresses van Dekkerswald hebben mij fantastisch geholpen bij de NACHT studie (hoofdstuk 4). Annemarie wil ik hier toch speciaal noemen als trekker van de NACHT-studie kar op de afdeling. Heel veel dank daarvoor. Femke Cuppen, bedankt voor het helpen met de data verzameling. Thy en Claudia, bedankt voor jullie geweldige steun met de logistiek om en rond mijn boekje!

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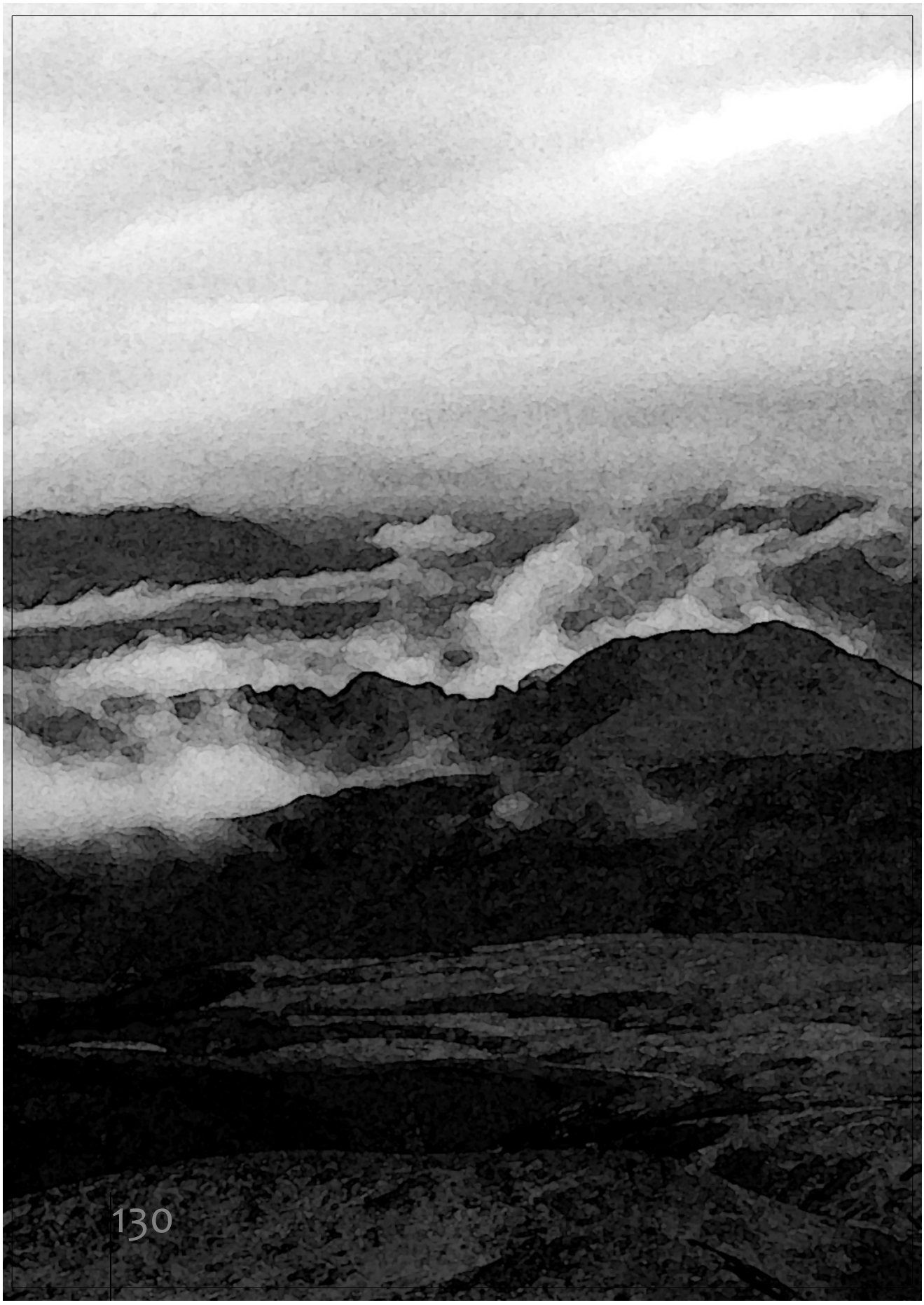
Lieve Marloes, jij staat altijd voor mij klaar. Dat is pas iets waar ik echt trots op mag zijn en ben!



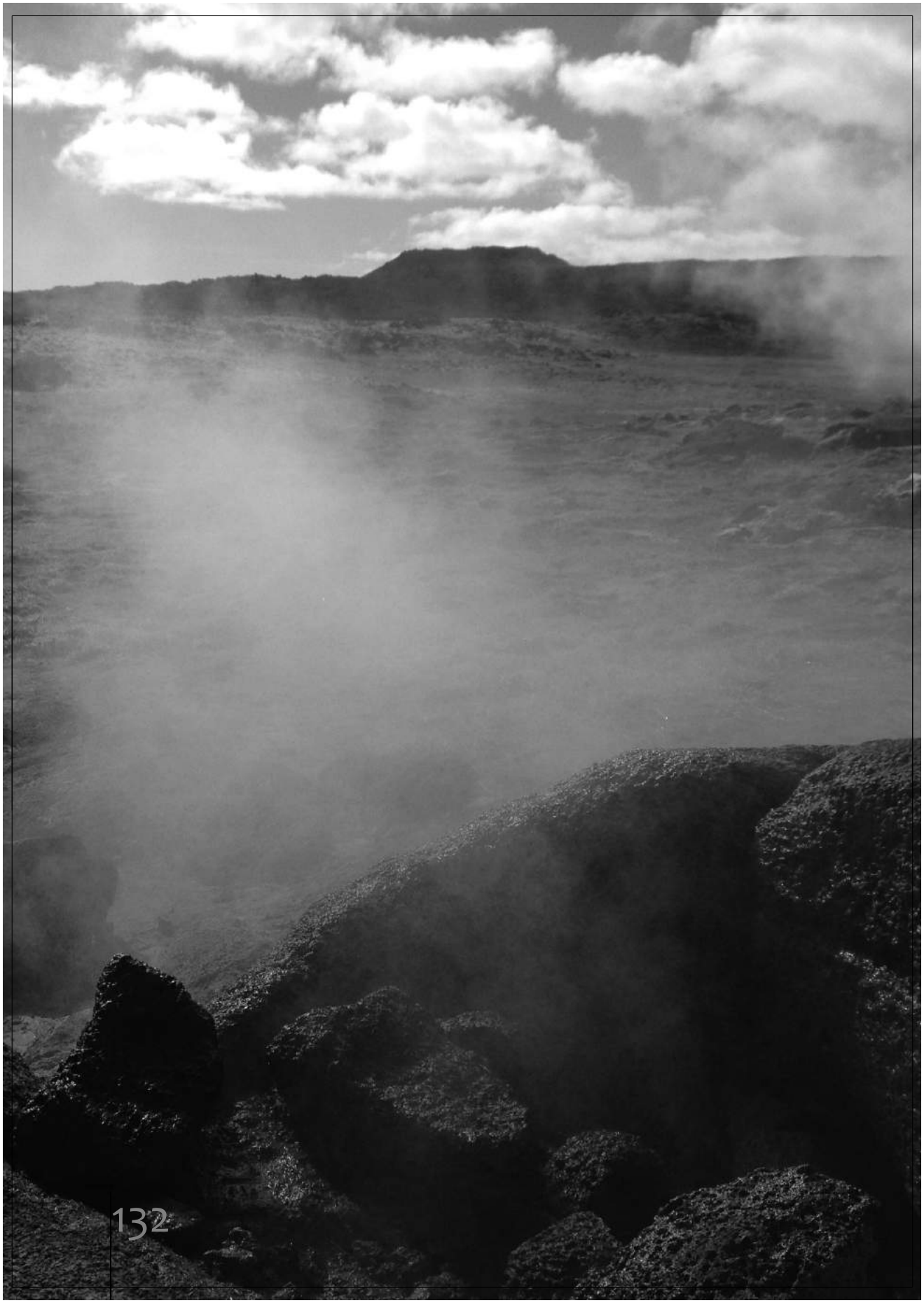
Biography IV

Wouter Hoefsloot was born on April 8th, 1978 in Krimpen a/d IJssel, the Netherlands. He grew up in a family with two older brothers. After completing secondary school in Doorwerth he started to study Biomedical Health Sciences at the Catholic University Nijmegen (KUN). He specialised in kinesiology. His first article was published in 2000 during his internship at the department of Physiology at the KUN.

He did his final internship at the department of Neurology of the KUN, where he studied mechanisms of fatique during a voluntary maximal contraction of the M. biceps brachii in healthy volunteers. In 2000 he received his master degree and decided to start studying Medicine at the Radboud University Nijmegen (RUN, former KUN). Waiting for his internship to start, he worked at the department of Geriatry on a systematic review on quantitative gait analysis in patients with dementia.

He did his final internship in Sumve District Designated Hospital, Sumve Tanzania. This internship was preceded by a short internship at the University Lung Hospital, Dekkerswald, one of the two specialised tuberculosis hospitals in the Netherlands. Thereafter, as a resident in pulmonary diseases, he participated in the research atypical mycobacteria (RAM) team and first focused on the nontuberculous mycobacteira *M. malmoense* as an important respiratory tract pathogen. His research work was supervised by Martin Boeree and Dick van Soolingen, in close conjunction with Jakko van Ingen who graduated on nontuberculous mycobacteria in 2009.

Since December 2011, Wouter Hoefsloot is working at the Radboud University Medical Center, Nijmegen, location University Center of Chronic Diseases, Dekkerswald (former University Lung Hospital, Dekkerswald). As a pulmonary phycisian he is specialised in tuberculosis, nontuberculous mycobacterial infections and cystic fibrosis. Since 2013 he is specialising in endoscopic ultrasound (EUS/EBUS) and interventional bronchoscopy in Radboud University Medical Center supervised by Dr. E. v.d. Heijden and Drs. O. Schuurbiers.



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