“Environmental transmission factors and host genetic polymorphisms influencing NTM infection in patients with no overt immunodeficiency”

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BSc (Hons)

A thesis submitted for the degree of Masters of Philosophy at
The University of Queensland in 2016
School of Medicine
Abstract

Pulmonary disease associated with non-tuberculous mycobacteria (NTM) is a poorly understood and difficult to treat condition. It is an increasingly common problem in a subset of older individuals, with NTM lung disease now reported more frequently than tuberculosis in Australia. Environmental exposure appears to be the primary route for pulmonary NTM infection, but specific risk factors remain poorly determined. A large subset of patients with pulmonary NTM disease appears to be immunocompetent, but the existence of an as yet unidentified deficiency in these patients’ immune response to NTM infection may influence their risk of infection.

This thesis begins with a review of the evidence that pathogenic species of NTM have been isolated from drinking water distribution systems, soil and house dust, so exposure may be very common. A defect in immune response appears critical to the development of disease. Ways by which genetic make-up may predispose some individuals to pulmonary NTM disease were addressed. This involved investigations of polymorphic alleles across \textit{IL10} and a group of three novel genes \textit{P2X4R}, \textit{P2X7R} and \textit{CAMKK2}.

DNA was obtained from 124 patients with pulmonary NTM disease attending Greenslopes Private Hospital, QLD, Australia and Prince Charles Hospital, QLD, Australia between 2005 and 2014. DNA from an additional 229 healthy control donors recruited from Western Australia (Temple et al., 2003) was provided by the Institute for Respiratory Health, Western Australia.

A strong association between pulmonary NTM disease and a polymorphism within \textit{IL10} (rs1518111), and a haplotype spanning the three genes \textit{P2X4R}, \textit{P2X7R} and \textit{CAMKK2} were identified. These results implicate these genes (or other genes in linkage disequilibrium with them) and their products as factors contributing to infection and disease. This information may prove critical in developing new strategies and therapies for patients with NTM disease and may provide critical insights into other mycobacterial diseases such as leprosy and tuberculosis.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature


Publications included in thesis


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**Paper 2:** Halstrom, S., Thomson, R., Goullee, H., Baltic, S., Allcock, R., Temple, S. E., & Price, P. 2017a. Susceptibility to non-tuberculous mycobacterial disease is influenced by rs1518111 in *IL10*. *Human Immunology*, 78(4), 391-393. PMID: 28174026

Incorporated as Chapter 4.

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## Contributions by others to the thesis

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Statement of parts of the thesis submitted to qualify for the award of another degree

None.
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Non-tuberculous mycobacteria, pulmonary disease, genetic polymorphism, epidemiology, environment, IL10

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Abbreviations

*ABCA3*: ATP binding cassette subfamily A member 3 (gene)
*AFLP*: Amplified fragment length polymorphism
*ATP*: Adenosine triphosphate
*BAT1*: HLA-B associated transcript 1 (gene)
*CAMKK2*: Calcium/calmodulin-dependent protein kinase kinase 2 (gene)
*CaMKK2*: Calcium/calmodulin-dependent protein kinase kinase 2 (protein)
*CCL2*: C-C Motif Chemokine Ligand 2 (gene)
*CD14*: Cluster of differentiation 14 (gene)
*CFU*: Colony forming unit
*COPD*: Chronic obstructive pulmonary disease
*CYBB*: Cytochrome B-245, beta polypeptide (gene)
*HPLC*: High performance liquid chromatography
*hsp65*: Heat-shock protein 65 (gene)
*HWE*: Hardy-Weinberg Equilibrium
*HP*: Hypersensitivity pneumonitis
*IFNGR1*: Interferon gamma receptor 1 (gene)
*IFNGR2*: Interferon gamma receptor 2 (gene)
*IL1A*: Interleukin 1A (gene)
*IL2*: Interleukin 2 (gene)
*IL4*: Interleukin 4 (gene)
*IL-4*: Interleukin 4 (protein)
*IL10*: Interleukin 10 (gene)
*IL-10*: Interleukin 10 (protein)
*IL12B*: Interleukin 12B (gene)
*IL12RB1*: Interleukin 12B1 (gene)
*IL18*: Interleukin 18 (gene)
*IL28B*: Interleukin 28B (gene)
iNKT cells: Innate-like natural killer T-cells
IRF8: Interferon regulatory factor 8 (gene)

ISG15: Interferon-stimulated protein, 15 KDa (gene)

MAC: *Mycobacterium avium* complex

MA: Minor allele

MAF: Minor allele frequency

MAIT cells: Mucosal-associated invariant T-cells

NEMO: NF-kappa-B essential modulator (gene)

NTM: Non-tuberculous Mycobacteria

P2X4R: P2X purinoceptor 4 (gene)

P2X4R: P2X purinoceptor 4 (protein)

P2X7R: P2X purinoceptor 7 (gene)

P2X7R: P2X purinoceptor 7 (protein)

PCR: Polymerase chain reaction

PFGE: Pulsed-field gel electrophoresis

RAPD - PCR: Random amplified polymorphic DNA - PCR

rDNA: Ribosomal DNA

Rep-PCR: Repetitive PCR

RFLP: Restriction fragment length polymorphism

rpoB: β subunit of bacterial RNA polymerase (gene)

RT - PCR: Reverse transcription - PCR

SFTPA/B/C/D: Pulmonary surfactant protein A/B/C/D (gene)

SLC11A1: Solute carrier family 11 member 1 (gene)

SNP: Single Nucleotide Polymorphism(s)

STAT1: Signal transducer and activator of transcription 1 (gene)

TLR2: Toll-like Receptor 2 (gene)

TLR2: Toll-like Receptor 2 (protein)

TNFA: Tumor necrosis factor alpha (gene)

VDR: Vitamin D (1,25- Dihydroxyvitamin D3) receptor (gene)
Chapter 1:
Introduction and literature review
1) **Introduction and Literature Review**

### 1.1) Pathology of Pulmonary NTM disease

Pulmonary disease associated with non-tuberculous mycobacteria (NTM) is an increasingly common problem in a subset of older individuals. Although frequency of reported NTM lung disease varies greatly around the world, it is now reported more frequently than tuberculosis in Queensland, Australia where it is a reportable condition (21.8 pulmonary NTM cases per 100,000 compared to 3.8 Tuberculosis cases per 100,000 in 2015) (Queensland Health, 2015a, Queensland Health, 2015b). Pulmonary NTM disease is thought to be primarily transmitted from the inhalation of pathogenic NTM from the surrounding environment. A full review of the literature analysing NTM disease epidemiology was published as a component of this project (See Chapter 3).

Pulmonary NTM disease is develops slowly and is difficult to eradicate, with patients experiencing bouts of active infection and periods of remission. Infections by different species have different expected outcomes. When the appropriate antimicrobial regimens are administered, the cure rate for *M. kansasii* is reported at around 90% (similar to *M. tuberculosis* infection) while *M. avium* is cured in 30-85% of patients. For patients with pulmonary *M. abscessus* infection complete recovery can be achieved but is unpredictable (Marušić and Janković, 2012).

The main mechanism of infection appears to be inhalation of aerosolised NTM. As intracellular pathogens, the NTM invade and replicate within host lung epithelia and alveolar phagocytes. In a majority of cases, it is thought that the innate immune system prevents the bacteria from exploiting local cells in this way. However, in a small subset of individuals, predominantly thin elderly women, an unknown susceptibility allows the development of fibronodular disease (Chan and Iseman, 2010, Thomson, 2010).

### 1.2) How are people exposed to NTM?

When aiming to identify major risk factors leading affecting an individual’s chances of pulmonary NTM disease, the first consideration is exposure and transmission of the pathogen. A review of the literature assessing confirmed cases of transmission of pathogenic NTM causing disease was written and published, exposing the knowledge gaps in research (See Chapter 3).

This review concludes that while aerosolisation of water appears to be the most reported and confirmed transmission route of pathogenic NTM, there is not enough data to say with certainty that
it is the primary method of transmission. However, it does appear that the majority of pathogenic NTM are not transmitted person-to-person, but exclusively from exposure to environmental sources such as water, soil and dust. As exposure to pathogenic NTM appears to be widespread, it is likely that risk of pulmonary NTM infection and disease is largely determined by differences in host susceptibility to these bacteria.

1.3) **Could a host genetic polymorphism influence susceptibility to NTM?**

Genetic polymorphism could help to explain the selective nature of pulmonary NTM infections in individuals with apparently healthy immune systems. Previous work from our team identified alleles of *TNFA, IL12B* and (most clearly) *IL10* affecting pulmonary NTM disease (Affandi et al., 2013). The study also found several alleles of genes encoding mediators of T-cell activation and inflammation that did not associate with NTM disease (See Table1.1), so here we have focussed on genes that may have a fundamental role in inflammation.

The most promising gene was *IL10*, as the (A) allele of rs1800896 has been significantly associated with pulmonary NTM disease and perturbations of the Th1/Th2 balance (Affandi et al., 2013). Previous studies have also observed high production of IL-10 in blood mononuclear cells from NTM patients (Lim et al., 2010). A more complete review of IL10s perceived relevance to pulmonary NTM disease is included as part of a short communication (Chapter 4). Affandi et al. also identified SNPs in *IL28B* and *TNFA* associated with pulmonary NTM disease, but were not investigated further in my project. SNPs affecting genes including *BAT1, IL1A, IL2, IL18, CCL2, CD14, IL-12B* and *SLC11A1* were investigated but did not appear associated with disease (Affandi et al., 2013). These genes were not investigated further. Although the Affandi review produced few strong leads, it highlights the perceived mechanisms capable of affecting susceptibility to pulmonary NTM disease (pattern-recognition molecules, surfactant proteins, signalling pathways, pro-inflammatory cytokines and the anti-inflammatory response), identifies SNP likely to affect these processes, and conveys the need for further effective genetic studies (Affandi et al., 2010). Genes investigated in this thesis are described here.
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<td>0.7-2.7</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>3</td>
<td>0</td>
<td>0.54</td>
<td>0.4-0.7</td>
<td>0</td>
<td>C_16043997_10</td>
<td>TT</td>
<td>42</td>
<td>18</td>
<td>1.20</td>
<td>0.5-2.7</td>
<td>0.65</td>
</tr>
<tr>
<td>IL1A+4845^f rs17561</td>
<td>CC</td>
<td>86</td>
<td>37</td>
<td>0.84</td>
<td>0.5-1.5</td>
<td>0.54</td>
<td>IL10-1082 d rs1800896</td>
<td>GA</td>
<td>109</td>
<td>33</td>
<td>0.40</td>
<td>0.2-0.7</td>
<td>0.004</td>
</tr>
<tr>
<td>C_9546471_10</td>
<td>AA</td>
<td>7</td>
<td>8</td>
<td>2.70</td>
<td>0.9-7.9</td>
<td>0.07</td>
<td>C_1747360_10</td>
<td>GG</td>
<td>41</td>
<td>17</td>
<td>0.54</td>
<td>0.3-1.1</td>
<td>0.11</td>
</tr>
<tr>
<td>IL2-330 d rs2069762</td>
<td>TT</td>
<td>92</td>
<td>36</td>
<td>1.29</td>
<td>0.8-2.2</td>
<td>0.35</td>
<td>IL12B 3'UTR^b rs3212227</td>
<td>AC</td>
<td>43</td>
<td>22</td>
<td>1.31</td>
<td>0.7-2.4</td>
<td>0.38</td>
</tr>
<tr>
<td>C_15859930_10</td>
<td>TG</td>
<td>77</td>
<td>39</td>
<td>1.44</td>
<td>0.5-2.0</td>
<td>0.91</td>
<td>C_2084293_10</td>
<td>CC</td>
<td>9</td>
<td>4</td>
<td>1.14</td>
<td>0.3-3.8</td>
<td>0.83</td>
</tr>
<tr>
<td>IL4-589^d rs2243250</td>
<td>CT</td>
<td>37</td>
<td>16</td>
<td>0.72</td>
<td>0.4-1.3</td>
<td>0.27</td>
<td>SLC11A1 rs17235409</td>
<td>AG</td>
<td>6</td>
<td>4</td>
<td>1.64</td>
<td>0.4-5.9</td>
<td>0.45</td>
</tr>
<tr>
<td>C_16176216_10</td>
<td>TT</td>
<td>5</td>
<td>3</td>
<td>0.77</td>
<td>0.3-1.9</td>
<td>0.59</td>
<td>C_25635296_10</td>
<td>AA</td>
<td>0</td>
<td>1</td>
<td>1.00</td>
<td>0.3-0.5</td>
<td>0</td>
</tr>
<tr>
<td>IL18-137^d rs187238</td>
<td>GC</td>
<td>65</td>
<td>25</td>
<td>1.20</td>
<td>0.4-2.3</td>
<td>0.48</td>
<td>IL28B SNPi^a rs8099917</td>
<td>TT</td>
<td>144</td>
<td>50</td>
<td>1.76</td>
<td>0.9-3.2</td>
<td>0.06</td>
</tr>
<tr>
<td>C_2408543_10</td>
<td>CC</td>
<td>17</td>
<td>7</td>
<td>0.77</td>
<td>0.3-1.9</td>
<td>0.59</td>
<td>C_11710096_10</td>
<td>GG</td>
<td>3</td>
<td>4</td>
<td>3.84</td>
<td>0.8-17</td>
<td>0.08</td>
</tr>
<tr>
<td>IL18-607^e rs1946518</td>
<td>TT</td>
<td>62</td>
<td>25</td>
<td>0.68</td>
<td>0.4-2.8</td>
<td>0.53</td>
<td>IL28B SNPy^a rs12979860</td>
<td>TT</td>
<td>10</td>
<td>0</td>
<td>1.00</td>
<td>0.3-0.6</td>
<td>0</td>
</tr>
<tr>
<td>C_2898460_10</td>
<td>TG</td>
<td>78</td>
<td>39</td>
<td>1.28</td>
<td>0.6-2.8</td>
<td>0.53</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

rs = reference SNP number, ^a OR = Odds ratio 95%, ^b CI = 95% Confidence interval, ^c Univariate P value calculated by logistic regression modelling (Significant P values ≤ 0.05), ^d Promoter region, ^e Intron, ^f Exon, ^g Nonsynonymous, ^h 3' Untranslated region.
1.3.1) **IL-10**

*IL10* is a target of interest for further genetic analysis based on previous work within our group (Affandi et al., 2013). The *IL10* SNP associated with NTM susceptibility associated with lower levels of IL-10, but we and others demonstrated higher than normal IL-10 production by mononuclear cells when cultured with mycobacterial antigen (Lim et al., 2010, Turner et al., 1997). Additional *IL10* SNPs and their potential associations with NTM disease are a promising area of investigation (See Chapter 4).

The three genes *P2X7R, P2X4R, and CAMKK2* are located adjacent to one another on chromosome 12, and as such are in linkage disequilibrium (see Figure 1). They represent a major new initiative in genetic studies of NTM as they have not been considered previously.

1.3.2) **P2X7R**

The *P2X7R* gene encodes the P2X7R protein, an ATP-gated ion channel which plays an important role in the innate immune response. When bound by its ligand, extracellular ATP, P2X7R enables the intracellular killing of phagocytosed bacteria (ie: NTM) in the presence of local inflammatory stress.

A loss of function polymorphism in the *P2X7R* gene (rs3751143) increases susceptibility to extrapulmonary tuberculosis but this occurrence has not been investigated in patients with pulmonary NTM disease (Fernando et al., 2007). This SNP is one of several within *P2X7R* being investigated in this project. Any one of these polymorphisms may alter function in the P2X7R channel, potentially allowing the evasion of a macrophage phagocytosis response by infectious agents.

1.3.3) **P2X4R**

*P2X4R* encodes the P2RX4 protein, a purinoreceptor activated by ATP allowing the transport of calcium ions across a membrane. It is generally co-expressed with P2X7R in intracellular lysosomal compartments of macrophages and microglia, but can be rapidly trafficked to the surface membrane via the process of endolysosomal secretion (Stokes and Surprenant, 2009).
P2X4R appears to be involved in the process of phagocytosis, lysosomal destruction and removal of pathogens taken up by macrophages, specifically post-fusion phase exocytosis (Miklavc et al., 2011). Polymorphisms in the P2X4R gene may allow exploitation of this system by nontuberculous mycobacterium, which can persist in macrophage phagosomes via inhibition of lysosome binding.

1.3.4) CAMKK2

CaMKK2 is a common calcium ion activated protein kinase involved in the function of many different pathways, potentially including lymphocyte activation. It may act downstream of P2RX4 in the process of exocytosis in macrophages that have taken up NTM. P2X4R allows calcium ions to traverse membranes activating CaMKK2 pathways. This function may be affected by loss of function mutations leading to an increased susceptibility to NTM.

CaMKK2 is selectively expressed in monocytes/macrophage (Racioppi et al., 2012). In knockout mice, CaMKK2-null macrophages displayed a deficiency to spread, phagocytose bacteria, and synthesize cytokines in response to the Toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS) (Racioppi et al., 2012). Loss of CaMKK2 action uncouples the TLR4 cascade from activation of PTK2B, thereby failing to properly activate the inflammatory response. The general macrophage weakening reported in the areas of migration, phagocytosis and cytokine production in the CAMKK2 KO-mice demonstrates the potential effect of deleterious mutations on NTM disease susceptibility.
Figure 1: Linkage disequilibrium plots for SNPS in *P2X7R*, *P2X4R* and *CAMKK2* found in the Caucasian (CEU) population. Solid red blocks indicate instances where two SNPs are invariably inherited together. The likelihood decreases with pink shading; white blocks show SNPs that are rarely inherited together. The blue blocks represent combinations where co-inheritance has not yet been investigated (Goullee et al., 2016). As IL10 is located on a different chromosome (chromosome 1) it has not been included in linkage analysis.
1.3.5) TLR2

TLR2 was investigated. SNP within TLR2 (rs4696480 and rs5743706) have been associated with an increased risk of pneumonia and gram-negative sepsis (Affandi et al., 2010, Lorenz et al., 2002, Sutherland et al., 2005). Immune processes involving TLR2 have been associated with pulmonary disease, raising the possibility of TLR2 polymorphism affecting pulmonary NTM disease. This is the case for pulmonary surfactant protein B polymorphism associating with risk of pneumonia and Myeloid differentiation factor 88 (signalling downstream of TLR2) polymorphism associating with decreased susceptibility to pneumococcal disease (Beutler, 2002, Griese et al., 2008). However, none of the SNP investigated within this gene (rs3804099, rs3804100 and rs5743704) associated significantly with pulmonary NTM disease in our cohort.

1.3.6) IL4

IL4 was also investigated. IL4 produces IL-4, a Th2 differentiating factor for naïve T-cells and suppressor of Th1 responses (Brown and Hural, 1997, Martinez et al., 1990). The four SNP investigated within IL4 in our study (rs2243250, rs2070874, rs2243282 and rs2227284) have previously been associated with other conditions including rheumatoid arthritis severity, and increased odds of asthma in Caucasians (Balsa et al., 2009, Baye et al., 2011). However, none displayed significant associations with pulmonary NTM disease.
Chapter 2:
Hypothesis development and aims of study
2) Hypothesis development and Aims of Study

Pulmonary NTM disease appears to be contracted primarily from environmental sources, with human to human transmission being a relatively rare transmission route. Unlike tuberculosis, most pulmonary NTM infections have no routes of transmission identified. NTM are opportunistic pathogens, but many patients have no overt immunodeficiency, suggesting the possibility of a more subtle or specific predisposing factor. What factors determine risk of infection and disease?

2.1) Hypotheses

This thesis addresses two hypotheses:

1) The primary route of pathogenic NTM transmission is via contaminated water.

And,

2) Frequencies of polymorphic SNP alleles previously implicated in tuberculosis and other infectious or pulmonary diseases differ in patients of pulmonary NTM disease.

2.2) Specific Aims

1) To determine how a majority of pathogenic NTM are transmitted to patients, assuming all are equally susceptible to infection. A comprehensive review of confirmed cases of transmission of pathogenic NTM from known sources resulting in infection and disease was constructed to determine the primary route of transmission (Chapter 3).

And,

2) Assuming exposure is common across the population, to determine if there are immunogenetic differences between apparently immunocompetent patients with pulmonary NTM disease and healthy controls? Genetic association studies were performed on SNP within the candidate genes \textit{IL10}, \textit{P2X4R}, \textit{P2X7R}, \textit{CAMKK2}, \textit{IL4} and \textit{TLR2} in patients with pulmonary NTM disease and healthy controls (Chapters 4 and 5). Candidate genes were chosen based on the reviewed literature (Chapters 1, 4 and 5).
Chapter 3: Review of pathogenic NTM transmission from environmental sources

This section of work consists of an extensive published review of the literature regarding transmission of pathogenic NTM from environmental sources. This paper is included as both a research component and major section of the literature review for this thesis.

3) **Review: How can we be sure that environmental isolates of nontuberculous mycobacteria are associated with human infection?**

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**Abstract**

Pulmonary infections with nontuberculous mycobacteria (NTM) are recognised as a problem in immunodeficient individuals and are increasingly common in older people with no known immune defects. NTM are found in soil and water, but factors influencing transmission from the environment to humans are mostly unknown. Studies of the epidemiology of NTM disease have matched some clinical isolates of NTM with isolates from the patient’s local environment. Definitive matching requires strain level differentiation based on molecular analyses including partial sequencing, PCR-restriction fragment length polymorphism (RFLP) analysis, random amplified polymorphic DNA (RAPD) PCR, repetitive element (rep-) PCR and pulsed field gel electrophoresis (PFGE) of large restriction fragments. These approaches have identified hospital and residential showers and faucets, hot-tubs and garden soil as sources of transmissible pathogenic NTM. However gaps exist in the literature, with many clinical isolates remaining unidentified within environments tested and few studies investigating NTM transmission in developing countries. To understand the environmental reservoirs and transmission routes of pathogenic NTM, more studies, investigating different environments, countries and climates are required.
3.1) Factors affecting the acquisition of pulmonary NTM

Pulmonary infections with nontuberculous mycobacteria (NTM) are an increasing problem in many countries. Symptoms include chronic cough, haemoptysis, weight loss, fever and progressive fatigue - similar to *Mycobacterium tuberculosis* pulmonary infection. Diagnoses are made with chest radiography, detection of acid fast bacilli in sputum and cultures from sputum and bronchoalveolar lavage fluids (Griffith et al., 2007). The increasing prevalence may reflect an aging population, as symptomatic infections are most common in post-menopausal women and older men. NTM infections can also manifest as lymphadenitis, disseminated disease, or skin, tissue or bone disease. Disseminated NTM disease is associated with genetic defects in the Th1 pathway - lymphadenitis is often seen in patients with advanced HIV disease, whilst skin, tissue and bone infections usually follow trauma at the site of infection. Pulmonary NTM infections in older individuals with no recognized immune defects carry a high morbidity and economic cost, as current treatments have multiple side effects and an intention-to-treat cure rate of <50%. Even in apparent cures, the relapse rate is 50% at 3 years (Thomson and Yew, 2009). Thus it is important to determine the source and mode of transmission of pathogenic strains in the environment for advancement of prophylactic measures, as subtle immune deficiencies are rarely evident prior to the diagnosis of NTM disease.

Pathogenic NTM comprise many species and strains found in water and soil. For a better understanding of NTM disease risk and to develop prophylactic measures, clinical and environmental NTM isolates need to be matched. This will identify critical environmental reservoirs and routes of transmission. Here we review studies where clinical and environmental strains of mycobacteria were matched and discuss limitations to the methodology employed.

3.2) NTM are common in many environments

NTM have been isolated from drinking water pipelines (Thomson et al., 2013b, Torvinen et al., 2004), water tanks (Tuffley and Holbeche, 1980), hot tubs (Mangione et al., 2001), residential faucets (Slosarek et al., 1993, Thomson et al., 2013a), hospital faucets and ice machines (Cooksey et al., 2008, Galassi et al., 2003), diagnostic laboratories (Chang et al., 2002), bottled and municipal water, commercial and hospital ice (Covert et al., 1999), potting soil (De Groote et al., 2006), house dust (Dawson, 1971, Torvinen et al., 2010), water damaged building materials (Torvinen et al., 2006), showerheads (Falkinham III et al., 2008), shower aerosols (Thomson et al., 2013a), hot-tub aerosols (Mangione et al., 2001), coniferous forest soils (Iivanainen et al., 1997), brook waters (Iivanainen et al., 1993), cigarettes (Eaton et al., 1995), livestock (Leite et al., 2003), coastal mosses
(Schröder et al., 1992) and seawater (Gruft et al., 1979). Reports span multiple countries (including the USA, Australia, the UK, France, the Netherlands, Denmark, Czechoslovakia, Italy, Finland, Germany, Madagascar, Tanzania, Taiwan, Japan and Korea) and climates. While many isolates were not directly associated with human disease, these reports show that NTM reside in a variety of natural and artificial environments.

3.3) Environments of interest are defined

Inhalation of aerosols appears to be the primary transmission route of NTM causing pulmonary disease. This usually occurs in artificial water environments such as hot-tubs and showers, but may involve garden soil and house dust. Mycobacteria may aerosolise more readily than other bacteria as they have highly hydrophobic cell walls. NTM have been isolated from natural water environments in which aerosolisation increases NTM concentration in the air (Parker et al., 1983, Wendt et al., 1980). However these studies will not be discussed here as the aerosols were not linked to NTM disease.

When aiming to identify NTM, the depth of identification should be considered. Species level is sufficient to determine presence or absence in an environment, but strain level identification is required when matching clinical and environmental isolates. For species level NTM identification, cheap lower resolution techniques include phenotypic and biochemical typing, RT-PCR, species specific DNA probe kits, HPLC of mycolic acids and sequencing of select genes (namely 16S, rpoB and/or hsp65). Serotyping (Tuffley and Holbeche, 1980) and phage typing (Engel et al., 1980) have been utilised, but are now less popular as more species of mycobacteria have been recognised.

With low resolution techniques, environments of interest can be selected based on assessments of NTM presence. Demonstrations of transmission causing disease require higher resolution typing techniques such as RFLP, PFGE, or rep-PCR. Although commonly used for species identification, RT-PCR or gene sequencing provide strain level identification if optimised correctly (Table 2). These techniques are discussed further in section 4. When clinical and environmental isolates are matched at species level only, conclusions should be treated with caution. False positive matches may occur when different strains of the same NTM species are present in different environmental locations (Cooksey et al., 2008).

Excessive exposure to NTM-containing aerosols can also result in hypersensitivity pneumonitis (HP) in people with no known immune deficiencies. This was documented in machine workers
following repeated inhalation of aerosolised contaminated metal removal fluids (Shelton et al., 1999). However the most frequently reported cause of NTM HP is aerosolisation of contaminated hot-tub and swimming pool water, with multiple reports describing patients who developed NTM HP from these sources after frequent use (Embil et al., 1997, Glazer et al., 2007, Kahana et al., 1997, Khoor et al., 2001, Marchetti et al., 2004, Rickman et al., 2002, Schwartzstein and Mark, 2000). Whilst patients with NTM HP can present acutely and be significantly unwell, most cases resolve after exposure to the contaminated aerosols is discontinued.

3.3.1) Water

The most credible routes for pulmonary NTM infection involve inhalation of aerosols generated by hot-tubs and shower-heads. Indeed the global increase in NTM infections may reflect the use of showers rather than bathing (O'Brien et al., 2000). In a study of particular interest, Feazel used quantitative molecular techniques to show that the showerhead environment enriches biofilm-forming organisms, including mycobacteria (Feazel et al., 2009). NTM organisms are common in treated water. Their innate resistance to chlorine and other disinfectants provides a growth advantage. The disinfectant resistance of NTM was demonstrated in Australia in a study of *M. avium* in hot-tubs (Lumb et al., 2004). Although the environmental bacterial load was kept at a low 1 CFU/ml by chlorination, the same samples yielded NTM numbering between $4.5 \times 10^3$ and $4.3 \times 10^4$ CFU/ml (Lumb et al., 2004).

Although not affecting the lungs, NTM furunculosis has also been linked to salon foot-baths used by two patients in the USA (Gira et al., 2004). The infections are attributed to the use of contaminated foot baths after shaving of the legs had breached the skin barrier.

NTM are also present in hospital environments. Earlier studies linking environmental and clinical NTM isolates found several NTM species were ubiquitous in hospital and residential taps and showers in the UK, the Netherlands and the Czech Republic (Engel et al., 1980, McSwiggan and Collins, 1974, Slosarek et al., 1993). Diagnosis of NTM disease can be confounded by false positive isolates due to contamination of diagnostic tests, as demonstrated in the USA (Goslee and Wolinsky, 1976) and Spain (Alvarez et al., 2008). NTM contaminating hospital materials can cause nosocomial outbreaks and pseudo-outbreaks (Panwalker and Fuhse, 1986, Wallace Jr et al., 1998). Prevention of nosocomial infections requires that medical instruments and patient wounds are not exposed to tap water (Griffith et al., 2007).
3.3.2) Soil

Studies of NTM in soil are less numerous than those of water, perhaps because soils are considered a less likely/common source of nosocomial infection and contain more overgrowing contaminants, complicating NTM isolation from culture. However NTM have been isolated from soil in multiple countries and climates, including Finland (Iivanainen et al., 1997), Japan (Ichiyama et al., 1988), the USA (Jones and Jenkins, 1965) and Uganda (Kankya et al., 2011). Some reports characterise isolates to the species level, but only one utilised molecular techniques to match clinical isolates with NTM isolated from garden soil (De Groote et al., 2006). Although the study matched 14 clinical isolates with isolates from the patient’s own gardens, only three were true matches at the strain level. The authors isolated the organisms by generating aerosols with the soil samples, establishing that the isolates had potential to be aerosolised to a respirable particle size.

3.3.3) House Dust

Many NTM species have been isolated from house dust (Dawson, 1971). Although these studies used only species specific analyses for detection and characterisation, they establish house dust as a source of NTM (Torvinen et al., 2010). As dust particles are commonly suspended in the air, inhalation of mycobacteria associated with dust particles is a plausible source of pulmonary infections. An early study from Australia used serotyping (one of the first strain typing techniques described) to match house dust and patient isolates (Reznikov and Dawson, 1971). Approximately 50% of these house dust strains were *M. intracellulare* matching the patient isolates. Further studies utilising more powerful techniques are needed to confirm these findings.
Table 2: Summary of popular molecular techniques for NTM strain characterisation.

<table>
<thead>
<tr>
<th>Name</th>
<th>Method of characterisation</th>
<th>Papers describing the method in use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partial Sequencing</td>
<td>Sequencing of particular genes and gene fragments (ie: 16S rDNA). Can directly compare SNPs of genomic DNA.</td>
<td>(Harmsen et al., 2003), (Turenne et al., 2006)</td>
</tr>
<tr>
<td>Repetitive sequence PCR (rep-PCR)</td>
<td>PCR of genomic DNA with primers specific to multiple repetitive sequences. Separate amplicons via gel electrophoresis. Produces unique fingerprint patterns from multiple bands of varying intensity.</td>
<td>(Thomson et al., 2014)</td>
</tr>
<tr>
<td>Random amplification of polymorphic DNA (RAPD)</td>
<td>PCR amplification, primers of random sequence utilised. Polyacrylamide gel electrophoresis performed on amplicons. Unique fingerprint generated.</td>
<td>(Zhang et al., 1997)</td>
</tr>
<tr>
<td>Restriction fragment length polymorphism (RFLP)</td>
<td>Perform PCR with primer of interest. Digest amplicons with restriction enzymes and determination of fragment length via gel electrophoresis. Discriminates DNA strands based on the locations of restriction enzyme sites (as band patterns).</td>
<td>(Lee et al., 2000)</td>
</tr>
<tr>
<td>Pulsed field gel electrophoresis (PFGE)</td>
<td>Amplify Genomic DNA by culture, digest into large restriction fragments and separate large restriction fragments via pulsed-field gel electrophoreses (ie: Electrophoresis where voltage periodically changes between three directions). Capable of accurate discrimination of sequences in long strands.</td>
<td>(Tenover et al., 1995, Zelazny et al., 2009)</td>
</tr>
<tr>
<td>Amplified fragment length polymorphism (AFLP)</td>
<td>Genomic DNA digested with restriction enzymes, adaptors ligated to restriction fragments, restriction fragments undergo PCR amplification, amplified fragments separated and visualised via polyacrylamide gel electrophoresis. Polymorphisms scored as present or absent from genome.</td>
<td>(Pfaller et al., 2007)</td>
</tr>
<tr>
<td>Multiplex PCR</td>
<td>Multiple primer sets used in a single PCR reaction, targeting multiple genes. Amplicon sizes determined by gel electrophoresis.</td>
<td>(Kim et al., 2011)</td>
</tr>
<tr>
<td>High performance liquid chromatography (HPLC)</td>
<td>Used to separate, identify, and quantify a mixture of components in liquid form. For NTM characterisation it is often used to fingerprint mycolic acid patterns.</td>
<td>(Galassi et al., 2003)</td>
</tr>
<tr>
<td>Identification kits</td>
<td>Pre-packaged kits designed to identify certain NTM species. Directions and techniques vary between kits and manufacturers.</td>
<td>(Thomson et al., 2014)</td>
</tr>
</tbody>
</table>

3.4) Molecular methods allow NTM from patients and their environment to be matched

Within environments identified by low resolution methods, environmental NTM have been compared using molecular techniques with isolates from patients. These techniques (Table 2) have been used as standalone procedures, and as part of a multistep fingerprinting process. Whilst strain differentiation within some individual species of NTM has been demonstrated using a single technique, this is not the case for all. Strain level differentiation across multiple NTM species requires a combination of differentiation techniques.

Molecular techniques used for characterisation of NTM at the strain level include high performance
liquid chromatography (HPLC), repetitive PCR (rep-PCR), random amplified polymorphic DNA PCR (RAPD-PCR), pulsed-field gel electrophoresis (PFGE) of large restriction fragments, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), partial gene sequencing, and multiplex PCR (ie: using hsp65, 16S rDNA, ropB etc.). Table 3 outlines studies in which these molecular techniques have been used to identify sources of clinical NTM isolates in the environment.

Many PCR-based analyses (eg: 16s rDNA sequencing) do not distinguish mycobacterial taxa at the species level as the genes are conserved across the genus (Cloud et al., 2002). For example; M. kansasii/M. gastri and M. abscessus/M. chelonae share identical 16s rDNA sequences. Similar problems also affect NTM differentiation at the strain level. This can be achieved with the use of multiple analyses in tandem (Picardeau et al., 1997). In one study, comprehensive analyses of the discriminatory power of 16s rDNA, hsp65, and rpoB gene sequencing, hsp65 RFLP, rep-PCR, RAPD PCR, and PFGE of large restriction fragments were performed on isolates of M. mucogenicium and M. phocaicum. PFGE analysis displayed the greatest differentiation power showing each environmental isolate as genetically unique (Cooksey et al., 2008). However PFGE cannot differentiate all NTM strains isolated (MacCannell, 2013) and should be used in combination with other techniques to achieve greater discrimination. It is also labour and time intensive.

Although not yet utilised in environmental and clinical isolate matching of NTM, it is becoming increasingly feasible to perform whole genome sequencing. Whilst offering definitive genomic differentiation of isolates, whole genome sequencing requires new stratagems in data management (MacCannell, 2013). When more sequences become available, targeted sequencing for accurate species identification will reduce costs and increase accessibility. Whole or targeted genome sequencing will likely replace PFGE as the gold standard for bacterial strain typing.
### Table 3: Summary of studies utilising molecular methods to differentiate and match NTM isolates from clinical and environmental isolates.

<table>
<thead>
<tr>
<th>Country</th>
<th>Clinical samples</th>
<th>Environment investigated</th>
<th>Environmental samples containing NTM: Number of environmental samples taken</th>
<th>Matched clinical and environmental NTM isolates: total environmental samples</th>
<th>Species matched</th>
<th>Species characterisation method</th>
<th>Strain characterisation method</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK</td>
<td>Patients (n=33) Isolates (n=36)</td>
<td>Hospital water faucets (n=97), tank (n=5)</td>
<td>57:97 (faucet) 3:5 (tank)</td>
<td>36:102</td>
<td><em>M. xenopi</em> (n=36)</td>
<td>Culture Unspecified methods</td>
<td>NA</td>
<td>(McSwiggan and Collins, 1974)</td>
</tr>
<tr>
<td>USA</td>
<td>AIDS patient stool, sputum and blood (n=36)</td>
<td>Environmental and municipal water sources</td>
<td>10:NA</td>
<td>5:NA</td>
<td><em>M. avium</em> (n=5)</td>
<td>Culture DNA probe kits (SNAP, Gen-Probe)</td>
<td>PFGE - AseI</td>
<td>(Von Reyn et al., 1994)</td>
</tr>
<tr>
<td>USA</td>
<td>Patient blood or bone marrow (n=40)</td>
<td>Hospital water (n=10) House water (n=58) Reservoir water (n=13)</td>
<td>10:10 (Hospitals) 54:58 (Houses) 12:13 (Reservoirs)</td>
<td>3:10 (Hospitals) 2:58 (Houses) 0:13 (Reservoirs)</td>
<td><em>M. avium</em> (n=5)</td>
<td>Culture Serotyping Species specific DNA probes (Syngene) Multilocus Enzyme Electrophoresis</td>
<td>NA</td>
<td>(Glover et al., 1994)</td>
</tr>
<tr>
<td>Canada</td>
<td>Patient sputum (n=1)</td>
<td>Hot tub water (n=1)</td>
<td>1:1</td>
<td>1:1</td>
<td><em>M. avium</em> (n=1)</td>
<td>Culture Multilocus Enzyme Electrophoresis</td>
<td>RFLP (IS1245)</td>
<td>(Kahana et al., 1997)</td>
</tr>
<tr>
<td>USA</td>
<td>Patients (n=17) Isolates (n=19)</td>
<td>Water reservoirs (n=13) Residential water (n=55) Commercial building water (n=31) Hospital water (n=15)</td>
<td>12:13 Water reservoirs 45:55 Residential water 31:31 Commercial building water 15:15 Hospital water</td>
<td>3:144</td>
<td><em>M. avium</em> (n=3)</td>
<td>Culture Biochemical analyses DNA probe kits (SNAP, AccuProbe) HPLC</td>
<td>PFGE - AseI</td>
<td>(Aronson et al., 1999)</td>
</tr>
<tr>
<td>USA</td>
<td>AIDS and non-AIDS patients (n=103 isolates)</td>
<td>Various vegetables (n=121)</td>
<td>25:121</td>
<td>1:121</td>
<td><em>M. avium</em> (n=1)</td>
<td>Culture DNA probe kits (Accuprobe, SNAP) RFLP</td>
<td>RT-PCR (IS 1245 and IS1311) (Avium only)</td>
<td>(Yoder et al., 1999)</td>
</tr>
<tr>
<td>USA</td>
<td>Patient biopsy tissue (n=2)</td>
<td>Nail salon whirlpool bath swabs (n=7)</td>
<td>3:7</td>
<td>2:7</td>
<td><em>M. mageritense</em> (n=2)</td>
<td>Culture Biochemical analyses PCR-RFLP (hsp65) HPLC (mycolic acid esters)</td>
<td>rep-PCR PFGE - Xbal</td>
<td>(Gira et al., 2004)</td>
</tr>
<tr>
<td>Country</td>
<td>Sample Type</td>
<td>Sources</td>
<td>Isolates</td>
<td>M. avium (n=85)</td>
<td>M. intracellulare (n=2)</td>
<td>M. mucogenicium (n=2)</td>
<td>PFGE - AsnI, XbaI</td>
<td>Serotyping RFLP (IS 1245 and IS 1311)</td>
</tr>
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</tr>
<tr>
<td>USA</td>
<td>Patient sputum</td>
<td>Hospital hot water system isolates (n=13)</td>
<td>13:13</td>
<td>85:13</td>
<td></td>
<td></td>
<td></td>
<td>Culture-PCR-RFLP (hsp65) Gen-probe DNA Probes</td>
</tr>
<tr>
<td></td>
<td>(n=131)</td>
<td></td>
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<tr>
<td></td>
<td>(n=161 isolates)</td>
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</tr>
<tr>
<td>Australia</td>
<td>Patient sputum</td>
<td>Spa water (n=2)</td>
<td>2:2</td>
<td>3:2</td>
<td></td>
<td></td>
<td></td>
<td>Culture-Phenotypic characteristics</td>
</tr>
<tr>
<td></td>
<td>(n=4)</td>
<td></td>
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<tr>
<td>Korea</td>
<td>Patient blood culture (n=12 isolates)</td>
<td>Hospital tap water n=100</td>
<td>50:100</td>
<td>2:100</td>
<td>M. mucogenicium (n=2)</td>
<td></td>
<td></td>
<td>PFGE - Asel rep-PCR (between IS1245 and IS1311)</td>
</tr>
<tr>
<td>USA</td>
<td>Patient sputum</td>
<td>Commercial potting soil aerosols (n=2) Patient garden soil aerosols (n=79)</td>
<td>21:21</td>
<td>3:79</td>
<td>M. intracellulare (n=2)</td>
<td>M. avium (n=1)</td>
<td></td>
<td>Culture-RFLP (ropB) - MspI, HaelII</td>
</tr>
<tr>
<td></td>
<td>(n=26)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Japan</td>
<td>Patient sputum</td>
<td>Patient residential bathrooms (showerhead water (n=46), kitchen tap water (n=48), bathtub tap (n=48), showerhead scale (n=37), drain slime (n=49) and air conditioners (n=45))</td>
<td>10:371 (all in bathrooms)</td>
<td>2:49</td>
<td>M. avium (n=2)</td>
<td></td>
<td></td>
<td>Partial sequencing (16s-23s RNA ITS region)</td>
</tr>
<tr>
<td></td>
<td>(n=30)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Serotyping RFLP (IS 1245 and IS 1311)</td>
</tr>
<tr>
<td>Country</td>
<td>Clinical samples</td>
<td>Environment investigated</td>
<td>Environmental samples containing NTM : Number of environmental samples taken</td>
<td>Matched clinical and environmental NTM isolates : total environmental samples</td>
<td>Species matched</td>
<td>Species characterisation method</td>
<td>Strain characterisation method</td>
<td>Source</td>
</tr>
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<tr>
<td>USA</td>
<td>Patient sputum (n=1)</td>
<td>Showerhead sediment (n=6), hot (n=6) and cold (n=6) bathroom tap water</td>
<td>6:6 (Hot water) 6:6 (Cold water) 6:6 (Sediment)</td>
<td>1:18</td>
<td><em>M. avium</em> (n=1)</td>
<td>Culture Partial sequencing (16s rDNA)</td>
<td>RFLP (<em>hsp65</em> and IS 1245/IS 1311) - BstEII, HaeIll</td>
<td>(Falkinham III et al., 2008)</td>
</tr>
<tr>
<td>USA</td>
<td>Patient blood (n=5)</td>
<td>Texas hospital sinks, showers (n=13) Municipal water tank swabs (n=2) Municipal water plant tanks (n=2) Hospital swabs (n=10) Ice machine ice (n=1)</td>
<td>12:27</td>
<td>1:27</td>
<td><em>M. phocaicum</em> (n=1)</td>
<td>Culture Partial sequencing (<em>hsp65</em>, 16S rRNA, and <em>rpoB</em>)</td>
<td>rep-PCR (Box A1R Primer) RAPD - PCR (RAPD1 primer) PFGE - Asel</td>
<td>(Cooksey et al., 2008)</td>
</tr>
<tr>
<td>USA</td>
<td>Patient isolates (n=74)</td>
<td>Residential water supplying patients homes (n=23)</td>
<td>23:23</td>
<td>1:23</td>
<td><em>M. avium</em> (n=1)</td>
<td>Culture Multilocus Enzyme Electrophoresis</td>
<td>PFGE - Xbal</td>
<td>(Hilborn et al., 2008)</td>
</tr>
<tr>
<td>Spain</td>
<td>Patients (n=59) Patient urine(n=23 isolates) Patient sputum (n=19 isolates)</td>
<td>Hospital tap water Urine collection bottles</td>
<td>6:NA (Hospital tap water) 11:NA (Urine bottles)</td>
<td>21:NA (Patient urine) 5:NA (Patient sputum)</td>
<td><em>M. avium</em> (n=26)</td>
<td>Culture Phenotypic characteristics DNA Probe kit (AccuProbe MAC culture identification kit)</td>
<td>rep-PCR (IS1245 and IS901) Partial sequencing (<em>hsp65</em>, 16S rDNA) RFLP (IS 1311) - HinfI, Msel PFGE - Xbal</td>
<td>(Alvarez et al., 2008)</td>
</tr>
<tr>
<td>USA</td>
<td>Patients (n=42)</td>
<td>Patient residential water systems (n=37) Total samples taken (n=394)</td>
<td>22:37 (houses) 109:394 (individual samples)</td>
<td>7:37</td>
<td><em>M. avium</em> (n=7)</td>
<td>Culture Partial sequencing (16s rDNA)</td>
<td>Rep-PCR</td>
<td>(Falkinham III, 2011)</td>
</tr>
<tr>
<td>USA</td>
<td>Patients (n=24) Patient samples (n=60)</td>
<td>Hospital water and ice machines (n=139) Household water (n=5 households)</td>
<td>112:139 (Hospital water and ice) 4:5 (Residential water)</td>
<td>5:139 (Hospital water and ice) 3:5 (Residential water)</td>
<td><em>M. porcinum</em> (n=8)</td>
<td>Culture HPLC of mycolic acids RFLP (<em>hsp65</em>)</td>
<td>PFGE - Xbal, Asel Partial sequencing (<em>hsp65</em>, <em>rpoB</em>, <em>recA</em> and 16s rDNA)</td>
<td>(Brown-Eliott et al., 2011)</td>
</tr>
<tr>
<td>Australia</td>
<td>Patients</td>
<td>Patient taps, swimming</td>
<td>19:20</td>
<td>7:20 (Species)</td>
<td><em>M. abscessus</em></td>
<td>Culture</td>
<td>rep-PCR</td>
<td>(Thomson et al., 2008)</td>
</tr>
<tr>
<td>Country</td>
<td>Study Details</td>
<td>Isolates</td>
<td>Isolates</td>
<td>Methods</td>
<td>References</td>
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<tr>
<td>Australia</td>
<td>Patients (n=68) - Isolates (n=72)</td>
<td>NTM patient residential water swabs and aerosols (n=16 isolates) Water from main, reservoir and distribution points (n=220)</td>
<td>n=16 isolates (NTM Patient residential water swabs and aerosols) 49:220 (Water from main, reservoir and distribution points)</td>
<td>6:65 (environmental isolates)</td>
<td>M. kansasii (n=6)</td>
<td>Culture Phenotypic characteristics Partial sequencing (hsp65, 16s rDNA) Multiplex PCR DNA probe kit (GenoType® Mycobacterium AS (additional species) kit) RFLP (16s-23s ITS rDNA) rep-PCR</td>
<td>(Thomson et al., 2014)</td>
<td></td>
</tr>
<tr>
<td>Australia</td>
<td>Patient samples (n=74)</td>
<td>Municipal water Tap water Tank water Swimming pool water</td>
<td>15:NA</td>
<td>6:15 (water isolates)</td>
<td>M. abscessus (n=6)</td>
<td>Culture Phenotypic characteristics Partial sequencing (16s rDNA, hsp65, rpoB) Multiplex PCR DNA probe kit (GenoType® Mycobacterium AS (additional species) kit) rep-PCR</td>
<td>(Thomson et al., 2013b)</td>
<td></td>
</tr>
</tbody>
</table>
3.5) **Factors determining clinically important sources of NTM**

3.5.1) **Geographic location**

Relevant studies have been conducted in different countries, with a majority from the USA and Australia (Table 3) and few from the developing world. The countries best represented in the literature have lower prevalence of *M. tuberculosis* infection, better diagnosis of NTM diseases and more funding available for research. The imbalance makes it difficult to address the role of climate and geographical location in the acquisition of NTM from water and soil. NTM does, however, appear to be widespread in most regions, as does NTM disease (Griffith et al., 2007, Hoefsloot et al., 2013). It remains unclear why the species causing disease vary between countries, but there is potential for climate to influence NTM environmental exposure through the prevalence of NTM in the environment (Reed et al., 2006).

3.5.2) **Patient exposure**

Many common activities expose individuals to pathogenic NTM. The most studied are hot-tub use (Kahana et al., 1997, Lumb et al., 2004, Mangione et al., 2001), showering (Cooksey et al., 2008, Falkinham III et al., 2008, Thomson et al., 2013a), faucet use (McSwiggan and Collins, 1974, Nishiuchi et al., 2007, Shin et al., 2007) and gardening (De Groote et al., 2006). These sources have been investigated by studies matching NTM strains between patient and the environment. NTM transmission has also been linked with exposure from commercial farming (Fujita et al., 2013), aerosolised metalworking fluids (Shelton et al., 1999) and tending of aquariums (Collier, 2002), but these routes have yet to be confirmed by matching clinical and environmental strains.

Sources of residential exposure to NTM have been investigated via questionnaire-based survey (Fujita et al., 2014, Maekawa et al., 2011, Reed et al., 2006). Exposure to soil was identified as a risk factor in all three studies and one showed an increase in mixed and polyclonal NTM infections in patients regularly exposed to soil, demonstrating the influence of environmental exposure on infection incidence within a cohort of NTM disease patients (Fujita et al., 2014). NTM disease did not correlate with exposure to food or pets (Maekawa et al., 2011, Reed et al., 2006).

Although useful data are provided, the use of surveys is flawed in that: 1) One exposure event may be enough for NTM colonisation, making accounts of activity frequency less relevant; 2) isolates were not matched to ascertain that the source implicated was responsible for NTM disease; 3) for a
study of the general population, a cohort of 800 is insufficient in an area with a NTM infection rate of 1.2/100,000 (Reed et al., 2006). Some studies have used sensitin skin testing as an indicator of NTM infection (Reed et al., 2006). Sensitin tests reflect both past and current infections, with potential for false positives, and are poor markers of clinical disease.

The low ratio of successful matches between environmental and clinical isolates is striking. While some studies matched most clinical isolates with their reservoir (Falkinham III et al., 2008, Lumb et al., 2004), this is likely due to low numbers of participating patients. Others achieved accurate matches with fewer than 20% of patient isolates, so any conclusions regarding relative environmental risk factors are broad extrapolations (Cooksey et al., 2008, De Groote et al., 2006, Nishiuchi et al., 2007, Shin et al., 2007). The reservoirs for most clinically infective NTM remain unknown, but many bacteria in the patient’s immediate environment are never identified and characterised. The missing pathogens may have originated from a known environment of interest (e.g.: showerhead) not sampled for the study, or may have been transmitted via a novel route. The significant lag time between acquisition of infection and diagnosis of disease (1-10 years) makes it difficult to pin-point potential sources, as the environment may have changed by the time of diagnosis.

Alternatively, human transmission may be responsible for more infections than previously suspected. Although this has rarely been observed, recent studies show links between isolates from multiple patients (Aitken et al., 2012, Bryant et al., 2013, Ricketts et al., 2014). These results do not prove human to human transmission, as patients may have acquired the pathogen from the same environmental reservoir, however, this route of NTM infection should not be dismissed without further study.

3.5.3) Species and strain infective potential vary

Species of NTM differ in habitat and in their potential for human infection. A summary compiled by the American Thoracic Society shows the relative risk of infection by different NTM species in the USA (Griffith et al., 2007). *M. kansasii* remains one of the most infective species whilst others (ie: *M. frederikbergense, M. flavescens, M. moriokaense*) appear to be non-pathogenic to humans (Griffith et al., 2007).

There is also evidence to suggest pathogenicity may vary between strains of the same species. In a study of clinical isolates of *M. kansas* (n=191), isolates from different subtypes differed in pathogenicity based on the percentage of patients infected (Taillard et al., 2003). Subtype 1
accounted for a majority of isolates and was considered pathogenic in 81% of cases, while subtype 3 was only pathogenic in 6% of cases and was generally considered a non-pathogen (Goy et al., 2007).

Antibiotics play a central role in the treatment of NTM disease, with individual NTM species and patients requiring different antibiotic cocktails. Difficulties prescribing the correct treatment arise from the limited ability of in vitro susceptibility tests to predict antimicrobial responses for all NTM species. For example, *M. kansasii* is susceptible to multiple antibiotics and *M. avium* is susceptible to the macrolide antimicrobials in vivo, but *M. abscessus subs abscessus* has inducible resistance to macrolides (Griffith et al., 2007). In rare cases, side effects of effective antibiotic treatment outweigh the consequences of the disease.

Strain typing of NTM is not routine clinical care, but could reveal patterns of pathogenicity and antibiotic resistance present in the population. For example, *M. abscessus* strains with a functional erythromycin ribosome methyltransferase (*erm*) gene are resistant to macrolide antibiotics. Strains lacking a functional *erm* gene are susceptible to macrolides in vivo and in vitro (Nash et al., 2009).

3.6) Understanding NTM transmission will facilitate targeted prophylactic measures

NTM are ubiquitous in the environment and vary in infectivity, pathogenicity and susceptibility to antibiotics. Although most studies using high resolution molecular techniques are from the USA and Australia, NTM have been isolated from similar sources in other countries and climates. The risk of a particular environmental source transmitting pathogenic NTM is determined by three variables. 1) The capacity for pathogenic NTM growth in the environment. 2) The frequency of human exposure from the source in question. 3) The method of patient exposure to pathogenic NTM from the source. Environments of interest are those with high NTM burdens in a form that is easily aerosolised, namely showers, hot tubs, garden soil and house dust.

From studies in which environmental NTM have been matched by strain to pathogenic isolates, key sources appear to be hot-tubs, faucets and showerheads, with similar frequencies of isolation from hospitals and private residences. Most private residences sampled, however, belonged to patients already infected with NTM. When private residences of uninfected individuals were sampled, NTM were recovered at a much lower frequency suggesting a relatively low risk of infection from household water (Nishiuchi et al., 2007).
Whilst many studies confirm that NTM is transmitted from water, this may simply reflect the large number of water-based studies. *M. avium* has a long-standing link to water environments, as does *M. abscessus* (Thomson et al., 2013a). Although *M. kansasii*, can be found in a tap water, few strains from tap water matched disease isolates. *M. intracellulare* infections were also associated with water, but these waterborne *M. intracellulare* isolates have since been reidentified as a novel species, *M. chimaera* (Tortoli et al., 2004). Although *M. intracellulare* infection is still commonly reported (Carter et al., 2014), since this development there remains no evidence linking water to *M. intracellulare* infection. Alternative transmission pathways for these mycobacteria may include soil exposure, which correlates with NTM pulmonary disease in multiple studies (Fujita et al., 2014, Ichiyama et al., 1988, Maekawa et al., 2011, Reed et al., 2006) although it has only been confirmed as a transmission route via strain-matching in one (De Groote et al., 2006).

Presently, prophylaxis of pulmonary NTM disease involves avoidance of exposure to possible NTM containing aerosols. Steps that can be made to reduce levels of NTM from water include maintaining hot water system temperatures at above 60°C and correct pH and chlorination in pools and spas. Nosocomial infections can be prevented by cleaning medical instruments using only sterile water and disinfectant before use, and use of personal protective equipment can aid in prevention of aerosol inhalation in areas of high exposure, e.g.: when gardening. These precautions can be recommended to individuals perceived to be at the greatest risk of infection. A more extensive understanding of NTM transmission will allow more targeted prophylactic measures.
Chapter 4: Do polymorphisms in IL10 influence pulmonary NTM disease susceptibility?

This section of work investigates SNP of interest within IL10 in patients of pulmonary disease and healthy controls. This association study follows on from previous work done by our lab (Affandi et al., 2013) and identifies two SNP alleles in LD associating significantly with disease.

4) Susceptibility to non-tuberculous mycobacterial disease is influenced by rs1518111 in *IL10*.

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⁵ Institute for Respiratory Health, University of Western Australia, Perth, W.A., Australia
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Abstract

Although exposure to potentially pathogenic non-tuberculous mycobacteria (NTM) via soil and domestic water supplies is common, pulmonary infection and disease are confined to a small proportion of older individuals. We have associated alleles of a polymorphism in *IL10* (rs1800896) with NTM disease and shown that blood leukocytes from patients with NTM produce high levels of IL-10. Here we investigate seven additional polymorphisms in *IL10* in a larger cohort of Caucasian controls and patients with pulmonary NTM disease. We show a significant association between pulmonary NTM disease and one polymorphism (rs1518111) in strong linkage disequilibrium with rs1800896.

4.1) Introduction
Pulmonary disease is the most common presentation of infections with non-tuberculous mycobacteria (NTM) in otherwise healthy adults, and is considered an emerging health risk in Australia and around the world (Thomson, 2010, Winthrop et al., 2010). Exposure through soil or water is common, but few people develop pulmonary NTM disease. Estimates from Queensland, Australia, suggest a frequency of 22.1 persons per 100 000 in the population (Thomson, 2010). Known risk factors for disease include smoking, excessive alcohol intake, past tuberculosis and underlying lung diseases such as COPD and bronchiectasis (Cassidy et al., 2009, Winthrop et al., 2010). However these factors do not account for all cases – a notable exception being patients with the "Lady Windermere" phenotype. These individuals are lean and tall, with higher than normal incidence of thoracic abnormalities including scoliosis, pectus excavatum and mitral valve prolapse. The majority of pulmonary NTM cases are Caucasian (91%) and female (95%), with an average age of 60 years (Kim et al., 2008). Behavioural questionnaires found no links between pulmonary NTM disease and patients’ activities, exposures or habits (Kim et al., 2008). Genetic factors should be investigated further.

Effective Th1 and Th17 cell-mediated immune responses are crucial for the clearance of NTM infection and can be inhibited by Th2 cytokines such as IL-10. Hence polymorphic alleles affecting either the expression or activity of IL10 may impact upon the Th1/Th2/Th17 balance/response in patients of pulmonary NTM disease. Our studies of cytokine production by blood leukocytes from patients with pulmonary NTM disease demonstrated comparatively high IL-10 responses (Lim et al., 2010). Interferon-γ/IL-10 ratios are also associated with disease severity in tuberculosis, further suggesting a role for IL-10 (Jamil et al., 2007).

Previously, we and others have linked differences in allele frequencies for single nucleotide polymorphisms (SNP) within the IL10 promoter with pulmonary NTM disease (rs1800896), asthma and tuberculosis (rs1800896, rs1800871, and rs1800872) (Affandi et al., 2013, Gao et al., 2015, Hsia et al., 2015, Liu et al., 2015). To further understand the influence of genetics on disease, we typed seven additional SNP within IL10 in an expanded cohort of patients with pulmonary NTM and healthy controls.
4.2) Materials and Methods

4.2.1) Patients and controls

DNA was obtained from 124 Caucasian patients with pulmonary NTM disease attending Greenslopes Private Hospital, QLD, Australia and Prince Charles Hospital, QLD, Australia between 2005 and 2014. NTM infection was diagnosed according to American Thoracic Society criteria (Griffith et al., 2007) using radiological and microbiological findings. All participants provided written informed consent and the project was approved by the Greenslopes Research and Ethics Committee (Protocol 12/12) in accordance with the National Statement on Ethical Conduct in Human Research. 229 control donors were recruited from Western Australia (Temple et al., 2003) and DNA samples were provided by the Institute for Respiratory Health, Western Australia, in accordance with a Royal Perth Hospital Human Research Ethics Approval. The cohort are considered to be representative of the Caucasian Australian population from which patients were also derived.

4.2.2) Genotyping

DNA samples were quantified by fluorometry with a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and adjusted to 50ng/uL. Genotyping was performed using custom TaqMan OpenArray Genotyping Plates (Life Technologies, Grand Island, NY, USA) (Goullee et al., 2016). DNA samples were diluted at 1:1 in TaqMan OpenArray Genotyping Master Mix for 50 cycles of PCR amplification. The output was viewed using OpenArray™ SNP Genotyping Analysis software, and genotypes were allocated manually. The SNP rs3024497 was excluded as it did not meet Hardy-Weinberg Equilibrium (HWE). The genotyping success rate for the remaining SNP averaged 94.4%.

4.2.3) Data analysis

Linkage between paired SNP alleles was determined with the use of Ensembl’s “Pairwise linkage disequilibrium data by population” tool with the 1000 genomes database. In this way, linkage is determined via both R^2 and D` values for each ethnicity with available data. Statistical analyses were performed in Stata 12 (StataCorp, Collage Station, TX, USA). Univariate analyses evaluating associations between pulmonary NTM lung disease and gender, age, SNP and haplotypes were
performed using two-tailed Fisher’s exact or Chi² tests (χ²) as appropriate. Multivariate analyses were performed using logistic regression modelling using all included factors associated with NTM in univariate analyses (p < 0.20 cut-off) followed by a stepwise removal procedure to obtain the model of best fit.

4.3) Results

4.3.1) The demographic profile of patients with pulmonary NTM disease

We studied 124 patients with pulmonary NTM disease and 229 healthy controls of Caucasian ethnicity. Seventy-three percent of patients and 55% of healthy controls were female. As this was a statistically significant difference (χ², p = 0.001), gender was included in logistic regression models with SNPs of interest.

NTM patients were older than healthy controls [median (range) 67 (25-89) vs. 44 (21-75) years, respectively]. Whilst some control donors may develop pulmonary NTM lung disease later in life, the impact on the data is likely to be minor as pulmonary NTM disease is rare. Patients were infected with *M. intracellulare* (n = 64), *M. avium* (n = 13), *M. abscessus* (n = 10), *M. kansasii* (n = 3), *M. triplex* (n = 2), *M. xenopi* (n = 1), *M. simiae* (n = 1), *M. interjectum* (n = 1), *M. shimoidei* (n = 1), *M. terrae* (n = 1), *M. gordonae* (n = 1), or *M. lentiflavum* (n = 1). Some individuals were infected with more than one mycobacterial species (n = 12).

4.3.2) Associations between SNP genotypes and pulmonary NTM disease

Of the seven SNP analysed, rs1518111 was the only marker showing a statistically significant association with pulmonary NTM disease (P =0.004) in univariate χ² analysis. This withstood Bonferroni correction for multiple comparisons (Table 4).
**Table 4**: *IL10* SNP associated with NTM disease in univariate analyses

<table>
<thead>
<tr>
<th>SNP</th>
<th>Chr. 1 Position</th>
<th>MA</th>
<th>MAF&lt;sup&gt;b&lt;/sup&gt; Control</th>
<th>MAF&lt;sup&gt;b&lt;/sup&gt; NTM</th>
<th>P-value&lt;sup&gt;c&lt;/sup&gt; (χ²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3024498</td>
<td>206941529</td>
<td>G</td>
<td>43.7%</td>
<td>54.9%</td>
<td>0.054</td>
</tr>
<tr>
<td>rs1518111</td>
<td>206944645</td>
<td>A</td>
<td>43.7%</td>
<td>27.6%</td>
<td><strong>0.004</strong></td>
</tr>
<tr>
<td>rs3021094</td>
<td>206944952</td>
<td>C</td>
<td>15.7%</td>
<td>18.5%</td>
<td>0.520</td>
</tr>
<tr>
<td>rs3024491</td>
<td>206945046</td>
<td>G</td>
<td>27.0%</td>
<td>22.1%</td>
<td>0.320</td>
</tr>
<tr>
<td>rs1800872</td>
<td>206946407</td>
<td>A</td>
<td>46.4%</td>
<td>35.8%</td>
<td>0.058</td>
</tr>
<tr>
<td>rs1800871</td>
<td>206946634</td>
<td>T</td>
<td>46.6%</td>
<td>38.5%</td>
<td>0.150</td>
</tr>
</tbody>
</table>

<sup>a</sup>SNP are shown in chromosomal order.

<sup>b</sup>Minor allele frequencies are based on individuals successfully genotyped for each SNP.

rs03024498 and rs1800872 displayed weaker associations with disease (χ², P < 0.06). These three SNP were included in logistic regression models with gender. After a stepwise removal process, the final model (model p < 0.001; R² = 0.05) included only gender and rs1518111 (Table 4.5). When gender was not included, the model was weakened but remained comparable, demonstrating the associations between the minor alleles of rs1518111 and rs3024498 with reduced and increased risk of disease (Table 5).

**Table 5**: Logistic regression models define gender and rs1518111 as predictors of NTM disease.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds Ratio</th>
<th>P-value</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model combining gender and SNP (n = 338&lt;sup&gt;a&lt;/sup&gt;, p &lt; 0.0001; R² = 0.05)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>0.440</td>
<td>0.001</td>
<td>0.266-0.718</td>
</tr>
<tr>
<td>rs1518111</td>
<td>0.507</td>
<td>0.007</td>
<td>0.309-0.830</td>
</tr>
<tr>
<td>Model combining SNP (n = 315&lt;sup&gt;a&lt;/sup&gt;, p = 0.0087; R² = 0.02)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs3024498</td>
<td>1.370</td>
<td>0.205</td>
<td>0.840-2.251</td>
</tr>
<tr>
<td>rs1518111</td>
<td>0.540</td>
<td>0.021</td>
<td>0.320-0.910</td>
</tr>
</tbody>
</table>

<sup>a</sup>excluding samples with genotyping failures.

We assessed whether radiological findings (nodular bronchiectasis, cavitary, or mixed) were associated with alleles of the rs1518111 SNP. Cavitary disease was more common in patients
carrying the minor allele (A) at rs1518111 ($\chi^2, P = 0.001$). The isolation of fast (n=9) or slow (n=86) growing mycobacteria from the lung were also tested for associations with alleles of rs1518111. A weak association was observed between fast growing NTM species and carriage of the minor allele ($\chi^2, P = 0.07$).

4.4) Discussion

The minor (A) allele of rs1518111 (previously denoted rs3748675) was associated with reduced risk of pulmonary NTM disease. This minor (A) allele has been linked to increased incidence of Behcet’s disease in a Turkish cohort (Remmers et al., 2010), reduced risk of prostate hyperplasia in a Korean cohort (Yoo et al., 2011), and increased risk of tuberculosis (as part of a three SNP haplotype) in an Ugandan cohort (Stein et al., 2007). The allele is also associated with lower IL-10 plasma concentrations, poor treatment outcome and enhanced systemic inflammation in patients with acute coronary syndrome in a Swedish cohort (Mälarstig et al., 2008). Studies investigating this allele as a contributing factor to disease identify associations with pro-inflammatory tendencies in patients, suggesting a modified IL-10 response. Our study implicates the minor (A) allele of rs1518111 in risk of pulmonary NTM disease in Caucasians.

Ethnicity is an important consideration because the frequency of the A allele of rs1518111 varies between different ethnicities, from 22% in Europeans (EUR) to 68% in East Asians (EAS) (Consortium, 2015). rs1518111 is in linkage disequilibrium with rs1800896 in all ethnicities covered by the 1000 Genomes database ($D' > 0.99$) (Consortium, 2015). However $R^2$ values differ between ethnicities, from 0.095 (Chinese Dai in Xishuangbanna, China) to 0.420 (Mandinka in Western Gambia). The minor (C) allele of rs1800896 is usually inherited with the minor (A) allele of rs1518111, but rs1800896 has a higher MAF. Both alleles have now been associated with NTM disease (Affandi et al., 2013), demonstrating the existence of an $IL10$ haplotype which could affect the ability of an individual to respond effectively to pulmonary NTM infection. In individuals from the population used in our study, a haplotype spanning the promoter region of $IL10$ affected transcription regulation in peripheral blood cells stimulated with *Streptococcus pneumonia* (Temple et al., 2003). The two SNP associated here with NTM disease (rs1800896 and rs1800872) were in complete linkage disequilibrium (Temple et al., 2003). In South African HIV patients, we have associated rs1800896 (Wadley et al., 2013) and rs1518111 (Goullee et al, unpublished data) with peripheral neuropathy, further demonstrating a link with inflammatory disease for these SNP alleles.
Radiological data was available for a subset of the patients used in this study. The majority (76.6\%) of patients presented with nodular bronchiectasis. Where this condition fits in the pathogenicity of NTM infection is still debated, but in many cases clear progression of radiological changes occurs after NTM is isolated, and very often in the absence of other pathogens. Very few of the patients were known to have had bronchiectasis before the diagnosis of pulmonary NTM infection, in the majority it is believed to have developed as a result of NTM infection rather than being a precursory condition. Carriage of the minor (A) allele at rs1518111 correlated with a presentation of cavitary disease (as opposed to nodular bronchiectasis) so the immunopathology of these presentations may differ. Infection with fast and slow growing NTM species is independent of radiological presentation and only weakly associated with the IL10 genotype.

In conclusion, when compared with a healthy control group, patients with pulmonary NTM disease were less likely to carry the minor (A) allele for the IL10 SNP rs1518111, which is in linkage disequilibrium with rs1800896 – a SNP previously associated with NTM disease. Our review of the literature associates these alleles with low levels of IL-10 and enhanced risk of inflammatory conditions.
Chapter 5
Do polymorphisms in the P2X7R-P2X4R-CAMKK2 region influence pulmonary NTM disease susceptibility?

This section of work consists of an investigation of genes in the P2X7R-P2X4R-CAMKK2 region in patients of pulmonary NTM disease and healthy controls. SNP genotypes are investigated individually and collectively within a multi-gene haplotype with the goal of identifying relevant associations between genotype and disease.

5) A haplotype spanning \(P2X7R\), \(P2X4R\) and \(CAMKK2\) may mark susceptibility to pulmonary non-tuberculous mycobacterial disease.

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\(^5\) School of Physiology, University of the Witwatersrand, Johannesburg, South Africa
\(^6\) Centre for Comparative Genomics, Murdoch University, Perth,
\(^7\) Harry Perkins Institute of Medical Research, Perth,
\(^8\) Institute for Respiratory Health, University of Western Australia, Perth
\(^9\) Translational Cancer Pathology Laboratory, PathWest Laboratory Medicine WA,
\(^10\) School of Pathology and Laboratory Medicine, University of Western Australia,

**Abstract**

Despite widespread exposure to potentially pathogenic mycobacteria present in the soil and in domestic water supplies, it is not clear why only a small proportion of older women contract pulmonary non-tuberculous mycobacterial (NTM) infections. Here we explore the impact of polymorphisms within three genes; \(P2X7R\), \(P2X4R\) and \(CAMKK2\) on susceptibility.

Thirty single nucleotide polymorphisms (SNP) were genotyped in NTM patients \((n = 124)\) and healthy controls \((n = 229)\). Weak associations were found between individual alleles in \(P2X7R\) and disease, but were not significant in multivariate analyses adjusted to account for gender. Haplotypes spanning the three genes were derived using the fastPHASE algorithm. This yielded 27 haplotypes with frequencies >1% and accounting for 63.3% of the combined cohort. In univariate analyses, seven of these haplotypes displayed associations with NTM disease above our preliminary cut-off \((p \leq 0.20)\). When these were carried forward in a logistic regression model, gender and one haplotype (SH95) were independently associated with disease (model \(p < 0.0001; R^2 = 0.05\)). Examination of individual alleles within these haplotypes implicated \(P2X7R\) and \(CAMKK2\) in pathways affecting pulmonary NTM disease.
5.1) Introduction

Pulmonary disease is the most common presentation of infections with non-tuberculous mycobacteria (NTM) in otherwise healthy adults. Hence NTM are considered an emerging public health risk in Australia and around the world (Thomson, 2010, Winthrop et al., 2010). Exposure through soil or water is extremely common but only a small subset of the population develops pulmonary NTM disease. Estimates in Queensland, Australia, suggest 22.1 persons per 100 000 in the population will develop disease (Thomson, 2010). Known risk factors include smoking, excessive alcohol intake, past tuberculosis, and underlying structural lung disease such as COPD and bronchiectasis (Cassidy et al., 2009, Winthrop et al., 2010). However these factors do not account for all cases – a notable exception being patients with the "Lady Windermere" phenotype. These individuals are lean and tall, with higher than normal incidence of thoracic abnormalities including scoliosis, pectus excavatum and mitral valve prolapse. The majority of cases are Caucasian (91%) and female (95%), with an average age of 60 years (Kim et al., 2008). Behavioural questionnaires found no links between pulmonary NTM disease and patients’ activities, exposures or habits (Kim et al., 2008). Genetic factors should be investigated further.

The risk of pulmonary NTM disease is increased in individuals carrying certain alleles of genes encoding cytokines including IL-10, TNFα and IL-28B (Affandi et al., 2013). Other studies have linked polymorphisms in natural-resistance-associated macrophage protein 1 (NRAMP1) and cystic fibrosis transmembrane conductance regulator (CFTR) to increased risk of NTM disease (Jang et al., 2013, Kim et al., 2008, Koh et al., 2005, Tanaka et al., 2007, Ziedalski et al., 2006). Mendelian susceptibility to mycobacterial disease (MSMD) is an identified syndrome and is associated with polymorphisms found in multiple genes involved in IFN-γ dependent immunity (IFNGRI, IFNGR2, STAT1, IL12B, IL12RB1, ISG15, IRF8, NEMO and CYBB) (Al-Muhsen and Casanova, 2008, Bustamante et al., 2014), but these polymorphisms have not been linked with pulmonary NTM disease. Genes of relevance showing no genetic associations with pulmonary NTM disease risk also include VDR, IFNGRI and TLR2 (Huang et al., 1998, Park et al., 2008a, Park et al., 2008b, Ryu et al., 2006). An intronic microsatellite repeat polymorphism in TLR2 (Yim et al., 2008) has been linked with pulmonary NTM disease.

No study yet has investigated possible associations between polymorphisms in P2X4R, P2X7R, CAMKK2 and pulmonary NTM disease risk. Their potential for modulation of the inflammatory immune response make them viable candidates. In particular, P2X7R has been associated with aggressive tuberculosis presentations, P2X4R and CAMKK2 are important in macrophage function and activated killing of phagocytosed bacteria (central to mycobacterial disease pathogenesis) and all three lie in a cluster on chromosome 12 exhibiting some measure of linkage disequilibrium.
The \textit{P2X7R} gene encodes the purinergic receptor P2X ligand gated ion channel 7 (P2X7R) protein, an ATP-gated ion channel which plays an important role in the innate immune response. When bound by its ligand, extracellular ATP, P2X7R enables the intracellular killing of phagocytosed bacteria (e.g. NTM) in the presence of local inflammatory stress. An important role for the P2X7R has been identified in aggressive forms of tuberculosis (Amaral et al., 2014, Ge and Chen, 2016, Wu et al., 2014). A \textit{P2X7R} knockout mouse model implicated \textit{P2X7R} in \textit{M. tuberculosis} infection (Santos et al., 2013). These data make \textit{P2X7R} a candidate for modulation of NTM disease.

\textit{P2X4R} plays a similar role to \textit{P2X7R} in immune regulation. The gene encodes the purinergic receptor P2X ligand gated ion channel 4 (P2X4R) protein, a purinoreceptor activated by ATP allowing the transport of calcium ions across a membrane. \textit{P2X4R} is generally co-expressed with \textit{P2X7R} in intracellular lysosomal compartments of macrophages and microglia, but can be rapidly trafficked to the surface membrane via the process of endolysosomal secretion (Stokes and Surprenant, 2009). \textit{P2X4R} appears to be involved in the process of phagocytosis, lysosomal destruction and removal of pathogens taken up by macrophages, specifically post-fusion phase exocytosis (Miklavc et al., 2011). Polymorphisms in \textit{P2X4R} may allow NTM to exploit this system, and so persist in macrophage phagosomes via inhibition of lysosome binding.

\textit{CAMKK2} encodes the Calcium/Calmodulin-Dependent Protein Kinase Kinase 2 beta (CaMKK2) protein, a calcium ion activated protein kinase involved in many different pathways, including lymphocyte activation. CaMKK2 may act downstream of \textit{P2X4R} in the process of exocytosis in macrophages that have taken up NTM, with \textit{P2X4R} allowing calcium ions to traverse cell membranes activating Calmodulin-dependant pathways. This function may be affected by total or partial loss-of-function mutations leading to an increased susceptibility to NTM. \textit{CAMKK2} is selectively expressed at higher than base levels in monocytes, macrophages and B-lymphocytes (Racioppi et al., 2012). In knockout mice, \textit{CAMKK2}-null macrophages displayed deficiencies in motility, phagocytosis of bacteria and synthesis of cytokines in response to the TLR4 agonist lipopolysaccharide (LPS) (Racioppi et al., 2012). Loss of CaMKK2 function uncouples the TLR4 cascade, thereby failing to properly activate the inflammatory response. Poor macrophage function in \textit{CAMKK2} knockout-mice establishes the potential for mutations in this gene to affect NTM disease.

Here, SNP and haplotypes spanning \textit{P2X7R}, \textit{P2X4R} and \textit{CAMKK2} are investigated for associations with the incidence of pulmonary NTM disease in Caucasians. Associations were corrected for the disproportionate incidence of pulmonary NTM disease between genders.
5.2) Materials and Methods

5.2.1) Patients and controls

DNA was obtained from Caucasian patients attending Greenslopes Private Hospital, and Prince Charles Hospital with pulmonary NTM disease between 2005 and 2014. All participants provided written informed consent to participate in this work, which was approved by the Greenslopes Research and Ethics Committee (Protocol 12/12) in accordance with the National Statement on Ethical Conduct in Human Research. Control samples were supplied by the Institute for Respiratory Health, Western Australia, in accordance with the Royal Perth Hospital Human Research Ethics Committee. This cohort is representative of the Caucasian Australian population from which patients were also derived.

5.2.2) Genotyping

DNA samples were quantified with Qubit Fluorometric Quantitation technology (Thermo Fisher Scientific, Waltham, MA, USA) and adjusted to 50 ng/µL. Genotyping was performed using TaqMan OpenArray Genotyping Plates (Life Technologies, Grand Island, NY, USA) (Goullee et al., 2016). DNA samples were diluted at 1:1 in TaqMan OpenArray Genotyping Master Mix for 50 cycles of PCR amplification. The output was viewed using OpenArray™ SNP Genotyping Analysis software, and genotypes were allocated manually. Several SNP were excluded from further analysis due to not meeting Hardy-Weinberg Equilibrium (HWE) or having a high percentage of no call readings (rs11065456, rs208294, rs208307, rs12299020, rs504677, rs10160951, rs2230912, rs2668252, rs11608486, rs1169719, rs11065502, rs11065503, rs11837114, rs1718120 and rs2686367).

5.2.3) Haplotype construction

Haplotypes were identified using the fastPHASE algorithm (Scheet and Stephens, 2006). As per convention, the most frequent allele in the population is denoted 0, with the “minor” allele denoted 1. This system is used to portray haplotypes in this study. During haplotype construction, multiple possible haplotypes were generated with fastPHASE for each individual based on their genotypes. The most probable combination of haplotypes for each were selected. From these, 188 different haplotypes were identified from the combined patient and control samples (n=353). Haplotypes with a combined frequency of 1% or less across patient and control populations were excluded from further analysis.

5.2.4) Statistical analyses
Statistical analyses were performed in Stata 12 (StataCorp, Collage Station, TX, USA). Univariate analyses evaluating associations between pulmonary NTM lung disease and gender, age, SNP and haplotypes were performed using two-tailed Fisher’s exact or Chi² tests (χ²) as appropriate. Multivariate analyses were performed using logistic regression modelling where all included factors associated weakly with NTM in univariate analysis (p < 0.20 cut-off chosen for this exploratory study) followed by a stepwise removal procedure to obtain the model of best fit.
5.3) Results

5.3.1) The demographic profile of patients with pulmonary NTM disease

Samples were available from 124 patients with pulmonary NTM disease (attending clinics at The Prince Charles and Greenslopes Private Hospitals, Queensland, Australia) and 229 healthy controls (resident in Western Australia). All participants were of Caucasian ethnicity. 73% of patients and 55% of healthy controls were female. As this is a significant difference ($\chi^2$, $p = 0.001$), gender was included in logistic regression models for SNP and haplotypes of interest.

NTM patients were older than healthy controls [median (range) 67 (25-89) vs. 44 (21-75) years respectively]. Whilst some control donors may develop pulmonary NTM lung disease later in life, the impact on the data is considered minor as pulmonary NTM disease is rare. Patients displayed infections from *M. intracellulare* (n = 64), *M. avium* (n = 13), *M. abscessus* (n = 10), *M. kansasii* (n = 3), *M. triplex* (n = 2), *M. xenopi* (n = 1), *M. simiae* (n = 1), *M. interjectum* (n = 1), *M. shimoidei* (n = 1), *M. terrae* (n = 1), *M. gordonae* (n = 1), or *M. lentiflavum* (n = 1). Some individuals were infected with more than one mycobacterial species (n = 12).

5.3.2) Associations between SNP genotypes and pulmonary NTM disease

Of the SNP tested, four in *P2X7R* (rs208288, rs1718125, rs1186055 and rs2857585) were weakly associated with pulmonary NTM disease ($P \leq 0.20$) in univariate $\chi^2$ analysis. These SNP and gender met our criteria for inclusion in logistic regression modelling (Tables 5.1 and 5.3). The optimal model (model $p = 0.0004$; $R^2$=0.027) did not retain any SNP, only gender. No SNP from *P2X4R* or *CAMKK2* were associated with NTM in univariate analyses ($P > 0.20$).
Table 6: No SNP associated with NTM in univariate analyses.

<table>
<thead>
<tr>
<th>SNP a</th>
<th>Position</th>
<th>Region</th>
<th>MA</th>
<th>MAF c</th>
<th>MAF c</th>
<th>P-value (χ²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X7R</td>
<td>rs10849849</td>
<td>121586395</td>
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<td>G</td>
<td>6.6 %</td>
<td>7.0 %</td>
</tr>
<tr>
<td></td>
<td>rs208288 b</td>
<td>121588088</td>
<td>3’dwns</td>
<td>G</td>
<td>19.5 %</td>
<td>12.8 %</td>
</tr>
<tr>
<td></td>
<td>rs17525767</td>
<td>121588125</td>
<td>3’dwns</td>
<td>T</td>
<td>21.2 %</td>
<td>21.7 %</td>
</tr>
<tr>
<td></td>
<td>rs1718125 b</td>
<td>121593019</td>
<td>Intronic</td>
<td>T</td>
<td>5.3 %</td>
<td>7.4 %</td>
</tr>
<tr>
<td></td>
<td>rs1169737</td>
<td>121600294</td>
<td>3’dwns</td>
<td>C</td>
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<td>39.2 %</td>
</tr>
<tr>
<td></td>
<td>rs1186055 b</td>
<td>121600529</td>
<td>3’dwns</td>
<td>G</td>
<td>14.3 %</td>
<td>9.4 %</td>
</tr>
<tr>
<td></td>
<td>rs2857585 b</td>
<td>121600953</td>
<td>3’dwns</td>
<td>G</td>
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<td>42.9 %</td>
</tr>
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<td>rs11065464</td>
<td>121601315</td>
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<td>50.9 %</td>
</tr>
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<td></td>
<td>rs503720</td>
<td>121605074</td>
<td>3’dwns</td>
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<td>40.0 %</td>
</tr>
<tr>
<td></td>
<td>rs1653609</td>
<td>121605919</td>
<td>3’dwns</td>
<td>C</td>
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<td>21.4 %</td>
</tr>
<tr>
<td></td>
<td>rs2230911</td>
<td>121615131</td>
<td>Exonic</td>
<td>G</td>
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<td>13.7 %</td>
</tr>
<tr>
<td></td>
<td>rs3751144</td>
<td>121622239</td>
<td>Exonic</td>
<td>T</td>
<td>11.2 %</td>
<td>14.4 %</td>
</tr>
<tr>
<td></td>
<td>rs3751143</td>
<td>121622304</td>
<td>Exonic</td>
<td>C</td>
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</tr>
<tr>
<td></td>
<td>rs12301635</td>
<td>121624108</td>
<td>3’dwns</td>
<td>G</td>
<td>20.0 %</td>
<td>14.6 %</td>
</tr>
<tr>
<td>P2X4R</td>
<td>rs2686387</td>
<td>121648870</td>
<td>5’ups</td>
<td>G</td>
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<td>43.3 %</td>
</tr>
<tr>
<td></td>
<td>rs2303998</td>
<td>12165063</td>
<td>Exonic</td>
<td>G</td>
<td>4.0 %</td>
<td>5.7 %</td>
</tr>
<tr>
<td></td>
<td>rs798368</td>
<td>121659684</td>
<td>5’ups</td>
<td>T</td>
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<td>33.3 %</td>
</tr>
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<td>rs25643</td>
<td>121660787</td>
<td>Exonic</td>
<td>T</td>
<td>31.5 %</td>
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<td></td>
<td>rs10849860</td>
<td>121668254</td>
<td>3’dwns</td>
<td>T</td>
<td>28.1 %</td>
<td>30.6 %</td>
</tr>
<tr>
<td>CaMKK2</td>
<td>rs1653587</td>
<td>121676232</td>
<td>3’utr</td>
<td>G</td>
<td>12.0 %</td>
<td>13.3 %</td>
</tr>
<tr>
<td></td>
<td>rs1653588</td>
<td>121676666</td>
<td>3’utr</td>
<td>T</td>
<td>11.6 %</td>
<td>11.4 %</td>
</tr>
<tr>
<td></td>
<td>rs7961979</td>
<td>121671261</td>
<td>3’dwns</td>
<td>C</td>
<td>27.0 %</td>
<td>24.8 %</td>
</tr>
<tr>
<td></td>
<td>rs11065504</td>
<td>121680460</td>
<td>3’dwns</td>
<td>G</td>
<td>44.3 %</td>
<td>46.2 %</td>
</tr>
<tr>
<td></td>
<td>rs7975295</td>
<td>121689101</td>
<td>3’dwns</td>
<td>T</td>
<td>20.7 %</td>
<td>21.5 %</td>
</tr>
<tr>
<td></td>
<td>rs2686344</td>
<td>121690548</td>
<td>Intronic</td>
<td>T</td>
<td>46.7 %</td>
<td>40.3 %</td>
</tr>
<tr>
<td></td>
<td>rs1560568</td>
<td>121690587</td>
<td>3’dwns</td>
<td>G</td>
<td>19.5 %</td>
<td>22.8 %</td>
</tr>
<tr>
<td></td>
<td>rs7314454</td>
<td>121698785</td>
<td>5’ups</td>
<td>T</td>
<td>19.1 %</td>
<td>16.4 %</td>
</tr>
<tr>
<td></td>
<td>rs3817190</td>
<td>121712077</td>
<td>Exonic</td>
<td>A</td>
<td>40.3 %</td>
<td>35.6 %</td>
</tr>
</tbody>
</table>

3’dwns: 3’ downstream region, 5’ups: 5’ upstream region, 3’utr: 3’ untranslated region, 5’utr: 5’ untranslated region, MA: minor allele in this cohort, MAF: minor allele frequency.

a SNP not in Hardy-Weinberg equilibrium were excluded. Remaining SNP are shown in chromosomal order (same as the order of appearance in the haplotypes).

b Four SNP carried forward into multivariable analyses are marked with shading.

a Minor allele frequencies generated are based on the number of individuals carrying each allele, so patients with no calls for a particular SNP are excluded from the frequency calculations.
5.3.3) Associations between haplotypes and pulmonary NTM disease

From the 30 SNP analysed, 188 unique haplotypes were generated by fastPHASE. Haplotypes present in less than 1% of the cohort were excluded, leaving 27 haplotypes of interest. One haplotype (SH95) was significantly associated with pulmonary NTM disease ($\chi^2$, $P = 0.019$) on univariate analysis (Table 7). Seven haplotypes (SH5, SH11, SH17, SH70, SH83, SH95 and SH97) displayed associations above our exploratory cut-off ($p \leq 0.20$) on univariate analysis and were included in logistic regression models with gender (Table 7). After a stepwise removal process, the final model (model $p < 0.0001$; $R^2 = 0.05$) included haplotypes SH95, SH70 and gender (Table 5.3). The haplotype most strongly associated with NTM disease in multivariate analysis was SH95. Associations between haplotypes and NTM disease were similar when gender was excluded as a variable (model $p = 0.0013$; $R^2 = 0.04$) (Table 5.3).

The results of patient radiological findings (nodular bronchiectasis, cavitary, or mixed) ($\chi^2$, $P = 0.631$) and mycobacterial isolates from the lung (fast or slow growing species) ($\chi^2$, $P = 0.590$) were tested for an elevated co-incidence with SH95. No associations were observed.
Table 7: No haplotype associated with NTM in univariate analyses.

<table>
<thead>
<tr>
<th>Haplotype (SH number)</th>
<th>Haplotype sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Frequency&lt;sup&gt;b&lt;/sup&gt; (Controls n=229)</th>
<th>Frequency&lt;sup&gt;b&lt;/sup&gt; NTM (n=124)</th>
<th>P-value (χ&lt;sup&gt;2&lt;/sup&gt;)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH6</td>
<td>000000000001000000100000011</td>
<td>45 (19.6 %)</td>
<td>22 (17.7 %)</td>
<td>0.662</td>
</tr>
<tr>
<td>SH11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>000000000000010000100000010000</td>
<td>31 (13.5 %)</td>
<td>11 (8.9 %)</td>
<td>0.196</td>
</tr>
<tr>
<td>SH1</td>
<td>000000011100000000000001000000</td>
<td>28 (12.2 %)</td>
<td>14 (11.3 %)</td>
<td>0.795</td>
</tr>
<tr>
<td>SH2</td>
<td>000000011000000000000001000000</td>
<td>27 (11.8 %)</td>
<td>13 (10.5 %)</td>
<td>0.712</td>
</tr>
<tr>
<td>SH5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>000000011100000000000001000011</td>
<td>22 (9.6 %)</td>
<td>18 (14.5 %)</td>
<td>0.165</td>
</tr>
<tr>
<td>SH9</td>
<td>001001100110001001100000010000</td>
<td>11 (4.8 %)</td>
<td>8 (6.4 %)</td>
<td>0.512</td>
</tr>
<tr>
<td>SH27</td>
<td>000000000001100000000001000000</td>
<td>10 (4.4 %)</td>
<td>7 (5.6 %)</td>
<td>0.592</td>
</tr>
<tr>
<td>SH47</td>
<td>000001010110000101101100000010</td>
<td>10 (4.4 %)</td>
<td>6 (4.8 %)</td>
<td>0.839</td>
</tr>
<tr>
<td>SH74</td>
<td>0010010001100010011000000100011</td>
<td>9 (3.9 %)</td>
<td>7 (5.6 %)</td>
<td>0.460</td>
</tr>
<tr>
<td>SH20</td>
<td>000000001000000000000010100000</td>
<td>8 (3.5 %)</td>
<td>6 (4.8 %)</td>
<td>0.536</td>
</tr>
<tr>
<td>SH83&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1001010000111001011010000101000</td>
<td>11 (4.8 %)</td>
<td>2 (1.6 %)</td>
<td>0.151</td>
</tr>
<tr>
<td>SH8</td>
<td>000000000000010101000001000010</td>
<td>6 (2.6 %)</td>
<td>6 (4.8 %)</td>
<td>0.272</td>
</tr>
<tr>
<td>SH19</td>
<td>00000000000010000100110000000000</td>
<td>7 (3.1 %)</td>
<td>5 (4.0 %)</td>
<td>0.629</td>
</tr>
<tr>
<td>SH97&lt;sup&gt;c&lt;/sup&gt;</td>
<td>000001100110001001000000010000</td>
<td>11 (4.8 %)</td>
<td>1 (0.8 %)</td>
<td>0.063</td>
</tr>
<tr>
<td>SH95&lt;sup&gt;c,e&lt;/sup&gt;</td>
<td>0000000000000100000000000100010</td>
<td>3 (1.3 %)</td>
<td>8 (6.4 %)</td>
<td>0.019</td>
</tr>
<tr>
<td>SH84</td>
<td>010111000000000000000000010000</td>
<td>7 (3.1 %)</td>
<td>4 (3.2 %)</td>
<td>1.00&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>SH104</td>
<td>01010000011000111011010000111</td>
<td>6 (2.6 %)</td>
<td>4 (3.2 %)</td>
<td>0.746</td>
</tr>
<tr>
<td>SH39</td>
<td>000000000000010011000000000111</td>
<td>6 (2.6 %)</td>
<td>3 (2.4 %)</td>
<td>1.00&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>SH10</td>
<td>000000001110000000000000010000</td>
<td>3 (1.3 %)</td>
<td>4 (3.2 %)</td>
<td>0.247</td>
</tr>
<tr>
<td>SH71</td>
<td>0000000000000000000000000100011</td>
<td>3 (1.3 %)</td>
<td>4 (3.2 %)</td>
<td>0.247</td>
</tr>
<tr>
<td>SH92</td>
<td>000001000000000000000001000011</td>
<td>6 (2.6 %)</td>
<td>1 (0.8 %)</td>
<td>0.429</td>
</tr>
<tr>
<td>SH70&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0000000000011101011010000101000</td>
<td>2 (0.9 %)</td>
<td>5 (4.0 %)</td>
<td>0.055</td>
</tr>
<tr>
<td>SH17&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0000000110000001010000000101000</td>
<td>2 (0.87 %)</td>
<td>4 (3.2 %)</td>
<td>0.190</td>
</tr>
<tr>
<td>SH34</td>
<td>0000000110100000101101100000010</td>
<td>3 (1.3 %)</td>
<td>3 (2.4 %)</td>
<td>0.428</td>
</tr>
<tr>
<td>SH41</td>
<td>00000001000001000000000100111</td>
<td>5 (2.2 %)</td>
<td>1 (0.80 %)</td>
<td>0.669</td>
</tr>
<tr>
<td>SH94</td>
<td>00100100011000010110110000000000</td>
<td>3 (1.3 %)</td>
<td>3 (2.4 %)</td>
<td>0.428</td>
</tr>
<tr>
<td>SH105</td>
<td>0101000001100010000000000100000</td>
<td>5 (2.2 %)</td>
<td>1 (0.8 %)</td>
<td>0.669</td>
</tr>
</tbody>
</table>

<sup>a</sup> Defined by alleles of SNP in the order shown in Table 6.<n><sup>b</sup> Frequency of patients with each haplotype based on the most probable assignment for each individual.<n><sup>c</sup> Seven haplotypes carried forward into multivariable analyses are marked with shading.<n><sup>d</sup> Two-tailed Fishers exact tests were substituted where any categorical frequencies fell below 5.<n><sup>e</sup> Only a single haplotype reached significance in univariate analysis.

**Table 5.3:** Logistic regression models define gender and haplotypes as predictors of NTM disease.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds Ratio</th>
<th>P-value</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model combining gender and haplotypes (n = 353&lt;sup&gt;a&lt;/sup&gt;, p &lt; 0.0001; R² = 0.05)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.43</td>
<td>0.001</td>
<td>0.267-0.70</td>
</tr>
<tr>
<td>SH95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.62</td>
<td>0.014</td>
<td>1.425-22.13</td>
</tr>
<tr>
<td>SH70</td>
<td>4.90</td>
<td>0.064</td>
<td>0.910-26.36</td>
</tr>
<tr>
<td><strong>Model combining haplotypes (n = 353&lt;sup&gt;a&lt;/sup&gt;, p = 0.0013; R² = 0.04)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SH97</td>
<td>0.18</td>
<td>0.104</td>
<td>0.023-1.420</td>
</tr>
<tr>
<td>SH95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.31</td>
<td>0.015</td>
<td>1.380-20.42</td>
</tr>
<tr>
<td>SH70</td>
<td>4.98</td>
<td>0.058</td>
<td>0.950-26.08</td>
</tr>
<tr>
<td>SH17</td>
<td>3.98</td>
<td>0.114</td>
<td>0.718-22.08</td>
</tr>
</tbody>
</table>

<sup>a</sup>Excluding samples with genotyping failures.

<sup>b</sup>Variables achieving significant association with pulmonary NTM disease within the logistic regression models.
5.4) Discussion

Exposure to potentially pathogenic mycobacteria from the environment is ubiquitous, so the mechanism behind the rare and selective distribution of infection in the population presents an apparent paradox. Genetic predisposition seems the most likely influence. Here polymorphisms within three genes involved in the inflammatory immune response were investigated. As pulmonary NTM disease generally develops during the later years of life (average patient age 60 years (Kim et al., 2008)) in individuals without histories of bacterial infection, it is unlikely that factors increasing susceptibility reflect a primary immunodeficiency. Hence loss-of function mutations in any of the genes of interest would be poor candidate polymorphisms. The SNP investigated in this study are likely to have more subtle effects. Here the clearest confounding factor was gender, with 59-95% of reported cases being in females (Huang et al., 1998, Kim et al., 2008, Winthrop et al., 2010). Gender was included in multivariate analyses here in order to confirm an independent effect from the genes under study.

Of the seven haplotypes meeting the $P < 0.20$ cut-off in univariate analyses, SH95 was most clearly associated with pulmonary NTM disease (Tables 5.2 and 5.3). None of the three SNP displaying minor alleles (rs12301635, rs7975295, and rs7314454) in this haplotype (present in $P2X7R$ and $CAMKK2$) were independently associated with disease (i.e.: none met the $P < 0.20$ exploratory cut-off).

SH70 and SH83 associated weakly with risk and protection (respectively) in univariate analysis and shared several SNP alleles. The three alleles (rs10849849, rs1718125 and rs1186055) distinguishing these haplotypes lie within $P2X7R$, and do not explain the associations observed at the haplotype level. We also compared SH97 (potentially protective) with the three haplotypes most closely linked with risk (SH95, SH70 and SH17). No minor alleles of SH97 were shared with either SH95 or SH17, but single minor allele was present in both SH97 and SH70, rs1653609 ($P2X7R$). As SH97 and SH70 are associated with protection and risk respectively, the impact of a minor allele at rs1653609 remains unclear.

These discrepancies between haplotypes and their contained SNP allele associations may be explained by linkage disequilibrium between the alleles identified and other as yet unidentified alleles. These may be within $P2X7R$ or $CAMKK2$ regions of the genome or in strong linkage disequilibrium with them. Alternatively, these alleles may require a second polymorphic allele in a pathway related gene before a contextual loss-of-function phenotype is observed. Further work is required to characterise the relationships between these haplotypes and pulmonary NTM disease.
While haplotypes displaying associations with disease were observed containing minor alleles present in $P2X7R$ and $CAMKK2$, there was no evidence of a similar relationship for $P2X4R$ alleles. No SNP alleles investigated in $P2X4R$ met the exploratory cut-off ($P < 0.20$) in univariate $\chi^2$ analysis, no haplotypes with significant association to disease contained minor alleles of any SNP within $P2X4R$, and no single allele differences between haplotypes with polarizing effects on disease risk were present.

This exploratory study implicates $P2X7R$ and perhaps $CAMKK2$ (and their protein products P2X7R and CaMKK2) in pathways affecting pulmonary NTM disease, whilst $P2X4R$ was not associated with disease protection or risk. Confirmatory studies in additional cohorts are required to confirm the results obtained. Future work would investigate $P2X7R$ and $CAMKK2$, genes in linkage disequilibrium with them, and genes encoding products in the same pathways in more depth and in larger cohorts.
Chapter 6:  
Conclusions and future studies
Conclusions and Future Studies

6.1) How do environmental conditions influence exposure to NTM?

NTM are prevalent in the environment. Although exposure to environmental NTM via aerosolised water, dust and/or soil is universal only a few people develop clinical disease. Risk of infection from environmental NTM is multifactorial involving host defence mechanisms, pathogen virulence and magnitude of exposure (Figure 2) (Mirsaeidi et al., 2014, Johnson and Odell, 2014).

![Diagram](image)

**Figure 2:** A proposed virulence-exposure-host model for NTM infection (adapted from Mirsaedi et al, 2014). Orange arrows represent pathways to infection and disease not demonstrated by Mirsaedi et al. Red arrows demonstrate a path to disease possible in our cohort of seemingly immunocompetent individuals possessing the *IL10* rs1518111 allele associating with disease risk.

6.2) The relevance of bacterial load

At least 40 species of NTM are associated with lung infections, with most caused by members of the *Mycobacterium avium* complex (MAC), *Mycobacterium kansasii*, and *Mycobacterium abscessus* (Johnson and Odell, 2014, Daley and Griffith, 2010). Although the infectious dose for NTM may vary between species and strains, it is estimated that 10-100 *M. bovis* organisms are enough to cause pulmonary infection (O'Reilly and Daborn, 1995). In mice exposed to $10^2$ and $10^3$ aerosolised *M. abscessus* organisms, $10^3$ organisms resulted in pulmonary infection but $10^2$ were...
insufficient (Ordway et al., 2008). These studies lend credibility to the theory that exposure to high environmental pathogen loads is a central component of infection. As of yet, there are no studies investigating the bacterial doses required for infection in *Mycobacterium avium* complex (MAC) and *Mycobacterium kansasii*.

### 6.3) Previous environmental studies do not address bacterial load

Previous studies have investigated environmental conditions associated with pathogenic mycobacterial presence in drinking water distribution systems (Liu et al., 2012, Dubrou et al., 2013). However these did not provide absolute abundances of pathogenic species at each point sampled, with no distinction made between a sample containing 10 viable pathogens per Litre and one containing $10^4$ pathogens per Litre. Future studies could achieve this with the use of sequencing methods such as multiplex PCR (see Table 2).

As a part of this study, a review paper summarising reported links confirmed between environmental NTM and pulmonary infection in the literature has been published (Chapter 3) (Halstrom et al., 2015). The degree to which pathogen loads in the environment are responsible for disease in local residents is a target of future studies.

### 6.4) The importance of human to human transmission

Since the publication of our review paper (Halstrom et al., 2015), findings indicating a major role for human to human transmission of *M. abscessus* lung disease have been published (Bryant et al., 2016). However, it should be noted that *M. abscessus* is known for its unique, more aggressive disease presentation when compared to other NTM species, and that it is classified as a fast-grower rather than a slow–grower. It remains to be seen whether human to human transmission is similarly important in the species primary responsible for NTM pulmonary disease (*M. avium* and *M. kansasii*, both being slow-growers).

### 6.5) The impact of *IL10* polymorphisms on disease

After investigation of seven SNP within *IL10*, one (rs1518111) demonstrated a significant association with incidence of pulmonary NTM disease in our cohort. This consolidates *IL10* and genes in strong linkage disequilibrium with it as genes of interest for future studies of pulmonary NTM disease.
Differences in likelihood of pulmonary NTM disease are observed between ethnicities with the highest being observed in Asian/Pacific Islanders in a multi-ethnicity study of NTM disease in the USA (Adjemian et al., 2012). Frequency of reported disease was lower in Caucasian, Hispanic and African ethnicities respectively (see Figure 4) (Adjemian et al., 2012). The frequency of SNP alleles associating with pulmonary NTM disease was therefore compared between cohorts of differing ethnicity where available. These comparisons were made with available online data from the 1000 genomes project (Figure 3) and with data generated by our lab where available. MAF for rs1518111 varies significantly between ethnic groups.

Figure 3: Allele frequencies from the 1000 Genomes project across all available ethnicities for our primary SNP of interest, rs1518111 (IL10) (Consortium, 2015). The abbreviations for the 5 super populations are AFR: African, AMR: Ad mixed American, EAS: East Asian, EUR: European and SAS: South Asian. The abbreviations for each subpopulation within the superpopulations are detailed in Table 8.
Table 8: Ethnicity population codes from “1000 genomes”, for reference with Figure 3.

<table>
<thead>
<tr>
<th>Super Population Code</th>
<th>Population description</th>
<th>Population code</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAS</td>
<td>Han Chinese in Beijing, China</td>
<td>CHB</td>
</tr>
<tr>
<td>EAS</td>
<td>Japanese in Tokyo, Japan</td>
<td>JPT</td>
</tr>
<tr>
<td>EAS</td>
<td>Southern Han Chinese</td>
<td>CHS</td>
</tr>
<tr>
<td>EAS</td>
<td>Chinese Dai in Xishuangbanna, China</td>
<td>CDX</td>
</tr>
<tr>
<td>EAS</td>
<td>Kinh in Ho Chi Minh City, Vietnam</td>
<td>KHV</td>
</tr>
<tr>
<td>EUR</td>
<td>Utah Residents (CEPH) with Northern and Western Ancestry</td>
<td>CEU</td>
</tr>
<tr>
<td>EUR</td>
<td>Toscani in Italia</td>
<td>TSI</td>
</tr>
<tr>
<td>EUR</td>
<td>Finnish in Finland</td>
<td>FIN</td>
</tr>
<tr>
<td>EUR</td>
<td>British in England and Scotland</td>
<td>GBR</td>
</tr>
<tr>
<td>EUR</td>
<td>Iberian Population in Spain</td>
<td>IBS</td>
</tr>
<tr>
<td>AFR</td>
<td>Yoruba in Ibadan, Nigeria</td>
<td>YRI</td>
</tr>
<tr>
<td>AFR</td>
<td>Luhya in Webuye, Kenya</td>
<td>LWK</td>
</tr>
<tr>
<td>AFR</td>
<td>Gambian in Western Divisions in the Gambia</td>
<td>GWD</td>
</tr>
<tr>
<td>AFR</td>
<td>Mende in Sierra Leone</td>
<td>MSL</td>
</tr>
<tr>
<td>AFR</td>
<td>Esan in Nigeria</td>
<td>ESN</td>
</tr>
<tr>
<td>AFR</td>
<td>Americans of African Ancestry in SW USA</td>
<td>ASW</td>
</tr>
<tr>
<td>AFR</td>
<td>African Caribbeans in Barbados</td>
<td>ACB</td>
</tr>
<tr>
<td>AMR</td>
<td>Mexican Ancestry from Los Angeles USA</td>
<td>MXL</td>
</tr>
<tr>
<td>AMR</td>
<td>Puerto Ricans from Puerto Rico</td>
<td>PUR</td>
</tr>
<tr>
<td>AMR</td>
<td>Colombians from Medellin, Colombia</td>
<td>CLM</td>
</tr>
<tr>
<td>AMR</td>
<td>Peruvians from Lima, Peru</td>
<td>PEL</td>
</tr>
<tr>
<td>SAS</td>
<td>Gujarati Indian from Houston, Texas</td>
<td>GIH</td>
</tr>
<tr>
<td>SAS</td>
<td>Punjabi from Lahore, Pakistan</td>
<td>PJJ</td>
</tr>
<tr>
<td>SAS</td>
<td>Bengali from Bangladesh</td>
<td>BEB</td>
</tr>
<tr>
<td>SAS</td>
<td>Sri Lankan Tamil from the UK</td>
<td>STU</td>
</tr>
<tr>
<td>SAS</td>
<td>Indian Telugu from the UK</td>
<td>ITU</td>
</tr>
</tbody>
</table>

With extensive data on allele frequencies of rs1518111 across so many ethnicities, our first thoughts were to use this data in a direct comparison with pulmonary NTM disease incidence between ethnicities to assess the extent of the allele’s role in ethnicity related predisposition to disease. However, due to unreliable reporting of NTM disease in countries where tuberculosis is endemic resulting in a lack of accurate data on disease incidence, this generalised statistical comparison was not plausible.

Frequency of the minor (A) allele for rs1518111 (which was associated with reduced risk of pulmonary NTM disease in this project) is lowest in our Caucasian cohort (MAF 0.15), and two-fold higher in the Indonesian and African cohorts (MAF 0.38 and 0.36 respectively) in data collected from our group and assayed with the microarray described herein (Dmello et al., 2017). This is consistent with available 1000 Genomes data (Figure 3). If a rough comparison is performed based on the findings of Adjemian et al. (Figure 4), there is no consistent pattern between risk due
to ethnicity and risk due to carriage of the minor A allele of rs1518111. This suggests a factor other than $IL10$ polymorphism as the cause of varying pulmonary NTM disease susceptibility between ethnicities. Nonetheless the association between the minor (A) allele of rs1518111 and disease may be the result of biological effect.

![Figure 4](image-url)

**Figure 4**: Period prevalence of pulmonary NTM cases among a sample of U.S. Medicare Part B enrollees aged 65 and older from 1997 to 2007 by sex and race/ethnicity (Adjemian et al., 2012).

To determine global significance of the SNP allele in terms of its effect on risk of contracting pulmonary NTM disease, studies investigating $IL10$ polymorphism associating with disease should be repeated on cohorts of different ethnicities. More accurate and representative reporting of pulmonary NTM disease in communities of different ethnicities will allow practical comparisons between ethnicity and genotype risk factors in the future. We note that this is problematic in populations with high rates of tuberculosis.

6.6) **The impact of $P2X4R$, $P2X7R$ and $CAMKK2$ polymorphisms on disease**

Unlike the investigation of $IL10$, no SNP investigated within the $P2X4R$, $P2X7R$, $CAMKK2$ block displayed alleles independently associated with pulmonary NTM disease. For this reason, haplotype analysis was performed.
One haplotype (SH95) was identified that occurred in 6.4% of the patient cohort and was significantly associated with disease. As no individual SNP within the haplotype significantly associated with disease, the haplotype may mark untyped SNP in these or adjacent genes. Alternatively there may be some interaction which only occurs with this specific combination of SNP alleles, increasing risk from pathogenic NTM. A fellow student (Ms Jessica Gaff, Curtin University) has documented significant (p < 0.05) differences in the minor allele frequency of 37 of the same SNP in the P2X4R, P2X7R and CAMKK2 region between Indonesians, Caucasian Australians, and South Africans (Gaff, 2017). For example; the minor allele of P2X7R SNP rs10160951 associated with sensory neuropathy in South African HIV patients but was not present in Indonesian HIV patients (irrespective of sensory neuropathy) or in any Australian participants, so could not be correlated with disease in these ethnicities. Several genotyped alleles (rs11065456, rs208294, rs208307, rs12299020, rs504677, rs10160951, rs2230912, rs2668252, rs11608486, rs1169719, rs11065502, rs11065503, rs11837114, rs1718120 and rs2686367) were excluded from the present analysis as alleles were not in HWE or were monomorphic in our Caucasian cohort, but they may be of interest in other ethnicities.

Studies of P2X7R allele associations with pulmonary and extrapulmonary tuberculosis have achieved mixed outcomes dependent on the ethnicity of the patients. For example; in Chinese Tibetans the “C” allele of rs656612, “A” allele of rs7958311, and “A” allele of rs208290 were associated with increased pulmonary tuberculosis risk (Zhu et al., 2016). The polymorphism A1513C (rs3751143) within P2X7R was also a significant risk factor for pulmonary tuberculosis in Asians, but not in Caucasians (Alshammari et al., 2016). These findings are not completely consistent with those of Ms Jessica Gaff for this SNP (rs3751143) for sensory neuropathy risk, with no link found with allele frequency and disease in the ethnicities investigated (including Indonesians) (Gaff, 2017). There were however differences in allele frequency observed for rs3751143 between Indonesians (0.23), South Africans (0.02) and Australians (0.18).

The variability of allele frequencies seen between ethnicities in these alleles demonstrates the importance of controlling for ethnicity in genotyping studies of this region. Further investigation of this gene cluster and polymorphic alleles in adjacent genes is warranted.

6.7) Genes for future study

Of the genes investigated in this project, IL10 appears to be the most promising, further implicating IL10 and other genes involved in inflammatory regulation/response (and possibly other genes in close proximity to IL10 on chromosome 1). Based on previous findings, other genes of interest for
future investigation of pulmonary NTM disease risk include *TNFA* and *IL28B* (identified SNP alleles in each of these associating with disease in a Caucasian cohort) (Affandi et al., 2013). Other groups have investigated *NRAMP1* and found heterozygosity in three different SNP to be associated with pulmonary NTM disease, warranting further investigation of this gene (Koh et al., 2005, Tanaka et al., 2007). Other genes identified as “of interest” but not yet investigated include *IL7*, *ABCA3*, and the pulmonary surfactant family of genes (*SFTPA/B/C/D*). *IL7* is a novel choice for investigation, as it has not yet been implicated in pulmonary disorders. However, it may be worth investigating due to its important central role in the functioning of the innate-like lymphoid cell response which is under investigated and may play a key role in inflammation and the clearance of bacterial infections of the lung (Spits and Di Santo, 2011). Polymorphisms in *ABCA3* and the pulmonary surfactant family of genes have been found to affect patient outcome in a range of pulmonary conditions, including COPD, Influenza A infection induced severe respiratory insufficiency and risk of bronchopulmonary dysplasia, but have not yet been investigated in relation to pulmonary NTM disease (Herrera-Ramos et al., 2014, Yang et al., 2014, Zhang et al., 2015).

To summarise our findings - Genes in which no correlation was found with NTM disease include *TLR2* and *IL4* (Table 9) and *BAT1, IL1A, IL2, IL4, IL18, CCL2, VDR, CD14, IL12B, IL28B* and *SLC11A1* typed previously (See table 1) (Affandi et al., 2013). Although we cannot rule out the influence of these genes further analyses are a low priority.

**Table 9:** *IL4* and *TLR2* SNP investigated were not associated with NTM disease in univariate analyses (unpublished data).

<table>
<thead>
<tr>
<th>SNP</th>
<th>Gene</th>
<th>MA</th>
<th>MAF Control (n = 229)</th>
<th>MAF NTM (n = 124)</th>
<th>P-value ($\chi^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3804099</td>
<td>TLR2</td>
<td>C</td>
<td>38.6%</td>
<td>34.7%</td>
<td>0.14</td>
</tr>
<tr>
<td>rs3804100</td>
<td>TLR2</td>
<td>C</td>
<td>7.0%</td>
<td>6.4%</td>
<td>0.91</td>
</tr>
<tr>
<td>rs5743704</td>
<td>TLR2</td>
<td>A</td>
<td>4.6%</td>
<td>4.8%</td>
<td>0.92</td>
</tr>
<tr>
<td>rs2243250</td>
<td>IL4</td>
<td>T</td>
<td>13.5%</td>
<td>14.1%</td>
<td>0.60</td>
</tr>
<tr>
<td>rs2070874</td>
<td>IL4</td>
<td>T</td>
<td>12.9%</td>
<td>16.1%</td>
<td>0.35</td>
</tr>
<tr>
<td>rs2243282</td>
<td>IL4</td>
<td>A</td>
<td>10.9%</td>
<td>12.1%</td>
<td>0.99</td>
</tr>
<tr>
<td>rs2227284</td>
<td>IL4</td>
<td>R</td>
<td>27.5%</td>
<td>29.4%</td>
<td>0.06</td>
</tr>
</tbody>
</table>
6.8) Directions for future work

Ultimately, it appears many environmental, genetic and physiological factors contribute to an individual’s risk of contracting pulmonary NTM disease. In this project we have reviewed the evidence linking environmental NTM exposures to disease. The most common known mode of pathogenic NTM transmission leading to infection in the literature is from aerosolisation and inhalation of contaminated water, but other sources of transmission (namely aerosolised soil and dust) require further investigation. We also highlighted the problems faced when investigating environmental factors and have offered suggestions for studies which would shed light on the primary sources of transmission from the environment. Namely, the need for studies matching patient isolates to environmental sources. We also investigated the associations between disease incidence and polymorphic alleles in select genes of interest, finding a relationship between IL10 polymorphism and disease incidence.

Future efforts to investigate the risk factors involved in contraction of pulmonary NTM disease should centre around immunological pathways crucial to a healthy immune response against the bacteria. IL10 was identified as a genetic target of interest from previous immunological studies of IL-10 levels in patients of pulmonary NTM disease (Lim et al., 2010) and this relevance has been confirmed via significant allele associations for a SNP within IL10. This workflow has proved successful in discovering a link with IL10, and may work again for other mechanisms affecting disease contraction.

A current area of interest requiring investigation in pulmonary NTM disease is the role of the unconventional innate-like lymphoid cell subsets and the role they play in a successful immune response to bacterial infection. These cells include MAIT cells, iNKT cells and γδ T-cells amongst others, and should be investigated in patient and control peripheral blood to determine any difference in proliferation or activation. Genetic polymorphisms affecting these responses may also be informative. Ideally, future immunological and genetics studies would match patients and healthy controls for perceived levels of exposure to NTM in the environment (gardening, spa-baths, humidity at residence etc.) as well as age and gender matching to eliminate as many confounding factors as possible. If any particular mechanism appears to differ significantly between patients and controls from these initial screens the genes involved would be targets of significant interest for follow up genetic and immunological studies.
Chapter 7: References
7) References


CHAN, E. D. & ISEMAN, M. D. 2010. Slender, older women appear to be more susceptible to nontuberculous mycobacterial lung disease. Gender medicine, 7, 5-18.


