INTESTINAL NONTUBERCULOUS MYCOBACTERIA AND ENVIRONMENTAL ENTEROPATHY IN ZAMBIA: HOSPITAL-BASED OBSERVATIONS

A thesis submitted to the University of Zambia School of Public Health in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Epidemiology

By

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DECLARATION

I hereby declare that all the work in this thesis is my own and has never been submitted for another degree in this or any other university or institution of higher learning.

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ABSTRACT

Background

Environmental enteropathy is prevalent in many low and middle-income countries. Although its aetiology is unknown, we hypothesised that nontuberculous mycobacteria (NTMs), which are ubiquitous in the environment and are known to cause disease in the lungs, the gut, skin, bloodstream and joints, could play a role in pathogenesis. We estimated the prevalence of intestinal carriage of NTMs and investigated whether *Mycobacterium avium* antigens could contribute to environmental enteropathy through matrix metalloproteinase (MMP)-mediated intestinal damage.

Methods

This was a sequential multimethod design comprising a hospital-based cross sectional study as phase one followed by a quasi-experimental post-test study as phase two. In the first phase, 97 patients scheduled for routine endoscopy were surveyed to determine the prevalence of NTM in the gut. Stool, intestinal lavage samples, and biopsy samples from the descending colon and caecum were collected. The samples were analysed using the Mycobacteria Growth Indicator Tube (MGIT) liquid culture method. In the second phase of the study, 48 participants were recruited for an *in-vitro* study to determine gut immune responses to *Mycobacterium avium.* Small intestinal biopsies and whole blood samples were stimulated with *M. avium* lysate over 24 hours. Unstimulated biopsy or blood samples served as negative controls, while stimulation with Staphylococcal enterotoxin B served as a positive control. Supernatants were used to quantify MMP-1, -2, -8 and -9 expression using ELISA, and interleukin 17A (IL-17A), IL-10, IL-6, IL-4, IL-2, IL-1β, interferon (IFN) gamma and tumour necrosis factor (TNF) alpha by flow cytometric assay. Questionnaires were used to collect demographic and clinical information in both phases.

Results

From the survey, out of the 97 patients, 52 (53.6%) were males. The mean age was 46.6 (±15.9), range (18–80) years. The prevalence of NTM was 7.2% (95% CI 1.9–12.4), while that of *Mycobacterium tuberculosis* (MTB) was 6.2% (95% CI 2.3–13.0). Carriage of NTM was not significantly associated with age, sex or presenting symptoms such as diarrhoea, abdominal pain, weight loss as well as HIV status. Descending colon samples were the most likely to be positive (9.8%, 95% CI 3.7, 15.8) followed by stool samples (6.8%, 95% CI 1.0–12.6), caecal biopsy (6.1%, 95% CI 0.3–11.8) and intestinal lavage samples (5.9%, 95% CI 0.3–

11.5). *In vitro* experiments using duodenal biopsies from 48 patients (21 men, 27 women, median age 35 years) demonstrated that *M. avium* lysate induced the expression of many Th1, Th2 and Th17 cytokines in peripheral blood but only IL-1β and IL-6 in duodenal tissue. *M. avium* lysate induced the expression of MMP-1 in duodenal tissue (p=0.004) compared to negative controls, but expression of MMPs 2, 8, or 9 were not significantly increased.

Discussion and Conclusion

These results have revealed a prevalence of NTMs of seven percent in this population, suggesting an environmental contamination of the gut by potentially pathogenic NTMs that was not associated with any symptoms or demographic status. We have also shown that *M. avium* induced the expression of MMP-1 in duodenal tissue and in peripheral blood. *M. avium* also induced the expression of a restricted set of cytokines in duodenal tissue, namely IL-1β and IL-6 as well as eliciting a Th1 and Th2 response in the blood. These findings suggest that NTM can no longer be dismissed as mere contaminants during investigations for other diseases. We speculate that the induction of these molecules by *M. avium* suggests a possible pathway through which NTMs, and *M. avium* in particular, could remodel the intestinal mucosa and lead to environmental enteropathy. As techniques for isolation of these organisms improve, coupled with a deeper understanding of the scope of disease caused by the organisms, the case can be made for mechanisms to improve both surveillance and diagnostic capacity in resource-poor settings.

Keywords: Nontuberculous Mycobacteria, *Mycobacterium avium*, cytokines, matrix metalloproteinases, environmental enteropathy

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CHAPTER ONE: INTRODUCTION

1.1 Background

Environmental enteropathy (EE), a chronic subclinical inflammatory condition of the gut mucosa, is widespread throughout the tropics (Haghighi and Wolf, 1997, Menzies et al., 1999) and is thought to result from constant faecal oral contamination (Korpe and Petri, 2012). Although its aetiology is unknown, it is associated with poverty and mucosal T-cell activation (Veitch et al., 2001, Prendergast and Kelly, 2012), as well as poor oral vaccine response (Levine, 2010). The morphological changes for this condition include villous atrophy, crypt hyperplasia, increased permeability and inflammatory cell infiltrate (Lionetti et al., 1993, Macdonald and Spencer, 1988). It has also been linked to malabsorption, malnutrition, stunting in children and poor oral vaccine responses (Campbell et al., 2003a, Campbell et al., 2003b, Levine, 2010).

Nontuberculous mycobacteria (NTMs) are widespread in the environment such as in soils, rivers and water systems. Nontuberculous mycobacteria from these sources can easily be passed to individuals during the course of everyday activities such as inhalation, inoculation and ingestion (Falkinham, 1996). Some of the NTMs are potentially pathogenic, especially in immunocompromised individuals. These organisms, like *Mycobacterium tuberculosis* (MTB), principally cause disease through activation of immune cells leading to accumulation of inflammatory infiltrate, disruption of epithelial surfaces and other changes, a situation which is not dissimilar to the way the changes due to EE are thought to

occur in the gut (Korpe and Petri, 2012, Veitch et al., 2001). Environmental enteropathy, though a widely studied condition, is still yet to be fully characterised. Although it is known to cause epithelial disruption and its severity is associated with carriage of *Citrobacter rodentium* and hookworm ova (Kelly et al., 2004), it is associated with nutritional and immunological changes in the gut mucosa, though its aetiology is unknown (Prendergast and Kelly, 2012). We, therefore, speculate that NTMs may play a significant role in the pathogenesis of EE. This study, therefore, explored the contribution of NTMs to environmental enteropathy in Zambians.

1.2 What is Environmental Enteropathy?

Environmental enteropathy is widespread throughout the tropics. It is a chronic inflammatory condition of the gut mucosa of unknown aetiology but is associated with mucosal T cell activation (Veitch et al., 2001). The condition is also associated with reduced expression of intestinal anti-microbial peptides (defensins) (Dhaliwal et al., 2003). It has been suggested that environmental enteropathy results from exposure to poor environmental sanitation, as is commonly found in many developing countries. There is chronic exposure to faecal pathogens, leading to repeated subclinical gastrointestinal (GI) infections with low-dose organisms that have some virulence (Kelly et al., 2004, Humphrey, 2009). This leads to inflammation, structural and functional changes in the small bowel (Korpe and Petri, 2012). This subclinical disease is characterised by villous atrophy, crypt hyperplasia, increased intestinal permeability, inflammatory cell infiltrate, and malabsorption (Humphrey, 2009). However, the immunological trigger for

environmental enteropathy is unknown. While *Citrobacter rodentium* and Hookworm infections have been associated with severity of the infection, its association with seasonality (Kelly et al., 2004) and the observation that it is more common in certain geographical regions (Menzies et al., 1999) (lower and middle income countries) suggests that is due to environmental factors.

Originally, environmental enteropathy was called ‘tropical enteropathy’ (Cook, 1980, Louis-Auguste and Kelly, 2017). Menzies et al. showed that residents living in affluent semitropical areas (such as El Paso, Texas and Doha, Qatar) with good sanitation had no significant differences in sugar absorption tests compared to residents of temperate countries. However, there were significant differences in sugar absorption tests (i.e. increased intestinal permeability and reduced absorptive capacity) between asymptomatic tropical residents and residents from tropical countries with enteropathy (Menzies et al., 1999). When residents from temperate countries visit the tropics, they soon acquire changes in their absorptive capacity similar to the local population but lose these changes when they return to their countries of origin (Lindenbaum et al., 1972). This regional association, coupled with the disease’s seasonality (Kelly et al., 2004) and reversibility (Gerson et al., 1971, Wood et al., 1991) suggests an environmental cause, particularly exposure to unsanitary conditions and has led to the name ‘environmental enteropathy’ (Korpe and Petri, 2012).

1.3 Environmental enteropathy as a cause of growth failure

The role of enteropathy in malnutrition, which accounts for up to 45 percent of all deaths in under-five children (Black et al., 2013), is not well understood. It appears that there is a relationship between undernutrition and enteropathy (Prendergast and Kelly, 2012). The gut inflammatory process such as is found in enteropathy is also associated with undernutrition in children and prolonged diarrhoea (Humphrey, 2009, Kau et al., 2011). At the same time, undernutrition, as is seen in children born stunted, has been found to influence enteropathy. Thus, enteropathy is known to be present before the appearance of protein-energy malnutrition and this may lead to impaired digestive, absorptive and barrier functions. Furthermore, these children are prone to instances of diarrhoea from infections such as *Cryptosporidium parvum*, *Escherichia coli* and *Entamoeba histolytica* infections in infancy (Campbell et al., 2003b, Mondal et al., 2012, Prendergast and Kelly, 2012).

In much of the developing world, one-third of the under-five children are stunted (United Nations Children's Fund (Unicef), 2009). This problem is much worse in Zambia, where in 2006, 54 percent of all under-five children in Zambia were stunted (Central Statistical Office, 2006), though the prevalence has recently dropped to 40 percent (Central Statistical Office (Cso) [Zambia] et al., 2014). Although stunting is largely attributed to inadequate food, nutrition programmes aimed at addressing this problem are only partially successful. Bhutta and others have modelled the use of all known nutrition promotion interventions, and shown that the use of these interventions in 99 percent of children worldwide would only

decrease stunting by 33 percent after one year of age, and 35.5 percent by three years of age (Bhutta et al., 2008). Stunting and growth failure are therefore not entirely due to the inadequacy of food, and it appears that enteropathy may play a role in their aetiology (Prendergast and Kelly, 2012).

There is some evidence from several studies suggesting that translocation of immunogenic luminal macromolecules (e.g bacterial lipopolysaccharide) or actual bacterial translocation across a compromised gut mucosa leads to stimulation of systemic immune/inflammatory processes and subsequent growth impairment (Campbell et al., 2003a, Campbell et al., 2003b, Humphrey, 2009) as shown in Figure 1-1.

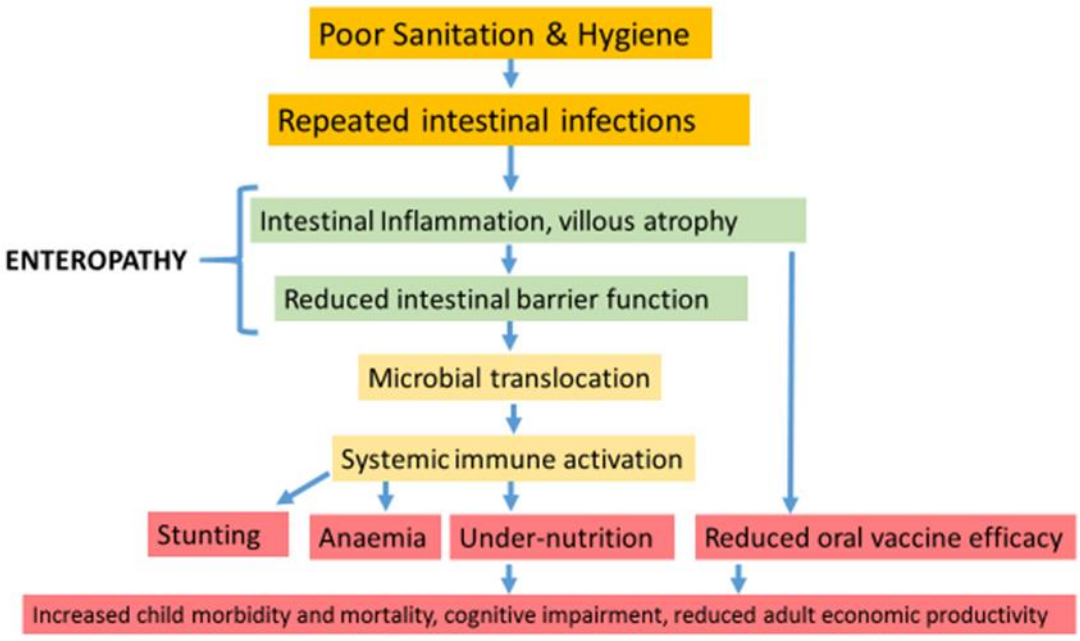


Figure 1-1 Mechanism for the development of environmental enteropathy, stunting and vaccine failure adapted from Prendergast & Kelly, 2012 and Korpe and Petri, 2012.

1.4 Enteropathy and Oral Vaccines

It has been shown in several trials testing the efficacy of oral vaccines including those against poliovirus, rotavirus, and cholera that there is lower efficacy of these vaccines in people from low-income countries when compared to individuals from high-income countries (Ferreira et al., 2010, Levine, 2010, Lagos et al., 1999, Patriarca et al., 1991). Oral rotavirus vaccine has an efficacy of 81-96 percent in children living in westernized societies but only 76.9 percent in South Africa and 49 percent in Malawi (Ruiz-Palacios et al., 2006, Vesikari et al., 2006, Linhares et al., 2008, Phua et al., 2009, Armah et al., 2010, Zaman et al., 2010, Madhi et al., 2010, World Health Organisation, 2009). A preliminary assessment of rotavirus vaccine efficacy in Zambia showed a vaccine effectiveness of 56% against hospitalisation and 48% against severe forms of the disease, but with only 389 participants, the study was not powered to detect effectiveness against milder forms of illness (Beres et al., 2016). Table 1-1 shows the studies that have been done on efficacy and safety of rotavirus vaccines from different parts of the world.

Table 1-1 Selected studies of rotavirus efficacy between 2006-2016

Year of Study	Country	Vaccine	Age of children	N	Efficacy (%)	Ref
2006	Finland, Latin America	Rotarix	2-12 months	63,225	85	(Ruiz-Palacios et al., 2006)
2006	Finland, USA	RotaTeq	1-13 months	68,038	95	(Vesikari et al., 2006)
2008	Brazil	Rotarix	2-24 months	15,183	81	(Linhares et al., 2008)
2009	Singapore, Hong Kong, Taiwan	Rotarix	2-24 months	10,708	96	(Phua et al., 2009)
2010	Ghana, Kenya, Mali	RotaTeq	0-24 months	5,468	39 (but 64% in the first year)	(Armah et al., 2010)
2010	Bangladesh, Vietnam	RotaTeq	0-21 months	2,036	43	(Zaman et al., 2010)
2010	South Africa, Malawi	Rotarix	2-12 months	4,939	77 South Africa, 49 Malawi	(Madhi et al., 2010)
2012	Malawi	RIX4414	1-3 months	1513	49.4 in the first year	(Cunliffe et al., 2012)
2014	India	Human-bovine (116E)	1 and a half months	6799	53.6 in the first year	(Bhandari et al., 2014)
2016	Zambia	Rotarix	6 months or older	389	48	(Beres et al., 2016)

Environmental enteropathy has been suggested as one of the causes of this phenomenon because the increased hyper-cellularity of the gut mucosa may indicate altered T-cell regulatory function leading to the dampened vaccine efficacy (Levine, 2010, Campbell et al., 2003b).

1.5 Mechanisms of enteropathy

1.5.1 T-cell activation

Although the aetiology of environmental enteropathy is unknown, its association with T-cell activation is well known (Veitch et al., 2001, Macdonald and Spencer, 1988). Veitch et al. compared mucosal T-cell activation, quantitated by dual colour immune-fluorescence staining for CD3 plus CD69 or HLA-DR, among healthy black Zambians from the University Teaching Hospital, Lusaka with black South Africans from Baragwanath Hospital in Soweto, and white South Africans (recruited from Johannesburg General Hospital and Verwoerd Hospital, Pretoria) and found that villous height was reduced, crypt depth and mitoses per crypt was increased among black Zambians (Veitch et al., 2001). It is thought that constant T-cell stimulation due to exposure to enteric pathogens in the intestinal lumen results in pathological changes in the small bowel. The structural and functional changes seen in EE may simply be the body's response to an over-stimulated environment (Korpe and Petri, 2012). Other studies have reported that the T-cell activation is responsible for diarrhoea among patients with enteropathy due to the disruption of the mucosal barrier and malabsorption (Musch, 2002, Clayburgh et al., 2005). Large numbers of activated macrophages have also been shown to result in mucosal tissue destruction (Lionetti et al., 1993).

1.5.2 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a group of Zn^{2+} dependent endopeptidases that are activated by calcium and share common functional domains and activation mechanisms but differ in terms of substrate specificity (Ravi et al., 2007, Birkedal-Hansen et al., 1993). Activated T-cells are known to increase the production of MMPs (Pender et al., 1997).

MMPs collectively degrade all components of the extracellular matrix at neutral pH. It is now known that MMPs are the predominant proteinases involved in the pathogenesis of inflammatory bowel disease via their influence on the function and migration of inflammatory cells, mucosal ulceration, as well as matrix deposition and proteolytic degradation (Ravi et al., 2007, Pender et al., 1997, Pender et al., 1996). Other ways in which extracellular matrix is degraded include the plasmin dependent pathway and polymorphonuclear cell serine proteinase pathway for interstitial connective tissues and

basement membranes, as well as an osteoclastic pathway for mineralized tissues such as bone, cementum and dentin (Pham, 2006, Birkedal-Hansen et al., 1993).

There are four major sub-classes of MMPs: collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11, -19), and membrane-type metalloproteinases (MT-MMP-1–5) (Ravi et al., 2007). Although much work is still to be done on MMPs, it is evident that the final outcome of the inflammatory response is dependent on the balance between anti-inflammatory MMPs (MMP-2, -10, MT1-MMP), pro-inflammatory MMPs (MMP-1, -3, -8, -9, -12, -13) and Tissue

inhibitors of MMPs (TIMPs) (Lee et al., 2014, Manicone and Mcguire, 2008, Ravi et al., 2007). A study by Ciccocioppo et al. (2005) found that MMP-1 and MMP-12 were significantly increased in patients with active celiac disease compared with controls. The role of MMPs in the inflammatory processes leading to environmental enteropathy is not well understood.

MMP-1, which is also called collagenase 1 or interstitial collagenase is thought to be involved in angiogenesis, epithelial remodelling as well as monocyte migration during active inflammation and gluten-sensitive enteropathy (Ravi et al., 2007, Mohamed et al., 2006, Wang and Keiser, 1998). It has also been suggested that it may have a role in potentiating the inflammatory response (Ravi et al., 2007) and has been shown to cause extensive tissue damage in human foetal gut explants (Pender et al., 1997). Its role in environmental enteropathy is not well elucidated.

Matrix metalloproteinase - 8, also known as collagenase 2 or neutrophil collagenase was once thought to be exclusively secreted by polymorphonuclear (PMN) leucocytes but is also expressed by mesenchymal and epithelial cells (Ravi et al., 2007). In the lung, it has been suggested that PMN-derived MMP-8 plays a critical role in tissue destruction through increased collagenase activity (measured by the DQ^{TM} collagen degradation assay) especially among patients with cavities (Ong et al., 2015). MMP-1 and 8 have also been shown to be over-expressed in chronic venous ulcers (Amato et al., 2015). Given that enteropathy is known to be associated with T-cell activation, it plausible that the tissue destruction seen in environmental enteropathy may be as a result of PMN derived MMP-8 activation.

Matrix metalloproteinase – 2 and 9 are the two known gelatinases, also called gelatinase-A and B respectively. These enzymes denature type IV collagen, which is a component of the basement membrane. Fascinatingly, they have an opposing effect on intestinal inflammation, with epithelial-derived MMP-2 playing a protective role, while MMP-9 secreted by epithelial cells mediating tissue damage (Ravi et al., 2007). It has been shown that MMP-2 is expressed in normal gut tissue, and its essential role in proper wound healing via angiogenesis and re-epithelialisation is well-established (Kirkegaard et al., 2004). MMP-9, which is activated by multiple cytokines including MMP-1, -2 and -7, inhibits wound healing possibly through defective re-epithelialisation, increased endothelial permeability as well as activation of a number of proteins such as fibrinogen, α1-proteinase inhibitor, interleukin 1β, IL-8 and transforming growth factor-β (Ravi et al., 2007, Atkinson and Senior, 2003, Opdenakker et al., 2001). MMP-9 together with MMP-2 have also been associated with the inflammatory response seen in mycobacterial infections (Lee et al., 2004, Quiding-Järbrink et al., 2001).

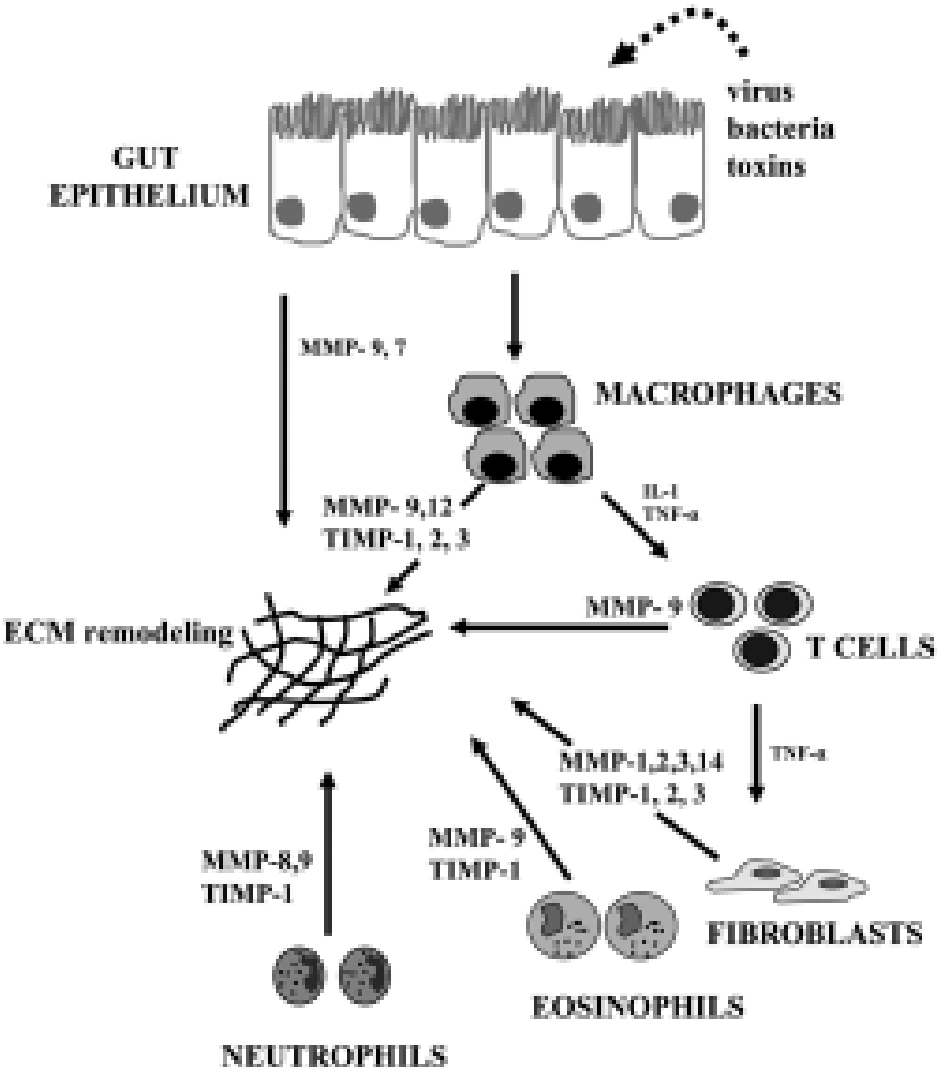


Figure 1-2 Summary of different cell types that produce MMPs and TIMPs in inflammatory bowel disease.

Figure 1-2 summarises the different ways in which MMPs are produced by various cell types (Medina and Radomski, 2006). From this figure, it can be seen that MMPs can be produced by macrophages, T-cells, fibroblasts, eosinophils and neutrophils.

1.6 Nontuberculous mycobacteria

Nontuberculous mycobacteria are a type of mycobacterium species that are separate from *Mycobacterium tuberculosis* complex (MTB), which cause Tuberculosis (TB) and the *M. leprae,* which causes leprosy. These mycobacteria have in the past been referred to as 'atypical mycobacteria', 'environmental' as well as 'mycobacteria other than tubercle bacilli' (MOTT) (Buijtels et al., 2009, Katoch, 2004, Lee et al., 2000). The prevalence of NTM infections has been on the increase worldwide (Donohue and Wymer, 2016, Marras et al., 2013, Adjemian et al., 2012, Winthrop et al., 2010).

Nontuberculous mycobacteria are ubiquitous in the environment with no evidence of human-to-human transmission. However, NTMs cause a variety of pathological changes in the lungs, lymph nodes, skin, wounds and bones. Bacteraemia is common in immunocompromised individuals, resulting in the recovery of these organisms from various foci (Muyoyeta et al., 2009, Katoch, 2004). In the gut, both *M. tuberculosis* and NTMs can cause abdominal pain, ascites, chronic diarrhoea and other clinical manifestations (Sinkala et al., 2009, Sharma et al., 2005).

Nontuberculous mycobacteria are increasingly being recognised as associated with human illness in both immune-competent and immune-compromised individuals (Wu et al., 2009). In the early nineties, NTMs emerged as a major cause of opportunistic infections in those who had AIDS (Covert et al., 1999). Although NTMs are found in patients in both developed and developing countries, the incidence of NTMs is higher in the developed world than in developing countries (Griffith et al., 2007, Falkinham, 1996). Generally, disease due to NTM is more

common in people with reduced local or systemic immunity than in those with normal immunity. Some of the conditions predisposing to NTMs include HIV, Chronic Obstructive Pulmonary Disease (COPD), emphysema, pneumoconiosis, cystic fibrosis, previous gastrectomy and chronic alcoholism (Katoch, 2004). When NTMs are isolated from normally sterile sites such as blood and bone marrow, diagnosis does not usually pose a challenge. However, isolates from non-sterile sources present the investigator with the difficulty of deciding whether the organisms may be from environmental contamination and colonisation (Buijtels et al., 2009). Other NTM species responsible for human disease include *M. kansasii, M. chelonae, M. abscessus, M xenopi, M. malmoense, M. fortuitum, M. Scrofulaceum and M. Ulcerans* (Falkinham, 1996). There is a gap in the knowledge as to whether NTMs are associated with enteropathy.

In the lungs, NTMs cause a Tuberculosis (TB)-like disease. In Zambia, as in many low-income countries, the mainstay of diagnosing TB is the detection of the acid-fast bacilli (AFB) using conventional light microscopy with Ziehl Neelsen (ZN) staining. This method does not differentiate between NTM and actual TB caused by MTB. Several studies have reported that some patients with NTMs end up being treated for MTB, using drugs to which they do not generally respond, and end up being misclassified as having drug-resistant TB (Hoza et al., 2016, Nasiri et al., 2015, Aliyu et al., 2013). This presents serious clinical and public health challenges that need to be resolved.

1.7 Epidemiology of Mycobacterium Avium Complex

Mycobacterium Avium Complex (MAC) refers to the two species *Mycobacterium avium* and *Mycobacterium intracellulare.* They are the most frequent NTMs associated with human disease (up to 73.3% of all pathogenic isolates). Nontuberculous mycobacterial disease in AIDS is caused primarily by MAC which has been isolated in cases of chronic diarrhoea in these patients (Kelly et al., 1996, Blanshard and Gazzard, 1995, Antony et al., 1988, Huh et al., 2008). In immunocompromised individuals, MAC causes disseminated disease especially when the CD4 count is less than 50cells/μl (Katoch, 2004, Falkinham, 1996). There has been an increased incidence of NTMs since the advent of HIV, partly due to HIV and to greater awareness (Henry et al., 2004).

There is marked variability in the prevalence of NTMs in the different parts of the world. The incidence in the USA ranges from 1 to 15 cases per 100,000 population. In Europe, the rates of isolation range from 0.9 to 6.6/100,000. In England and Wales, there has been a significant increase in rates of isolation from 0.9 in 1995 to 2.9/100,000 in 2006. In Finland, the rates of isolation were 6.6/100,000 between 1991 and 1993. In Ghana, a national search in 1999 for Buruli ulcer, which is caused by *Mycobacterium ulcerans*, revealed an incidence of 20.7/100,000 population, but it was as high as 150.8/100,000 population in the worst affected district. Two studies among black South Africans between 1979 and 1980 showed very high rates of NTM isolation of 1,400-6,700/100,000. Table 1-2 shows a summary of incidence of NTM isolation in USA, Canada, some European countries, Ghana and South Africa.

Table 1-2 Prevalence of NTM isolation in selected areas

Country/region	Year(s)	Prevalence per 100,000 population	References
Canada (Ontario)	1998-2010	29.1-41.3	(Marras et al., 2013, Marras et al., 2007)
England, Wales & NI	1995-2006	0.9-2.9	(Moore et al., 2010)
Finland	1991-1993	6.6	(Buijtels et al., 2009)
Ghana	1999	20.7	(Amofah et al., 2002)
Netherlands	2002-2006	2.9	(Buijtels et al., 2009)
South Africa	1979-1980	1,400-6700	(Kleeberg, 1981)
USA	1998-2005	15 -300	(Marras and Daley, 2002, Buijtels et al., 2009, Adjemian et al., 2017, Adjemian et al., 2012, Mohamed et al., 2006)
Zambia	2013-14	1,477/100,000	(Chanda-Kapata et al., 2015)

Previous reports have given different estimates of the burden of NTMs in Zambia. Using a nationally representative sample, the national TB prevalence survey found that out of 6,123 individuals with presumptive TB, 923 (15.1%) had NTMs, equivalent to a prevalence of 1,477/100,000 population (Chanda-Kapata et al., 2015). A report from three hospitals found that 56% of chronically ill patients presenting with a variety of complaints yielded positive NTM sputum cultures. In this study, two of the hospitals had rates of 74 and 77% respectively (Buijtels et al., 2010). In contrast, Ayles et al. (2005, unpublished) isolated NTMs from sputum

in 4 percent of 8044 healthy adults. Out of these, 55 percent were due to MAC. In the pre-ART era, Kelly et al. found that 97 percent of hospitalised patients with persistent diarrhoea were HIV infected, with most of them (81%) carrying potentially pathogenic parasites, including *Mycobacterium tuberculosis* (five percent) and MAC (three percent) (Kelly et al., 1996). As most of the available information about NTMs relates to respiratory carriage, the contribution of mycobacteria and NTMs to enteropathy is not well understood.

1.8 Microbiology of NTMs

Many NTMs are free-living organisms which have been isolated in a wide range of environments, including water, soil, dust, and aerosols. Mycobacterium avium complex bacilli are slow growing, acid-fast, thermophilic (grow at 41°C) and Periodic Acid–Schiff (PAS) positive. The niacin test is one of the biochemical methods used to identify mycobacterial species. In contrast to *M. tuberculosis* which is niacin positive, MAC is niacin negative (Inderlied et al., 1993). However, this method of identifying NTMs is extremely slow and laborious. Rapid commercial tests such as High-Pressure Liquid Chromatography (HPLC) and species-specific DNA probes, sequencing of housekeeping genes and PCR restriction analysis (PRA) are now available (Da Costa et al., 2010, Wu et al., 2009).

1.9 Clinical features and disseminated disease

Patients with AIDS and those with profound immune deficiency often have disseminated disease. This is characterised by fever, night sweats, weight loss, diarrhoea and anaemia. Elevated serum levels of alkaline phosphatase (one-third

of the cases) and anaemia are the most commonly found laboratory abnormalities (Benson, 1994).

In the early 90s, it was observed that some *M. avium* serovars (4 and 8), though rare in the environment, cause most of the cases of disseminated disease in AIDS patients (Inderlied et al., 1993, Griffith et al., 2007). However, other studies have shown that there is no difference in the type of serovars isolated in these patients, and the prevalence of *M. avium* strains appears to depend on the geographical distribution of the serovars (Schorey and Sweet, 2008). Disseminated disease is also associated with specific genetic disorders such as mutations in interferon (IFN)-γ and interleukin (IL)-12 synthesis (Gopinath and Singh, 2010).

Studies conducted in Uganda and Côte d'Ivoire have revealed that disseminated MAC disease is rare in African patients with AIDS (Morrissey et al., 1992, Okello et al., 1990). This is despite the presence of NTMs with similar plasmid characteristics as those in Europe and America (Eaton et al., 1995). However, a study among HIV infected black South Africans found a point prevalence of 10% disseminated MAC disease (Pettipher et al., 2001), which was comparable to the prevalence in developed countries. Disseminated disease is more common in patients whose CD4 count is below 50 cells/mm^3 (Chaisson et al., 1992, Nightingale et al., 1992), which with the current availability of effective antiretroviral therapy, is a rare situation.

1.10 Gastrointestinal disease

Gastrointestinal (GI) transmission occurs through ingestion of environmental MAC. It may also occur through swallowing sputum from lung disease (Hellyer et al., 1991). Gastrointestinal disease involves infiltration of Peyer's patches and the mesenteric lymph nodes of the small intestine, leading to foamy histiocytes and mycobacteria laden macrophages within the lamina propria of the gut mucosa. Diffuse abdominal pain, diarrhoea, weight loss and fever are some of the symptoms of GI disease. Endoscopy may show normal-appearing mucosa, but can also have multiple raised nodules, ulceration, erythema, oedema, reduced mucosal vascularity, increased friability, stricture, and aphthous erosions (Bhaijee et al., 2011, Huh et al., 2008). In immune compromised patients, granulomas are poorly formed (Jarzembowski and Young, 2008, Torriani et al., 1996).

1.11 NTMs, enteropathy and the immune system

Several studies have suggested a link between *Mycobacterium avium paratuberculosis* (MAP) which causes Johne's disease in animals, and Crohn's disease (a chronic inflammatory disease of the intestine) in humans. This link is however disputed (Pierce, 2010, Uzoigwe et al., 2007, Katoch, 2004, Chiodini, 1989). Our literature search did not reveal any studies that have been done to link environmental enteropathy, which is another type of chronic inflammatory disease, with nontuberculous mycobacteria.

The immunological response to Mycobacterium tuberculosis is well documented. Phagocytosis of the antigen by macrophages and B lymphocytes leads to a specific cellular immune response, resulting in the activation of T-helper cells,

macrophages, T-cytotoxic cells and NK cells. In a recent review, Lake (2016) has summarised the immune response to mycobacteria. The review reports that ingestion of the mycobacterium leads to the production of IL-12 by the infected mononuclear phagocyte. The phagocyte binds to the IL-12 receptor on T or natural killer (NK) cells, which leads to the production of IFN-γ. The binding of the IFNγ to its receptor on the phagocyte leads to JAK-STAT signalling, resulting in macrophage activation, further release of IL-12 release, tumour necrosis factor alpha (TNF-α) and IL-1. There is increased phagosome maturation, increased killing of intracellular pathogens and upregulated antigen presentation by the activated macrophages, in the process triggering the activation of Th1-phenotype T-cells to proliferate and release further IFN-γ (Lake et al., 2016). This is consistent with other many previous reports that have identified the upregulation of IL-1, IL-2, IL-12, TNF-α, and IFN-γ as the predominant immune response to mycobacterium (Cooper, 2009, Van Crevel et al., 2002). Other cytokines involved in mycobacterial immune response include IL-17, IL-18, IL10 and Transforming Growth Factor – beta (TGF-β) (Lake et al., 2016).

Much less is known about the immune responses triggered by infection with NTMs. A study by Shin et al. in 2008 (Shin et al., 2008) showed that infections with *Mycobacterium abscessus*, a rapidly growing NTMs, activates the macrophage innate immune response via a physical and functional interaction between Toll-Like Receptor 2 (TLR 2) and dectin-1. The study also showed that *M. abscessus* induced the secretion of TNF-α, interleukin (IL)-6 and IL-12p40 in murine macrophages. Bermudez and Young have suggested that TNF, alone or in

combination with IL-2, but not IFN-γ, is associated with the killing of Mycobacterium avium by macrophages (Bermudez and Young, 1988). However, Appelberg et al. found that IFN-γ and TNF-α act in an additive or synergistic fashion in the induction of bacteriostasis and that IFN-γ is also involved in priming TNF-α secretion (Appelberg et al., 1994).

Blanchard et al. showed that IL-6 is produced by macrophages and NK cells when exposed to *M. avium* in vitro, and may have a role in the pathogenesis of infection (Blanchard et al., 1991). IL-6 has been shown to suppress TNF-α release by mononuclear phagocytes, and decrease the ability of *M. avium* infected human macrophages to respond to TNF-α stimulation and to down-regulate TNF-α receptors on macrophages. TNF-α can stimulate human and murine macrophages to inhibit intracellular growth of *M. avium* (Bermudez et al., 1992).

NTMs are common causative agents of immune reconstitution inflammatory syndrome (IRIS) in both adults and children with AIDS. IRIS has a variety of clinical presentations and severity. Presentations include abdominal, pulmonary, lymphadenitis, disseminated disease, articular and skin conditions. IRIS is commonly found in patients whose baseline CD4+ cell count is lower than 100 at the commencement of antiretroviral therapy (Puthanakit et al., 2006, Phillips et al., 2005).

1.12 Theoretical Framework

Figure 1-1 was been modified to create Figure 1-3 in order to illustrate a theoretical basis for this study. Given the ubiquity of nontuberculous mycobacteria in the

environment, their isolation in various parts of the body and association with human disease under specific circumstances, the immune response to mycobacteria that is largely characterised by a T-cell response, coupled with matrix metalloproteinase involvement, we speculated that these organisms may trigger the type of immune responses that is typically seen a patient with environmental enteropathy.

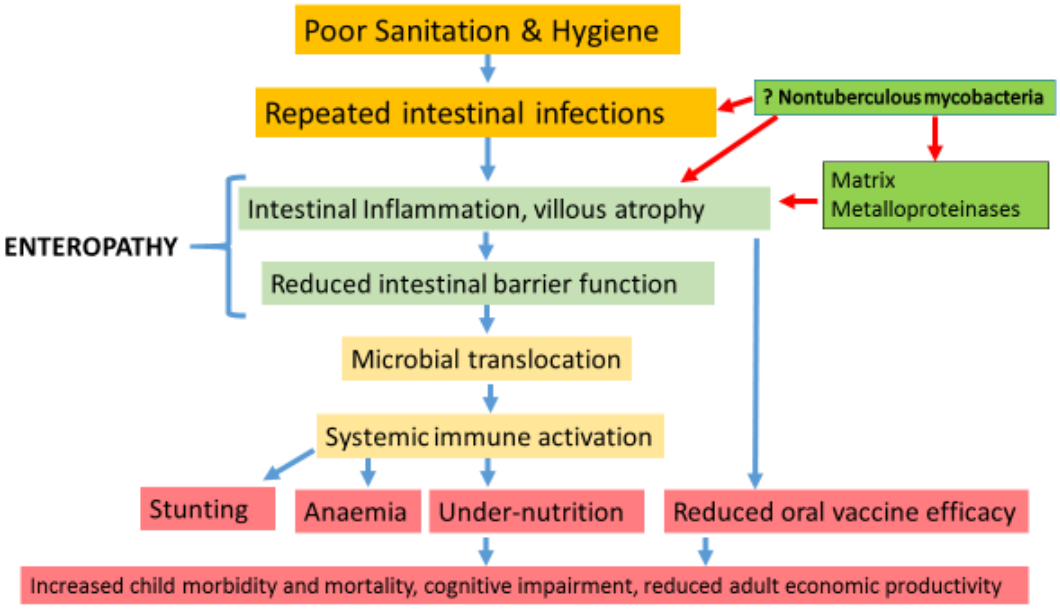


Figure 1-3 Theoretical framework for the study to evaluate nontuberculous mycobacteria as a trigger for cytokine responses seen in patients with environmental enteropathy

1.13 Summary

This review of the literature has shown that environmental enteropathy, as the name suggests, is thought to result from poor environmental sanitation that causes

a cascade of events in the intestinal mucosa leading to inflammation, malabsorption, stunting in children and reduced vaccine response. It is yet to be established what the exact causative agent of enteropathy is. Further, the mechanism that leads to enteropathy is also not yet fully understood, though Korpe and Petri have suggested a mechanism that is currently widely accepted (Korpe and Petri, 2012). The possibility that intestinal carriage of environmental mycobacteria such as *M. avium* might contribute to enteric T-cell stimulation in the lamina propria and activation of macrophages, leading to enteropathy, has not been explored. We speculate that NTMs may play a significant role in the pathogenesis of environmental enteropathy, through their activation of cytokines and matrix metalloproteinases. This study will determine the association of NTMs, particularly *M. avium,* with environmental enteropathy in Zambian adults and children.

CHAPTER TWO: THE MYCOBACTERIA – ENTEROPATHY STUDY

2.1 Statement of the Problem

Nontuberculous mycobacteria are known to cause a variety of diseases in both immunocompromised and healthy individuals and are ubiquitous in the environment such as in soil and water. The role of NTMs in the aetiology of environmental enteropathy, which has been suggested as a cause of poor vaccine efficacy as well as a cause of growth failure in children in developing countries (Prendergast and Kelly, 2012), has not been investigated. Furthermore, the relationship between carriage of NTMs and the severity of enteropathy, prevalence of NTMs among endoscopy patients, risk factors for carriage as well as the understanding of the associated immune responses to mycobacteria in the gut mucosa of patients with enteropathy remains limited. In addition, there is a dearth of knowledge on how this knowledge gap contributes to the welfare of patients and apparently healthy individuals, justifying the need to build knowledge bases in the understanding of environmental enteropathy.

2.2 Rationale

Although NTMs are a group of environmental bacteria and rarely cause clinical disease, they have gained increasing importance in AIDS patients. Little is known about the significance of these organisms in Zambia. This study aimed to investigate the extent to which NTMs could trigger the inflammatory changes that are seen in environmental enteropathy among patients and apparently healthy individuals in Lusaka, Zambia. The study was done to generate data about the risk factors for gastrointestinal NTM disease, cytokine expression as well as diagnostic

yield of different samples from patients with NTMs. The evidence generated from this study will be useful for policymaking related to environmental risks posed by NTMs, in addition to identifying areas needing further research. The study was also warranted because understanding the mechanism for environmental enteropathy, which is associated with stunting in children and poor vaccine efficacy, would provide a way through which these serious ailments would be alleviated.

2.3 Goal and Objectives

2.3.1 Goal

The goal of this research was to improve the overall understanding of the burden of NTMs and whether these organisms could plausibly lead to the kind of gut immune responses that is seen in environmental enteropathy.

2.3.2 Main Objective

To estimate the carriage and distribution of risk factors of intestinal mycobacteria and explore intestinal mucosal immune responses to *M. avium* among endoscopy patients seen in Lusaka, Zambia.

2.3.3 Specific Objectives

1. To estimate the prevalence and risk factors for carriage of NTMs and MTB among endoscopy patients seen at the UTH endoscopy clinic in Lusaka.
2. To compare the mycobacterial yields from stool, colonic biopsy, gut lavage samples from endoscopy patients.
3. To measure whole blood and gut immune responses to *M. avium* in biopsies from endoscopy patients.

Ultimately, the goal of the study is to demonstrate whether the immune responses to *M. avium* could plausibly lead to the kind of T-cell changes that are seen in environmental enteropathy patients.

CHAPTER THREE: METHODS

3.1 Study site and population

The study was conducted at the University Teaching Hospital (UTH) in Lusaka, Zambia among patients who were booked for lower gastrointestinal endoscopy, i.e. either sigmoidoscopy, defined as endoscopy of the rectum, sigmoid and descending colon, or a full colonoscopy which is an endoscopy that goes as far as the caecum. The UTH is the biggest referral hospital in Zambia, with a capacity of 2480 beds. This tertiary hospital caters for both inpatient and outpatient care and is also the main specialist hospital with patients coming from across the country, which had a population of 15 million in 2014 (Central Statistical Office (Cso) [Zambia], 2011). Since 2017, the government unbundled the hospital into five (5) separate teaching hospitals namely: Adult Hospital, Women and New-born Hospital, Children's Hospital, Eye Hospital, and the Cancer Diseases Hospital.

3.2 Study Design

This was a multimethod design comprising a hospital-based cross-sectional study as phase one followed by a lab-based quasi-experimental post-test only study as phase two. The survey aim was to determine carriage rates of nontuberculous mycobacteria among patients coming for endoscopy in Lusaka, understand risk factors for carriage and characterise the mycobacterial species commonly found in this population. The survey was also used to estimate the yields of different intestinal specimens, namely stool, intestinal lavage, caecal biopsy (only for those undergoing colonoscopy) and descending colon biopsy for NTMs. The second part of the study was an *in-vitro* quasi-experimental study (post-test only with controls)

to estimate cytokine responses of duodenal tissue samples after stimulation with a lysate of *Mycobacterium avium* and is described in detail in Section 3.5.1. In both cases, the patients were recruited from the endoscopy unit of the University Teaching Hospital, Lusaka.

3.3 Sampling procedures

Participants were sampled from among patients booked for routine endoscopy procedures. On each day of recruitment, the endoscopy list, which normally had between 10-20 patients, was collected from the nursing staff and scrutinised. Using the list and after checking the patient files, participants who met the inclusion and exclusion criteria were identified and a new list was prepared, which constituted the sampling frame for that day. Simple random sampling using random numbers generated in Microsoft Excel was then used to pick patients who were requested to take part in the study. On each recruitment day, which was once a week, two to three patients were enrolled into the study after obtaining informed consent. Data collection took one year to complete.

We will now describe how Specific Objectives 1 and 2, which dealt with the burden of NTMs and associated factors, were investigated. This is described in detail in Section 3.4. In Section 3.5, we will describe how we examined the gut immune responses to *M. avium*.

3.4 Survey to estimate burden of intestinal NTMs and associated factors

This section was done to answer objectives 1 and 2, which were to estimate the prevalence and factors associated carriage of NTMs in the abdomen, as well as diagnostic yields of various abdominal samples from patients attending the endoscopy clinic at the University Teaching Hospital, Lusaka. (See section 2.3.3)

3.4.1 Research Question

In this phase of the study, the research question was: what is the burden and associated risk factors of intestinal mycobacteria in patients undergoing routine endoscopy at UTH?

3.4.2 Procedure for recruitment for the survey

Patients were selected at random from a list of booked patients for each day (number of booked patients ranged from 10 to 20 per day). This was followed by the explanation of the study by the principal investigator, after which the patients were requested to take part. If the patient accepted to take part, they were requested to sign a consent form. After the consent process, a questionnaire (Appendix 1) was used by the investigator to explore the risk factors for carriage of *M. avium* and other mycobacteria among endoscopy patients.

3.4.3 Inclusion and exclusion criteria

Patients were included in the study if they were 18 years and older, willing to undergo an HIV test and having normal endoscopy findings (no lesions, ulcers or other overt signs). Patients were excluded if they were receiving treatment for tuberculosis, were severely ill (Patients who had a Karnofsky performance scale

equivalent to or below 60, which means they required considerable assistance and frequent medical care) or had tumours or inflammatory lesions on endoscopy.

3.4.4 Sample size estimation for the prevalence survey

The sample size was estimated assuming that the carriage of mycobacteria in stool (expected prevalence) was 3% in the target population (Kelly et al., 1996). Clinic records showed that over 2000 patients are seen per year in the endoscopy unit at the UTH adult hospital. Using the OpenEpi statistical software, and given a confidence level of 95%, a sample size of 70 patients was needed to provide 80% power to detect a prevalence of 3% with a precision of 4%. After adjusting for a 20% non-response, the estimated sample size was 85 participants.

3.4.5 Data analysis for the prevalence survey

The data for the survey was collected using a structured questionnaire designed to collect demographic characteristics, symptoms and drug history. After collection, data was entered into Epidata statistical software (EpiData Association Odense, Denmark) on a panel that matched the unique identifier for the individual's demographic data, then cleaned and analysed using STATA (Version 14, Stata-Corp, College Station, Texas). Carriage of any nontuberculous mycobacteria was the primary outcome variable, while carriage of MTB was the secondary outcome variable. Sample descriptions were expressed as means with their respective standard deviations; whereas proportions (e.g. prevalence) were expressed as percentages with corresponding 95% confidence intervals. In this study, the term "carriage" and "prevalence" were used

interchangeably. For cross tabulations, Pearson's or Fisher's exact Chi square tests were used where appropriate. Normality testing for age and other continuous variables was done using the Shapiro–Wilks test. Multivariable logistic regression was used to measure factors associated with the carriage of mycobacteria and to control for confounding. The independent variables used in the model included age, occupation, residence, level of education as well as presenting symptoms such as abdominal pain, diarrhoea, vomiting, fever and weight loss. The Akaike (AIC) and Bayesian (BIC) information criteria were used for model selection. A $p < 0.05$ was considered statistically significant.

3.4.6 Collection of stool and intestinal lavage fluids

After the questionnaire was administered, patients were handed specimen containers to provide stool or intestinal lavage specimens depending on the stage of readiness for endoscopy. In preparation for the endoscopy procedure, each patient received 2-4 litres of Klean Prep® (Norgine Ltd, Middlesex, UK), an oral fluid used for bowel preparation. The Kleen Prep came as a white powder containing polyethylene glycol 3350 (PEG 3350) and sodium chloride which was reconstituted using tap water. The rate of intake was adjusted depending on the readiness of the patient for endoscopy and if the patient vomited or felt nauseated. Patients were requested to provide a 3-5 ml stool sample before being asked to take the Kleen Prep. When the fluid became clear and watery, a 3-5 ml lavage sample was collected.

3.4.7 Collection of biopsy samples

Biopsy specimens for the prevalence study were collected from the caecum and descending colon for those who came for a colonoscopy and from the descending colon only for those patients booked for flexible sigmoidoscopy (for anatomy of the bowel (see Figure 3-1). The endoscopists included the academic supervisor and two other physicians from the Department of Internal Medicine, University Teaching Hospital. The endoscopy procedures were done under sedation with diazepam and pethidine using high-resolution endoscopes from Pentax (EC 3490Li), while the collection of biopsies was done by way of a Radial Jaw® biopsy forceps (Boston Scientific, Natick, MA). Four biopsies per patient were collected into a labelled 2ml cryovial filled with 1ml culture media. These samples were immediately taken to the lab for processing.

Source: http://www.thewellingtongiunit.com/

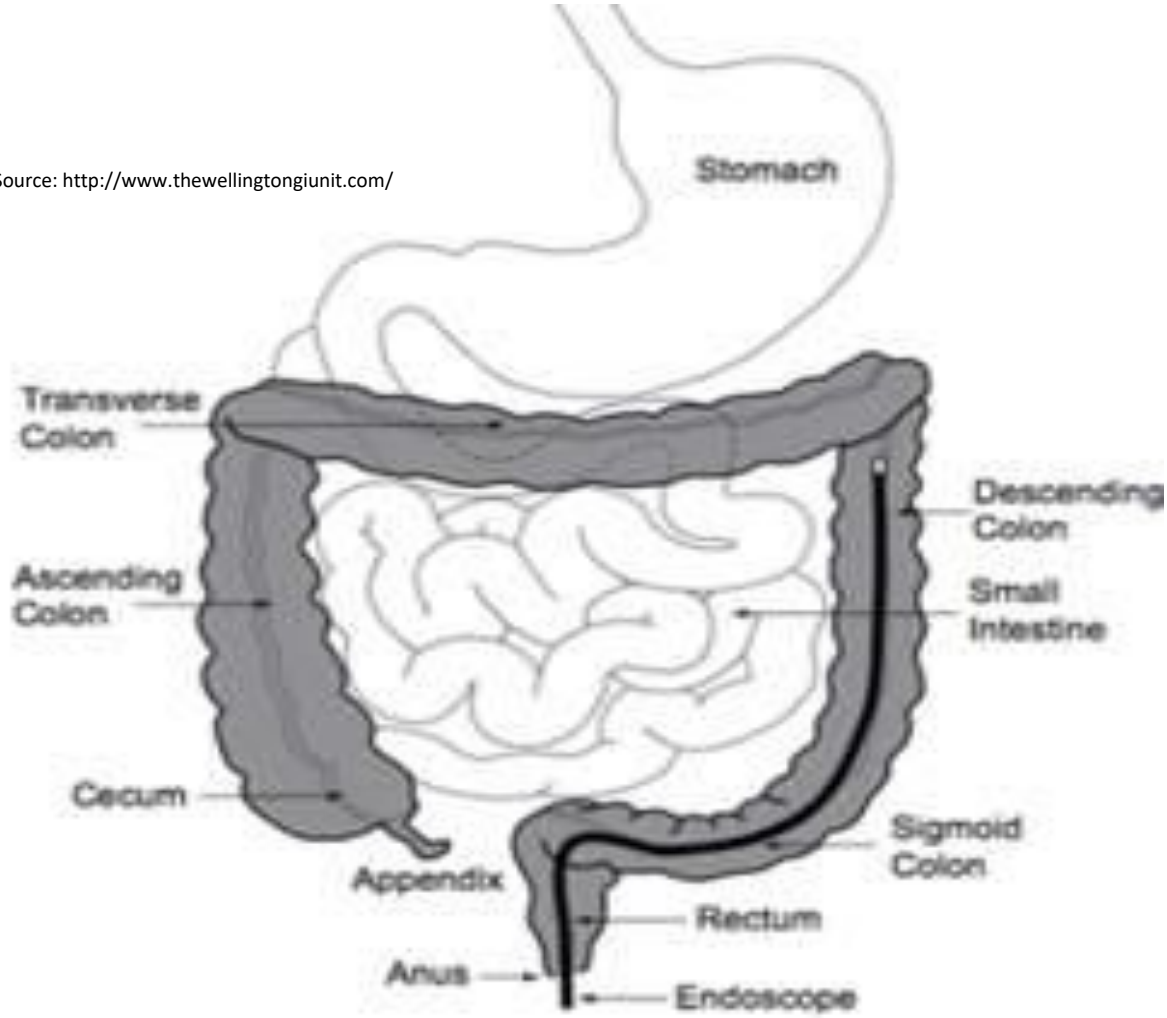


Figure 3-1 Showing the anatomy of the bowel with sites of flexible endoscopy conducted on participants

3.4.8 Laboratory Procedures for the prevalence survey

3.4.8.1 HIV testing

HIV testing was done using the Zambian national testing algorithm, which follows the standard two-test algorithm that begins with the Allere Determine® HIV1/2 test kit (Abbott, Japan) followed by the Uni-Gold™ Recombigen HIV-1/2 (Trinity Biotech) if reactive. In case of a discrepancy between the two tests, a tiebreaker

test such as Bioline® HIV-1/2 test (Standard Diagnostic), is performed or an enzyme-linked immunosorbent assay (ELISA) testing is done (Ministry of Health [Zambia], 2010). There were no discordant results in all our HIV test results.

3.4.8.2 Culture for NTMs

Stool, intestinal lavage as well as biopsy samples obtained from eligible patients were cultured in the University Teaching Hospital Tuberculosis Reference Laboratory in Lusaka to look for mycobacteria species. The culture was done using the standard procedures for mycobacteria culture and is described in the following section.

3.4.8.3 Preparation of decontamination and digestion fluid

To prepare a working solution of decontamination and digestion fluid (sodium hydroxide (NaOH)/ N-Acetyl-L-Cysteine (NALC) mixture), 30g of 6% NaOH and 10g of NALC powder were mixed in a beaker containing 500mls of distilled water as shown in Table 3-1. To prepare a 2.9 percent solution of sodium citrate, 14.5g of Sodium citrate powder was dissolved in 500ml distilled water and the two solutions were mixed together to achieve a final concentration of 3 percent NaOH in 1000 ml. The preparation was autoclaved at 121ºC for 20 minutes and allowed to cool before being used.

Table 3-1 Preparation of the decontamination and digestion fluid for TB culture.

Volume	NaOH/NALC Solution		Volume	Sodium Citrate (2.9%)	Final Volume
	NaOH (6%)	NALC			
50 ml	3g	1g	50 ml	1.45g	100 ml
100 ml	6g	2g	100 ml	2.9g	200 ml
500 ml	30g	10g	500 ml	14.5g	1000

3.4.8.4 Decontamination and digestion

Working in a class II biosafety cabinet, 3-5ml of sample fluid was added to a 50ml labelled Falcon tube. To this was added an equivalent amount of a freshly prepared solution containing 6 percent sodium hydroxide (NaOH) which acts as both a decontaminant as well as an emulsifier, N-Acetyl-L-Cysteine (NALC) for digesting the mucoid components of the specimen as well as sodium citrate which helps to liquefy the sample (Global Laboratory Initiative, 2014). The mixture was vortexed thoroughly and then allowed to stand at room temperature for 15-20 minutes. The mixture was then neutralized by filling the 50 ml Falcon tube with phosphate buffer of pH 6.8 up to the 48 ml mark. This mixture was then placed in a refrigerated centrifuge and centrifuged at 3000*g* for 15 minutes. The supernatant was then discarded in a safety cabinet into a flask containing a disinfectant. The pellet was re-suspended in 2mls of pH 6.8 phosphate buffer, ready for inoculation into both liquid and solid culture media.

In addition to the clinical samples, ten water samples each were collected in duplicate using from the Endoscopy clinic and the TB laboratory using 50mL

Falcon tubes. These samples were centrifuged at 3000g for 15 minutes, the pellet was thereafter decontaminated with NaOH and inoculated into liquid culture media (Edirisinghe et al., 2014) in the same manner as the clinical samples.

3.4.8.5 Preparation of the 7 mL MGIT tubes for inoculation

The MGIT tubes were placed in a rack and labelled with unique specimen numbers. Five hundred µL of PANTA was added to each labelled tube. PANTA is a lyophilized mixture of antibiotics, namely Polymixin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocilin; (see Table 3-2 for concentrations) that has been reconstituted with the OADC growth supplement fluid containing Oleic acid, Bovine Albumin, Dextrose, Catalase and polyethylene stearate was added (Becton Dickinson & Company, 2013).

Table 3-2 Concentrations for Growth Supplement and the antibiotic mixture used in decontamination of samples for TB culture.

Growth Supplement		**MGIT PANTA**	
Name	**Concentration**	**Name**	**Concentration**
Oleic Acid	0.1 gm	Polymixin B	6000 units
Bovine Albumin	50.0 gm	Amphotericin B	600 µg
Dextrose	20 gm	Nalidixic Acid	2400 µg
Catalase	0.03 gm	Trimethoprim	600 µg
Polyethylene stearate	1.1 gm	Azlocilin	600

The inoculation was done by adding 500 µL of the decontaminated specimen to the 7 mL MGIT tubes which had been enriched with the OADC supplement and

the PANTA antibiotics as described above. The tubes were then incubated at 37ºC in a BACTEC™ MGIT™ 960 machine for up to 42 days.

3.4.8.6 Processing of positive MGIT tubes

Whenever the MGIT machine flagged a positive specimen tube, the tube was collected, and the sample was inoculated on blood agar plates to look for contamination (which was defined as a complete overgrowth of organisms on the plate), as well as on microscopy slides for auramine staining. This enabled us to identify the acid-fast bacilli on microscopy. The sample was also tested using the Tauns Capilia TB Neo assay (Figure 3-2). This test is based on the detection of the MPB 64 protein, which is specific to the *Mycobacterium tuberculosis* complex (consisting of *M. tuberculosis, M. bovis, M. bovis* BCG, *M. africanum, M. caprae* and *M. cannetti*) (Muchwa et al., 2012). The Capilia test was used to identify MTB. In the case where the smear microscopy was positive for AAFB but Capilia negative, the isolate was deemed to be a possible NTM and examined using a line probe assay as described in section 3.4.8.7.

Figure 3-2 Picture of Capilia TB assay used in MTB identification

3.4.8.7 Species Identification

The GenoType Mycobacterium CM assay (Hain Lifesciences, GmbH) was used to identify the nontuberculous mycobacteria. This assay is based on DNA STRIP technology and is designed to identify the most common clinically relevant mycobacteria species including *M. avium* complex*, M. chelonae, M. abscessus, M. fortuitum, M. gordonae, M. intracellulare, M scrofulaceum, M. interjectum, M. Szulgai, M. kansasii, M. malmoense, M. peregrinum, M. marinum/M. ulcerans, M. xenopi and the M. tuberculosis* complex (Hain Life Science, 2014). The assay involves the amplification of the 23S rRNA gene region, followed by reverse hybridization of nucleotide probes embedded on membrane strips (Singh et al., 2013). An additional kit, called GenoType Mycobacterium AS assay, can detect 16 species including *M*. *genavense, M. simiae, M. lentiflavum, M. mucogenicum and M. smegmatis (Singh et al., 2013).* The assay has three main steps: extraction

of DNA from the MGIT tubes, amplification and a reverse hybridization, as described in section 3.5.7.8 - 11.

3.4.8.8 DNA extraction

For this step, care was taken to ensure that work was done in an area free from any amplified DNA to prevent contamination. To extract DNA, a 1ml aliquot from a culture positive MGIT tube was put into a container and centrifuged in an aerosol tight rotor at 10,000 g for 15 minutes in a class II safety cabinet. The supernatant was discarded, and the pellet was re-suspended in 300 µl of molecular grade water. Following that, the suspension was incubated in a heating block at 95 ˚C for 20 minutes, followed by sonication for 15 minutes as per manufacturer instructions. This was then spun down for 5 minutes after which 5 µl was used for the amplification stage.

3.4.8.9 Amplification

The following were the requirements for preparing the amplification master mix per sample:

35 µl primer nucleotide mix (PNM)

5 µl 10x polymerase chain reaction (PCR) Buffer for HotStar Taq (contained 15 mM $MgCl_2$)

2 µl 25 mM $MgCl_2$

0.2 µl (1 U) HotStar Taq (Qiagen GmBH, Germany)

3 µl molecular grade water

For each run, we used a positive control containing an MTB DNA as well as a (technical) negative control containing molecular biology grade water. The master mix was prepared by combining the above reagents (except the DNA solution) in a 50 ml Falcon tube and then mixing thoroughly. A 45 µl aliquot of the master mix was then added to each 5 µl from sample DNA solution (prepared from a separate area) in a prepared PCR tube and then placed in a thermocycler with the following conditions:

15 minutes at 95 ˚C	1 cycle
30 seconds at 95 ˚C	10 cycles
2 minutes at 58 ˚C	
25 seconds 95 ˚C	20 cycles
40 seconds at 53 ˚C	
40 seconds at 70 ˚C	
8 minutes at 70 ˚C	1 cycle

After completion of these cycles, the amplification products were stored at -20 ˚C.

3.4.8.10 Hybridization

This step was done at the ZAMBART Laboratory using a TwinCubator (Hain Lifescience GmbH, Nehren, Germany). In preparation for the assay, The TwinCubator was pre-warmed to 45 ˚C, whereas the hybridization buffer, the stringent wash solution and the rinsing solution which are normally kept at room temperature, were pre-warmed to between 37-45 ˚C if they showed signs of precipitates. Other reagents except for the conjugate concentrate and the substrate concentrate were warmed up to room temperature. Next, the conjugate concentrate and the substrate concentrate were diluted with the conjugate buffer and the substrate buffer in a ratio of 1:100 respectively, as required. While wearing gloves, the Mycobacterium CM strips were taken from the tube using forceps and labelled using a laundry marker.

The assay starts by dispensing 20 µl of denaturation solution into the corners of the wells on a tray supplied with the kit, according to the number of specimens and controls. To each well, 20 µl of the amplification product was added using DNase/RNase-free filter tips and mixed well by pipetting. This solution was then incubated at room temperature for 5 minutes. After this, 1 ml of the pre-warmed hybridization buffer was added to each well followed by gentle mixing (shaking), taking care not to spill it onto the neighbouring well. The previously labelled membranes strips coated with specific probes were thereafter placed into each well using tweezers, ensuring that the strips were facing upwards and that they were completely covered by the solution. The tray was placed on the TwinCubator and incubated at 45 ˚C for 30 minutes.

After the incubation, the hybridization buffer was completely aspirated using Pasteur pipettes followed by the addition of 1 ml of stringent wash solution to each strip. This was incubated at 45 ˚C in a TwinCubator for 15 minutes. The stringent wash solution was then completely removed by pouring it out and then turning the tray upside down and gently stroking it on absorbent paper. After this, the strips were rinsed for one minute with 1 ml of rinse solution in a TwinCubator at room temperature. The rinse solution was then discarded by pouring it out. One millilitre of the previously diluted conjugate solution was added to each strip and incubated in a TwinCubator at room temperature for 30 minutes after which it was completely removed. The strips were next washed twice, using 1 ml rinse solution for one minute followed by 1 ml distilled water for one minute on a TwinCubator at room temperature, ensuring to pour out the solution after each rinse. The previously diluted substrate solution was next to be added (1 ml per well), and this step was followed by incubation in the dark without shaking for six minutes. This reaction was stopped by rinsing twice with distilled water, then removing and drying the strips between layers of absorbent paper. With this step, the strips were ready for reading and interpretation.

3.4.8.11 Interpretation of membrane strips

The dry strips were pasted into designated fields of the Mycobacterium CM Evaluation Sheet as shown in Figure 3-3. Each strip had a total of 17 reaction zones (Figure 3-4). In order to interpret the strips, the CC and UC bands were aligned with the respective lines on the sheet. The positive signals were noted down in the appropriate columns, and an interpretation chart was used to

determine species, whose name was entered in the last column. The Interpretation template Figure 3-5 also served as an aid for evaluation and was used to by aligning it with the CC and UC bands of the strip as well. The strips were stored in a box file protected from light.

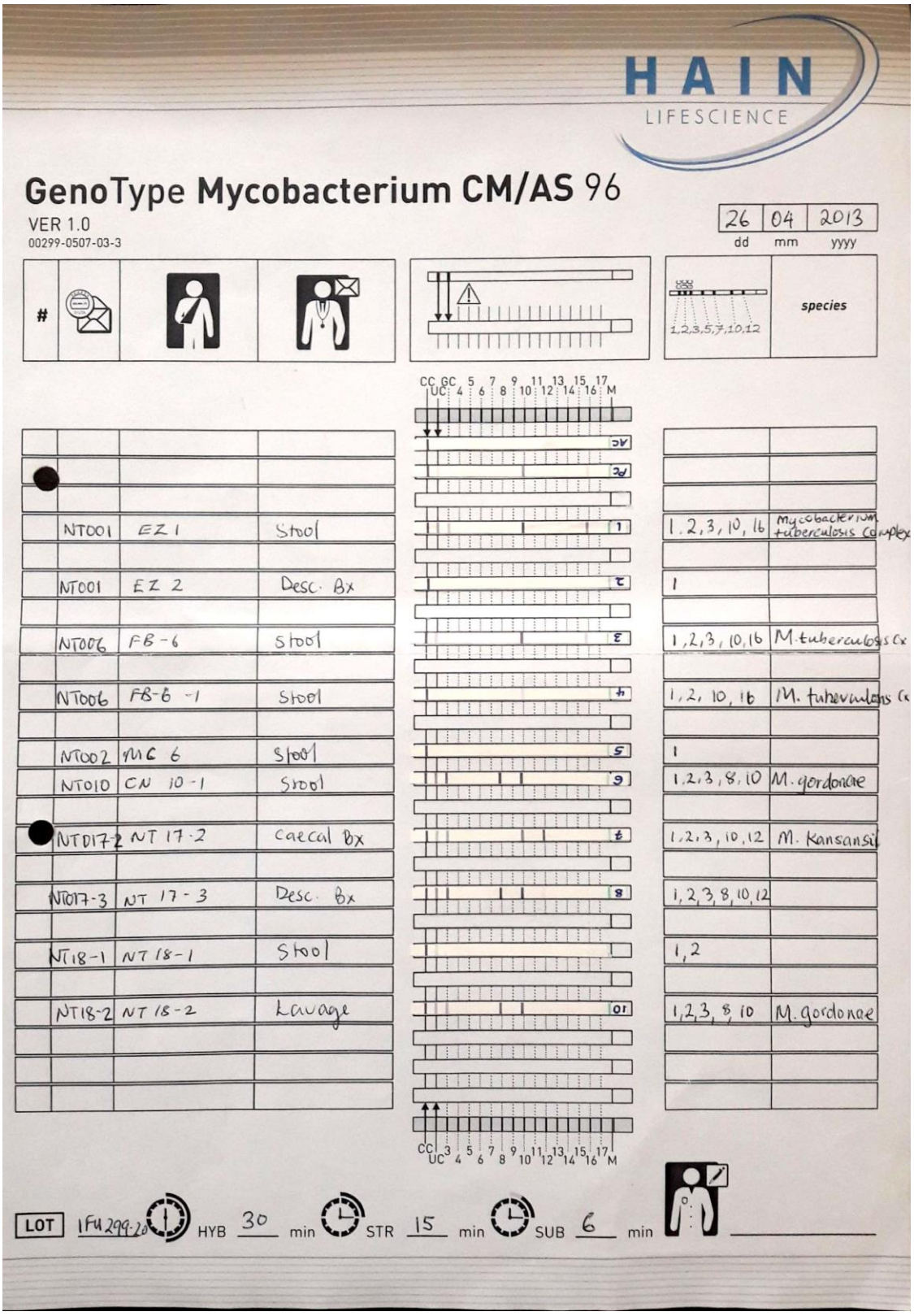


Figure 3-3 Results of the Hain GenoType Mycobacterium CM/AS assay

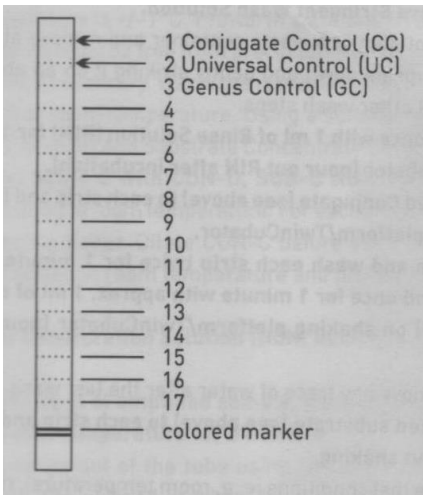
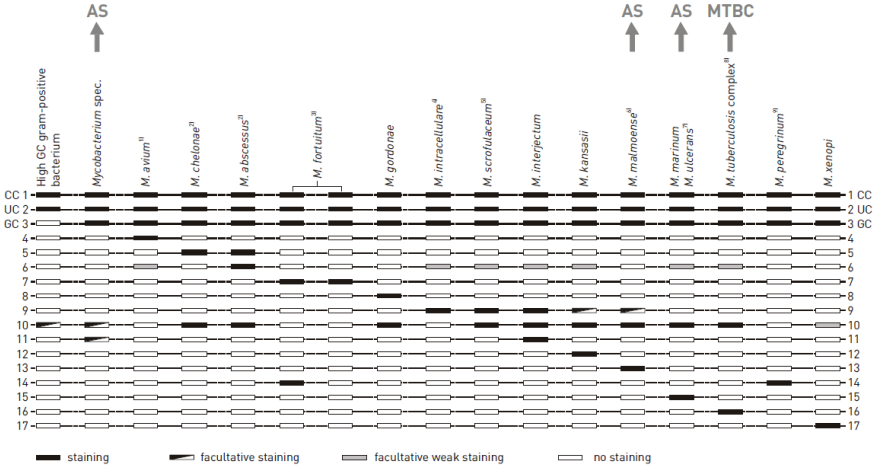


Figure 3-4 Card showing reaction zones for the HAIN mycobacterium CM assay

Interpretation Chart



Band No. 1 (CC): Conjugate Control
Band No. 2 (UC): Universal Control
Band No. 3 (GC): Genus Control
AS: Species may possibly be identified with the **GenoType Mycobacterium AS** kit.
MTBC: For further differentiation use the **GenoType MTBC** kit.

1) Does not include other species of the *M. avium* complex.
2) *M. immunogenum* (belongs to the *M. abscessus/M. chelonae* group) shows the same banding pattern as *M. chelonae* or *M. abscessus*. In case the quality and/or quantity of the extracted DNA does not allow an efficient amplification, the amplicon hybridizing both to the Genus Control and to band 6 may have been supplanted due to competition of the single reactions during amplification. In this case, *M. abscessus* shows the banding pattern identifying *M. chelonae*. However, as long as the specifications given in these instructions for use are observed and the DNA polymerase used for performance evaluation is applied, this does not occur.
3) Due to sequence variations within the species two different *M. fortuitum* banding patterns do occur. *M. mageritense* shows the *M. fortuitum* banding pattern as depicted in the right column.
4) *M. chimaera* shows the same banding pattern as *M. intracellulare*.
5) *M. paraffinicum* and *M. parascrofulaceum* show the same banding pattern as *M. scrofulaceum*.
6) *M haemophilum*, *M. palustre*, and *M. nebraskense* show the same banding pattern as *M. malmoense*. *M. haemophilum/M. nebraskense* can be identified with the **GenoType Mycobacterium AS** kit.
7) *M. ulcerans* can be identified with the **GenoType Mycobacterium AS** kit.
8) If band 15 has also stained positive, additional detection methods must be applied.
9) *M. alvei* and *M. septicum* show the same banding pattern as *M. peregrinum*.

Figure 3-5 Mycobacterium CM/AS interpretation chart

3.5 In-vitro study for intestinal *Mycobacterium avium* immune response

This section was done in order to answer the third objective, which reads as follows:

"To measure whole blood and gut immune responses to M. avium in biopsies from endoscopy patients" (See section 2.3.3)

3.5.1 Hypothesis: Could a lysate of *M. avium* lead to duodenal mucosal disruption seen in enteropathy?

This section describes the procedures that were done to test the hypothesis that NTMs, particularly *M. avium*, could induce cytokine and matrix metalloproteinase expression in duodenal tissue and ultimately lead to the kind of changes seen in patients with environmental enteropathy.

3.5.2 Study design and sampling for the *in-vitro* study

Using *in vitro* culture experiments, we studied the impact of heat-killed mycobacteria on cytokine expression in small intestinal biopsies using cytometric bead array and ELISA to quantify cytokine expression. In particular, levels of pro-inflammatory factors including interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and several interleukins (IL1β, IL-2, IL-4, IL-6, IL10, IL-12 and IL-17A) were measured after exposure of the duodenal biopsies to mycobacteria. We also assessed mucosal release of Zinc-based enzymes called matrix metalloproteinases (MMP-1, 2, -8, and -9) in response to mycobacteria. For each set of experiments, four biopsies from each participant were used (see below).

The sampled population for this component were patients booked for upper gastrointestinal (GI) endoscopy at UTH in Lusaka, in contrast to the first part of the

study where we recruited patients booked for lower GI endoscopy (see section 3.1). Patients were recruited into the study after endoscopy of the duodenum was deemed to be normal by the endoscopists.

3.5.3 Sample size estimation for the *in-vitro* study

We used the Power and Sample size calculator in STATA 13 to estimate the expression of cytokines in the gut and whole blood after stimulation of gut tissue with *M. avium*. Using the sample size for the means of two paired samples option, we assumed that unstimulated samples were going to express 152.7 picograms of interleukin 6 [standard deviation (SD) 249.1] while stimulated samples were going to express 313 picograms (SD 348.6). We further assumed that the correlation between the samples was 0.3, power was 80% and alpha was 0.05 in a two tailed test. Using the foregoing assumptions, the estimated sample size was 42 participants. These figures were based on means obtained after a pilot study with the first ten participants.

3.5.4 Data analysis for the *in-vitro* study

Data on the expression cytokines and MMPs after stimulation by Mycobacterium avium was expressed in picograms per ml for cytokines and nanograms per ml for MMPs. The data was entered and cleaned in Microsoft Excel® and analysed using Stata version 14. Using the Shapiro–Wilk test for normality, it was established that most of the cytokine data were non-normally distributed, so the Wilcoxon signed rank test was used to compare cytokines and MMP concentrations in supernatants from M. avium stimulated samples and unstimulated control samples from the

same individuals. To check for associations between and among the different cytokines and MMPs, Spearman's correlation was used. Secretion of cytokines as well as MMPs was stratified by HIV status, having a previous Bacillus Calmette–Guerin (BCG) vaccination (verified by a scar on the arm) and self-reported history of TB.

The cytokine data was paired in that at least two samples from the same patient were tested, one of which was stimulated with *M. avium* while the other was a negative control. In order to take into account the hierarchical nature of the data, the differences in cytokine secretion between each *M. avium*-stimulated sample and their negative control was estimated using panel random effects multivariable linear regression analysis with maximum likelihood estimation (xtreg in Stata), using an investigator-led backward regression. Akaike and Bayesian information criteria were used for model selection, and a p-value of less than 0.05 was considered significant. Although positive controls were included in all experiments, they were used only to confirm the viability of each experiment and were not included in pairwise comparisons.

Because the study involved making multiple inferences involving a number of cytokines, there was a chance that some inferences could be statistically significance by chance alone, leading to type 1 error. In order to account for this multiplicity problem, while avoiding the possibility of increasing the type 2 error rates that can be caused by a stringent method such as the commonly used Bonferroni correction (Mcdonald, 2009), we adopted the use of the Benjamini-

Hochberg procedure to control the false discovery rate (Benjamini and Hochberg, 1995). The false discovery rate for this study was set at 0.2 (Mcdonald, 2009).

3.5.5 Recruitment of participants for the *in-vitro* study

The recruitment of participants for this arm of the study was similar to the method described for the prevalence study (see section 3.4.2). The difference, in this case, was that the biopsies were collected from the first or second part of the duodenum during an upper gastrointestinal (GI) endoscopy. Stool and lavage fluid were not collected, although blood for in-vitro tissue culture experiments was also collected. Briefly, patients were selected at random (using Excel generated random numbers) from a list of patients booked for upper GI endoscopy for each day (number of booked patients ranged from ten to fifteen per day). After an explanation of the study and the consent process (Appendix 3), a questionnaire (Appendix 2) was used by the investigator to collect data on the participants in order to explore the risk factors for expression of cytokines after stimulation with mycobacteria among patients in endoscopy. If during the endoscopy it became apparent that pathology was present, no samples from the patient were collected; only tissues from patients with normal endoscopy were used in the study.

3.5.6 Inclusion and exclusion criteria

Consenting patients 18 years and older with normal endoscopy findings were recruited. The criteria for recruitment included a willingness to have an HIV test. Patients on anti-TB medications, those who were severely ill (Karnofsky performance scale < 60) and those who had tumours or inflammatory lesions on the endoscopy were excluded.

3.5.7 Collection of Biopsy Samples

Biopsy specimens for the *in-vitro* study were collected from the first and second part of the duodenum. The endoscopy procedures were done under sedation with diazepam and pethidine using high-resolution endoscopes from Pentax (EG2990i), while the collection of biopsies was done by way of a Radial Jaw® biopsy forceps (Boston Scientific, Natick, MA). Four biopsies per patient were collected into a labelled 2ml cryovial filled with 1ml culture media (see Table 3-3). These samples were immediately taken to the lab for processing.

3.5.8 In-vitro culture of intestinal mucosal tissue

Four biopsies from the duodenum from each patient coming for endoscopy were collected into culture medium and established in culture within 2 hours, at 37 ˚C in an atmosphere of 95 percent O_2/ 5 percent CO_2 according to the method previously described by Dhaliwal (Dhaliwal et al., 2009, Dhaliwal et al., 2003). The culture medium consisted of 5 volumes National Cancer Tissue Culture-135 medium, 5 volumes Dulbecco's modified Eagle's medium and 1 volume new-born calf serum (all from Sigma). After reconstitution, the culture medium was kept in a 2-8 ˚C fridge until use.

As shown in Table 3-3, for each participant, the first biopsy was placed in a culture dish with no stimulant added and served as a negative control. The second biopsy was stimulated with 10 µL of *Mycobacterium avium* lysate, while the third biopsy was stimulated with 10 µL of *Staphylococcus* enterotoxin B antigen (SEB) and the fourth one was stimulated with 10 µL of *Salmonella typhimurium* lipopolysaccharide (LPS). In the results section, only SEB results are shown for

clarity, but the results of LPS were generally consistent with those of SEB. The lysate was from the same batch and had a concentration of 28pg/ml, measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, United States).

Table 3-3 Stimulation of tissue biopsy samples for expression of cytokines

Biopsy No.	**Stimulant**	**Designation**
1	Media only (no stimulant added)	Negative Control
2	Media + *M. avium* lysate	Experiment
3	Media + Staphylococcus Enterotoxin B (SEB)	Positive control
4	Media + Salmonella Typhimurium Lipopolysaccharide (LPS)	Positive Control

Each set of biopsies was cultured for 24 hours in 1·5 ml sterile culture medium supported on a sterile RNase-free metal mesh (Expanded Metal Co, Hartlepool, UK). For activation of resident immune cells, we used *Mycobacterium avium* lysate. Figure 3-6 illustrates how the experiment was set up. After 24 hours, the samples were removed from the incubator, and the supernatant was aspirated using pipettes and stored in sealed 2 mL containers at -80 ˚C awaiting analysis.

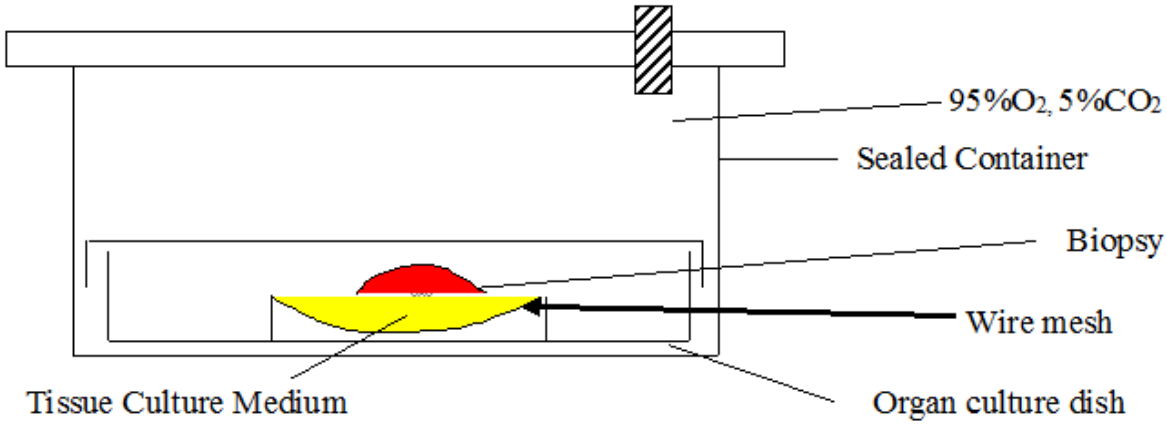


Figure 3-6 Set up of in-vitro tissue culture

3.5.9 Flow cytometry procedures for Cytometric bead array

Part of the supernatant from the in-vitro tissue culture described in section 3.5.8 was used to determine the expression of Th1, Th2 and Th7 cytokines using the human cytometric bead array (CBA) kit (Becton, Dickinson and Company, San Jose, California, USA). For the materials required to run this kit, check Appendix 4. This assay permits the capturing of multiple cytokines with beads of known size and fluorescence, allowing the detection of analytes using flow cytometry. In the BD CBA kit, each capture bead is bound to a specific antibody. Seven bead populations each with their own fluorescence intensities have been coated with capture antibodies specific for Tumour Necrosis Factor (TNF), Interferon Gamma (IFN gamma), interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 17A (IL-17A) proteins. The detection reagent used in this kit is phycoerythrin (PE), which provides a fluorescent signal in

proportion to the amount of bound analyte. During the assay procedure, the capture beads and detector reagent are incubated with an unknown sample (our supernatant) to form complexes. Flow cytometry identifies particles with fluorescence characteristics of both the bead and the detector (Becton Dickinson & Company, 2012). The FCAP Array™ software was used to generate results in tabular and excel format. The detailed procedure for the CBA assay is described below.

3.5.10 Preparing human Th1/Th2/Th17 cytokine standards

One vial of lyophilized Human Th1/Th2/Th17 Standards was opened, and the spheres were transferred to a 15-mL conical, polypropylene tube. The tube was labelled “Top Standard.” The standards in this tube were reconstituted with 2.0 mL of Assay Diluent fluid from the kit and allowed to equilibrate for at least 15 minutes at room temperature. The standards were gently mixed using only a pipette. Eight 12 × 75-mm tubes were labelled and arranged in the following order: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256 after which 300 µL of Assay Diluent was added to each of them. A serial dilution was performed by transferring 300 µL from the Top Standard to the 1:2 dilution tube and mixing thoroughly by pipet only. The serial dilutions were continued by transferring 300 µL from the 1:2 tube to the 1:4 tube and so on to the 1:256 tube (see Figure 3-7) and mixing thoroughly by pipet only and avoiding the use of the vortex. One 12 x 75-mm tube containing only Assay Diluent was prepared to serve as the 0-pg/mL negative control (Becton Dickinson & Company, 2012).

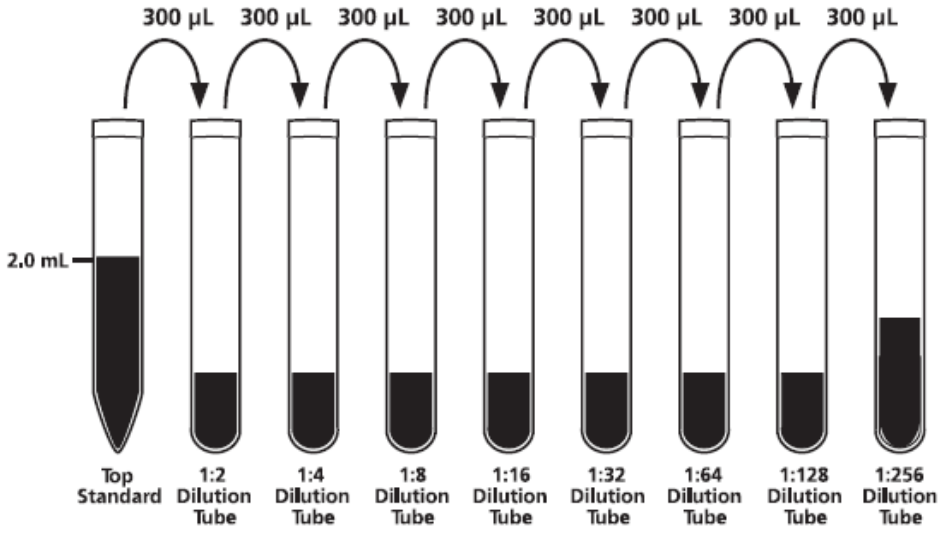


Figure 3-7 Serial Dilution for the cytometric bead array assay

The standards resulting from the above serial dilutions had concentrations as shown in Table 3.4.

Table 3-4 Showing the concentrations of standards after serial dilutions

Cytokine Standard Dilution	Concentration
Top standard	5,000
1:2	2,500
1:4	1,250
1:8	625
1:16	312.5
1:32	156
1:64	80
1:128	40
1:256	20
Assay Diluent Only	0 (negative control)

3.5.11 Mixing human Th1/Th2/Th17 capture beads

The first step was to determine the number of assay tubes (including standards and controls) that were required for the experiment (for example, 50 unknowns [either the supernatant or serum], 9 cytokine standard dilutions, and 1 negative control = 60 assay tubes). Each Capture Bead suspension from the kit was vigorously vortexed for a few seconds before mixing. A 10-µL aliquot of each Capture Bead was added, for each assay tube to be analyzed, into a single tube labelled “mixed Capture Beads” (for example, 10 µL of IL-2 Capture Beads × 60 assay tubes = 600 µL of IL-2 Capture Beads were required). The bead mixture was thoroughly vortexed. In the case were serum or plasma samples were being tested, the capture beads were incubated in Serum Enhancement Buffer by centrifuging the mixed Capture Beads at 200g for 5 minutes, after which the supernatant was carefully aspirated and discarded. The capture beads pellet was then re-suspended in Serum Enhancement Buffer equal to the volume removed in the aspiration and discarding step and the mixture was thoroughly vortexed. The mixed capture beads were then incubated for 30 minutes at room temperature in the dark. This procedure was necessary for serum and plasma samples but was optional for other sample types (Becton Dickinson & Company, 2012).

3.5.12 Performing the human Th1/Th2/Th17 cytokine assay

The Cytometric Bead Array (CBA) assay kit allows the simultaneous detection of multiple Th1, Th2 and Th17A cytokines from a single specimen of tissue culture supernatant (see 3.5.9 for details). Prior to performing this step, the steps in section 3.5.10 and 3.5.11 would already have been done. The mixed capture

beads were vortexed, and 50 µL was added to all appropriately labelled 2 mL microcentrifuge assay tubes (i.e. the unknowns such as the supernatant or serum, including the standards). The assay tubes for the standards were labelled from 1-10 with the negative standard labelled 1 and the top standard having a label of 10. To each of the tubes for the standards, 50 µL of standard dilutions were added accordingly. The unknown samples (50 µL) were also added to the tubes containing the mixed capture beads. This was followed by adding 50 µL of detection reagent to all the assay tubes and incubation in the dark for 3 hours at room temperature. After incubation, 1 mL of wash buffer was added to each assay tube and centrifuged at 200g for 5 minutes. Supernatant from this step was carefully aspirated and discarded followed by re-suspending of the bead pellet in 300 µL of wash buffer. The resulting bead pellet was acquired on a BD FACSverse flow cytometer as described in section 3.5.13 (Becton Dickinson & Company, 2012).

3.5.13 Acquiring samples on a FACSverse Machine

The machine setup was performed as per manufacturer instructions (see Figure 3-8). Three drops of setup buffer was added to 500µL sheath fluid. In the assay and tube setup mode, a performance QC was performed. After this, the in-built CBA kit standards and samples protocol was selected, and under the cytometer, the samples were acquired manually in a worklist. After the acquisition of samples, the files were saved in FCS format and analysed using BD FCAP array software.

Figure 3-8 Acquisition of samples on the BD FACSverse flow cytometer

3.5.14 Analysis of Data using FCAP software

After the acquisition of samples in the FACSVerse flow cytometer, the results were saved as FCS files and imported into FCAP software (BD Biosciences) for analysis. This software was used to fit standard curves to the data for each cytokine obtained from the CBA analysis. The software then used each standard curve for the respective cytokine to determine absolute concentrations of each sample. The cytokine concentrations were expressed in picograms per millilitre (pg/mL). Appendix 11-23 are examples of standard curves that were fitted using some of the data from the current study.

3.5.15 In-vitro culture of whole blood with mycobacteria for expression of Th1/Th2/Th17 cytokines

Blood samples from patients recruited for cytokine expression were stimulated with *M. avium* in much the same way as described for biopsy samples in section 3.5.8. Briefly, whole blood was incubated for 24 hours in 95 percent O_2 and 5 percent CO_2 at 37 ºC with various stimuli as shown in Table 3-5. After 24 hours, the samples were centrifuged at 5000 rpm for 5 minutes, and serum was obtained and

stored at -80 ºC in fresh 2 ml vials. The serum samples were then used to quantify cytokine expression using CBA.

The procedures for the quantification of cytokines using the CBA assay were as described for the biopsies in section 3.5.14, except that since serum was used instead of supernatant from a biopsy, when mixing the beads an additional step where the mixed capture beads were re-suspended in serum enhancement buffer after centrifuging them at 200g for five minutes was followed. After re-suspending, the beads were incubated at room temperature for 30 minutes, protected from light. At this stage, the beads were ready to use, and the procedures already described from 3.5.9 to 3.5.14 above were followed.

Table 3-5 Stimulation of whole blood samples for expression of cytokines

Blood Specimen No.	**Stimulant**	**Designation**
1	Whole Blood (WB)	Negative Control
2	Serum Only	Negative Control
3	WB + Mycobacterium Avium lysate	Experiment
4	WB + Staphylococcus Enterotoxin B (SEB)	Positive control
5	WB + Salmonella Typhimurium Lipopolysaccharide (LPS)	Positive Control

The blood was collected in green lithium heparin bottles and processed immediately.

3.5.16 Elisa for Interleukin 1 beta

Materials: See Appendix 5

ELISA Method

Before the procedure, reagents and standards were prepared according to manufacturer's instructions (R&D Systems, 2013a). Excess microplate strips were removed from the plate frame and resealed. Next, 100 µL of assay diluent RD1-83 was added to each 96 plate well. Thereafter, 100 µL of samples, controls and standards were added in duplicate, covered with an adhesive strip and incubated for two hours at room temperature. After this, each well was aspirated and washed three times by filling each well with 400 µL wash buffer using a squirt bottle. At the end of the last wash, the remaining wash buffer was removed by decanting and inversion of the plate and blotting against clean paper towels. IL-1β conjugate amounting to 200 µL was added to each well, covered with a new adhesive strip and incubated for one hour at room temperature, followed by aspiration and washing three times. To each well, 200 µL of substrate solution was added and incubated at room temperature for 20 minutes, protected from light. Finally, 50 µL of stop solution was added to each well leading to a colour change from blue to yellow, and reading was done using a microplate reader at 450 nm within 30 minutes. Wavelength correction was set at 540 nm (R&D Systems, 2013a).

3.5.17 Elisa for Interleukin 12

Materials: See Appendix 6

ELISA Method

Before the procedure, reagents and standards were prepared according to manufacturer's instructions. Next, 100 µL of assay diluent RD1W was added to each 96 plate well. Thereafter, 100 µL of samples, controls and standards were added in duplicate, covered with an adhesive strip and incubated for two hours. After this, each well was aspirated and washed four times by filling each well with 400 µL wash buffer using a squirt bottle. At the end of the last wash, the remaining wash buffer was removed by decanting and inversion of the plate and blotting against clean paper towels. IL-12/IL-23 p40 conjugate amounting to 200 µL was added to each well, covered with a new adhesive strip and incubated for one hour, followed by aspiration and washing four times. To each well was added 200 µL substrate solution and incubated at room temperature for 30 minutes, protected from light. Finally, 50 µL of stop solution was added to each well leading to a colour change from blue to yellow, and reading was done using a microplate reader at 450 nm within 30 minutes. Wavelength correction was set at 540 nm (R&D Systems, 2013b)

3.5.18 Elisa for Matrix Metalloproteinases

The supernatant from the in-vitro experiment was also assessed for expression of matrix metalloproteinases (MMPs). The experimental samples were produced as a result of stimulation with *Mycobacterium avium* complex lysate. *Staphylococcus* Enterotoxin B antigen (SEB) was used as the positive control. A sample in which

a biopsy was incubated in medium only was used as a negative control (see Table 3-3).

3.5.18.1 Elisa for Total MMP-1

Materials: See Appendix 7

ELISA Method

In preparation for the assay, 360 µg/mL capture antibody was diluted to the working concentration of 2.0 µL/mL in PBS without carrier protein, and 100 µL of this solution was immediately used to coat a 96 well plate, which was incubated overnight at room temperature. Each well was aspirated and washed with wash buffer (0.05 percent Tween, in PBS, pH 7.2-7.4) three times, taking care to completely remove all fluids at each wash step. After the last wash, the remaining fluid was removed by aspiration and blotting on clean paper towels. The plates were blocked by adding 300 µL of reagent diluent to each well, followed by incubation at room temperature for at least one hour. The plates were again aspirated and washed three times as described above. The plates were now ready for sample addition.

One hundred µL of the sample or prepared standards was added to each well in reagent diluent, covered with adhesive strip provided and incubated for two hours at room temperature. Each well was aspirated and washed with wash buffer three times as described in the preparation step above, taking care to completely remove all fluids at each wash step. This was followed by adding 100 µL of detection antibody diluted in reagent diluent, covering with an adhesive strip and incubating

at room temperature for two hours. The aspiration and wash step described above was repeated, followed by adding 100 µL of the working dilution of Streptavidin-HRP to each well. The plates well covered and incubated at room temperature for 20 minutes, avoiding direct sunlight. To stop the reaction, 50 µL of stop solution was added to each well, tapping gently to ensure thorough mixing. The optical density was determined immediately, using a microplate reader set to 450 nm, with wavelength correction set to 540 nm. The readings for 540 nm were subtracted from the readings for 450 nm to get the optical density for each sample, which in turn was used to calculate the actual MMP-1 concentration from the standard curves (R&D Systems, 2011a).

3.5.18.2 Elisa for Total MMP-2

Materials: See Appendix 8

ELISA Method

A 96 well plate with microplates that were pre-coated with an antibody specific for total MMP-2 was used for this assay. To each well, 50 µL of assay diluent RD1-116 was added. After this, 50 µL of standard, controls or samples were pipetted into the wells in duplicate and covered with the adhesive strip provided. This mixture was incubated for 2 hours at room temperature on a horizontal microplate shaker set at 500 rpm. Each well was aspirated and washed for a total of four washes by adding 400 µL wash buffer using a squirt bottle. After the last wash, any remaining wash buffer was removed by aspiration or decanting followed by inverting and blotting the plate against clean paper towels. To each well was added

200 µL of Total MMP-2 conjugate, covered with an adhesive strip and incubated for two hours at room temperature on the shaker. The wash steps above were repeated, and then 200 µL of the substrate solution was added to each well and incubated for 30 minutes at room temperature on the benchtop, protected from light. Stop solution in the amount of 50 µL was added to each well. After the colour change was observed, the plate was transferred to a microplate reader and optical density determined at 450 nm, with a wavelength correction set at 540 nm (R&D Systems, 2013c).

3.5.18.3 Elisa for MMP-8

Materials: See Appendix 9

ELISA Method

A 96 well plate with microplates that were pre-coated with an antibody specific for MMP-8 was used for this assay. To each well, 150 µL of assay diluent was added. After this, 50 µL of standard, controls or samples were pipetted into the wells in duplicate and covered with the adhesive strip provided. This mixture was incubated for 2 hours at room temperature on a horizontal microplate shaker set at 500 rpm. Each well was aspirated and washed for a total of four washes by adding 400 µL wash buffer using a squirt bottle. After the last wash, any remaining wash buffer was removed by aspiration or decanting followed by inverting and blotting the plate against clean paper towels. To each well was added 200 µL of the MMP-8 conjugate, covered with an adhesive strip and incubated for two hours at room temperature on the shaker. The wash steps above were repeated, and then 200

µL of the substrate solution was added to each well and incubated for 30 minutes at room temperature on the benchtop, protected from light. Stop solution in the amount of 50 µL was added to each well. To ensure thorough mixing and uniform colour change, the plate was tapped gently. Plates were read using a microplate reader at 450 nm wavelength as described in section 3.5.18.1 above (R&D Systems, 2011b).

3.5.18.4 Elisa for MMP-9

Materials: See Appendix 10

ELISA Method

Using a 96 well plate with microplates pre-coated with antibody specific for MMP-9, 100 µL of assay diluent RD1-34 was added. After this, 100 µL of standard, controls or samples were pipetted into the wells in duplicate and covered with the adhesive strip provided. This mixture was incubated for 2 hours at room temperature on a horizontal microplate shaker set at 500 rpm. Each well was aspirated and washed for a total of four washes by adding 400 µL wash buffer using a squirt bottle. After the last wash, any remaining wash buffer was removed by aspiration or decanting followed by inverting and blotting the plate against clean paper towels. To each well was added 200 µL of the MMP-9 conjugate, covered with an adhesive strip and incubated for one hour at room temperature on the shaker. The wash steps above were repeated, and then 200 µL of the substrate solution was added to each well and incubated for 30 minutes at room temperature on the benchtop, protected from light. Stop solution in the amount of 50 µL was

added to each well. To ensure thorough mixing and uniform colour change, the plate was tapped gently and transferred to a microplate reader to determine the optical density at 450 nm, with wavelength correction set at 540 nm (R&D Systems, 2014).

3.6 Ethical Considerations

This study was approved by the University of Zambia Biomedical Research Ethics Committee (Reference no. 015-07-12). The participants were being recruited because they had a complaint or symptoms that required routine endoscopy. Ideally, in order to increase the external validity of the study, we would have loved to recruit participants whether or not they needed an endoscopy, but we refrained from doing so because subjecting participants to an unpleasant procedure such as endoscopy that they did not need merely to facilitate collection of samples for our study was deemed unwarranted. Therefore asking for biopsies after routine endoscopy was considered less invasive overall than asking for patients who didn't need an endoscopy to go through it merely for the study samples. Furthermore, the participants were requested to submit biopsy samples after it was determined that their endoscopy was normal. To ensure participant autonomy, this request for biopsies and other samples was fully explained to the participants during the consenting process and was included in the information sheet. The participants provided written informed consent before participating in the study. Privacy of the participants was respected by ensuring that interviews took place in a private environment. There was strict adherence to the recruitment criteria in accordance with the principle of justice and fair selection of participants (Beauchamp, 2008).

Participants who wanted to know their HIV status were counselled according to standard national guidelines before results were shared with them. Data collected from the participants was de-identified and entered into a password-protected computer whose access was restricted only to the principal investigator to ensure confidentiality.

CHAPTER FOUR: RESULTS

4.1 NTM Prevalence and associated factors.

In this part of the study, the aim was to understand the prevalence of nontuberculous mycobacteria among colonoscopy patients, to determine the risk factors for carriage of NTMs and MTB and to compare the yields from stool microscopy, colonic biopsy, gut lavage samples and culture for mycobacteria. The patients were recruited over a period of one year, starting in October 2012.

4.1.1 Patient characteristics

We recruited 97 patients from among those who had been booked for routine endoscopic procedures. Out of these, 45 (46.4%) of the patients recruited were female, and 52 (53.6%) were male, with overall mean age being 46.6 years (±15.9). The age distribution by sex was similar in both females and males (mean 49.1±16.7 versus 44.4± 14.9 years, p = 0.15) respectively. Just over half of the participants came from low-cost or high-density residential areas. The refusal rate was 4%. The most common indication for colonoscopy was abdominal pain (60.8%, 95% CI 50.4-70.6), followed by passing blood in stool (39.2%, 95% CI= 29.4-49.6), and diarrhoea (23.7%, 95% CI 15.6-33.4) as shown in Table 4-1. The mean haemoglobin was 11.9 g/dl (±3.0), whereas the overall HIV positivity status was 19.3 % (95% CI 10.5-26.6). There were no significant differences between males and females in terms of presenting symptoms, HIV status and mean haemoglobin levels (Chongwe et al., 2017), as shown in Table 4-1.

Table 4-1 Demographic characteristics and presenting symptoms of study participants, stratified by gender

	Overall	**Female** n (%)	**Male** n (%)	**P***
Gender [n (%)]	97	45 (46.4)	52 (53.6)	0.54
Age in years [Median (IQR)]	43 (34, 59)	48 (36, 63)	40.4 (33, 54)	0.15
Area of residence [n (%)]				0.22
Low cost	49 (50.5)	22 (48.9)	27 (51.9)	
Medium cost	27 (27.8)	10 (22.2)	17 (32.7)	
High cost	21 (21.7)	13 (28.9)	8 (15.4)	
Occupation status				0.01
Unemployed	52 (53.6)	31 (68.9)	21 (40.4)	
Employed	45 (46.4)	14 (31.1)	31 (59.6)	
Presenting symptoms				
Abdominal pain				0.88
Yes	59 (60.8)	27 (60.0)	32 (61.5)	
No	23 (39.2)	18 (40.0)	20 (38.5)	
Diarrhoea				0.80
Yes	23 (23.7)	10 (22.2)	13 (25.0)	
No	74 (76.3)	35 (77.8)	39 (75.0)	
Vomiting				0.39
Yes	16 (16.5)	9 (20.0)	7 (13.5)	
No	81 (83.5)	36 (80.0)	45 (86.5)	
Weight loss				0.08
Yes	18 (18.6)	5 (11.1)	13 (25.0)	
No	79 (81.4)	40 (88.9)	39 (75.0)	
Fever				0.72
Yes	8 (8.2)	3 (6.7)	5 (9.6)	
No	87 (91.8)	42 (93.3)	47 (90.4)	
HIV				0.95
Yes	17 (19.3)	8 (19.1)	9 (19.6)	
No	71 (80.7)	34 (80.9)	37 (80.4)	
Anaemia				0.98
Yes	44 (51.2)	20 (51.3)	24(51.1)	
No	42 (48.8)	19 (48.7)	23 (48.9)	

* Except for the age, where a T-test was used, the p-value was derived with the chi-square test, including Fisher's exact test where appropriate. IQR = Interquartile range

4.1.2 Carriage rates (prevalence) and diagnostic yield

Mycobacteria species were isolated in 13 participants (13.4%, 95% CI 6.5-20.3) out of which NTMs were isolated in 7 (7.2%, 95% CI 3.4, 14.5) of the participants and *Mycobacterium tuberculosis* was isolated in six participants (6.2%, 95% CI 2.3-13.0). There were no differences in carriage rates between males and females. Descending colon samples were the most likely to be positive (9.8%, 95% CI 3.7, 15.8) followed by stool samples (6.8%, 95% CI 1.0-12.6), caecal biopsy (6.1%, 95% CI 0.3-11.8) and intestinal lavage samples (5.9 %, 95% CI 0.3-11.5) as shown in Table 4-2. The contamination rate was 17.6% (Chongwe et al., 2017).

Table 4-2 Diagnostic yield for Mycobacterial spp. from abdominal specimens among endoscopy patients attending endoscopy clinic at University Teaching Hospital in Zambia

Specimen	Total number isolated (%)	Species isolated	Diagnostic yield (%)
Descending colon biopsy	9	4 MTB 2 *Mycobacterium gordonae* *2 Mycobacterium kansasii* *1 Mycobacterium genavense*	9.8 (95% CI 3.7-15.8)
Stool	5	3 MTB 2 *Mycobacterium gordonae*	6.8 (95% CI 1.0-12.6)
Caecal biopsy	4	1 MTB 1 *Mycobacterium kansasii* *2 Mycobacterium gordonae*	6.1 (95% CI 0.3-11.8)
Intestinal lavage	3	2 MTB 1 *Mycobacterium gordonae*	5.9 (95% CI 0.3-11.5)

Notes: 1. Sample size for the participants was n=97
2. Sampling was random among consenting respondents

Overall, six (6.2%) of the participants had *M. tuberculosis*, four (4.1%) participants had *M. gordonae*, two (2.1%) had *M. kansasii* while *M. genavense* was found in only one participant (1%). No participant was found with more than one species of mycobacteria at a time. However, in three participants (4.3%, n=69) we isolated mycobacteria in both stool and descending colon biopsy. Three other patients also produced mycobacterium isolates from both caecal biopsies and descending biopsy specimens (4.3%, n=69). In one patient, MTB was isolated in all the four samples. Through multivariable logistic regression, it was found that intestinal carriage of mycobacteria was not associated with age, sex or presenting symptoms such as diarrhoea, abdominal pain, weight loss, rectal bleeding, anaemia as well as HIV status (Chongwe et al., 2017) as shown in Table 4-3.

Table 4-3 Relationship between carriage of Nontuberculous mycobacteria and Mycobacterium tuberculosis and characteristics of endoscopy patients at the University Teaching Hospital, Lusaka

		Nontuberculous Mycobacteria				Mycobacterium tuberculosis			
Variable	N=97	Crude OR* OR (95% CI)	P-value	Adjusted OR OR	P-value	Crude OR* OR (95% CI)	P-value	Adjusted OR OR	P-value
Sex									
Female	45	1		1		1		1	
Male	52	0.63 (0.13-3.00)	0.55	0.28 (0.04-1.94)	0.20	1.79 (0.31-10.40)	0.98	0.34 (0.03-3.89)	0.38
Age									
Below 45 years	50	1		1		1		1	
Above 45 years	47	0.16 (0.02-1.45)	0.06	0.22 (0.02-2.08)	0.26	5.11 (0.55-47.4)	0.11	-	-
Occupational Status									
Unemployed	52	1		1		1		1	
Employed	45	3.12 (0.56-17.41)	0.17	3.64 (0.53-24.99)	0.37	2.44 (0.42-14.26)	0.31	7.05 (0.31-162.40)	0.22
Weight loss									
No	79	1		1		1		1	
Yes	18	0.72 (0.08-6.42)	0.76	1.67 (0.14-20.38)	0.69	0.87 (0.09-8.03)	0.90	-	
Anaemia									
No	42	1		1		1		1	
Yes	44	6.47 (0.70-59.77)	0.06	5.75 (0.54-61.01)	0.15	1.17 (0.01-1.62)	0.08	0.08 (0.01-1.20)	0.07
HIV									
No	80	1		1		1		1	
Yes	17	0.68 (0.07-6.11	0.73	1.35 (0.12-14.96)	0.81	1.42 (0.14-14.74)	0.77	4.84 (0.22-04.87)	0.31

4.2 Expression of cytokines in the gut and peripheral blood

This constituted the second phase of the study and was concerned with understanding the gut immune responses to nontuberculous mycobacteria among the patients.

4.2.1 Patient characteristics

We recruited 48 patients, 54.2% of whom were female. The median age overall was 35 years old (IQR 27.5, 50.5) while that for females was 39.5 years (IQR 27, 53) and 34 years (IQR 28, 40) for males. Seven (15.6%) of the participants were HIV positive. Most of the participants (77.4%) had evidence of previous BCG vaccination, while 17.0% had a previous history of TB. The most common presenting symptom was abdominal pain in 39 patients (81%), out of which 54% were female and 46% male. Other common presenting symptoms were vomiting (16.7%), diarrhoea (14.6%) and cough (6.2%) as shown in Table 4-4.

Table 4-4 Characteristics of participants recruited for the in-vitro study to determine the expression of cytokines and MMPs in duodenal samples from 48 individuals

Variable		Overall n(%)	Female n(%)	Male n(%)
Sex		48	26 (54.2)	22 (45.8)
Age [M(SD)]*		38.8 (15.5)*	41.2 (17.0)*	36.0 (13.3)*
BCG scar				
	Yes	24 (77.4)	10 (71.4)	14 (82.4)
	No		4 (28.6)	3 (17.6)
History of TB				
	Yes	8 (16.7)	4 (15.4)	4 (18.2)
	No		22 (84.6)	18 (81.8)
HIV status				
	Yes	8 (16.7)	6 (23.1)	2 (9.10)
	No		20 (76.9)	20 (90.9)
Presenting Symptoms				
Abdominal pain				
	Yes	39 (81.2)	21 (80.8)	18 (81.8)
	No	9 (18.8)	5 (19.2)	4 (18.2)
Vomiting				
	Yes	8 (16.7)	5 (19.2)	3 (13.6)
	No	40 (83.3)	21 (80.8)	19 (86.4)
Diarrhoea				
	Yes	7 (14.6)	3 (11.5)	4 (18.2)
	No	41 (85.4)	23 (18.5)	18 (81.8)
Night sweats				
	Yes	4 (8.30)	1 (3.80)	3 (13.6)
	No	44 (91.7)	25 (96.2)	19 (86.4)
Cough				
	Yes	3 (6.20)	2 (7.70)	1 (4.40)
	No	45 (93.8)	24 (92.3)	21 (95.6)
Weight loss				
	Yes	2 (4.20)	0 (0.0)	2 (9.10)
	No	46 (95.8)	26 (100)	20 (90.9)
Fever				
	Yes	1 (2.10)	0 (0.0)	1 (4.60)
	No	47 (97.9)	26 (100)	21 (95.4)

***Notes:** Showing Mean and standard deviation. The rest of the figures are numbers with a percentage in parentheses.

4.2.2 *M. avium* induces IL-1β and IL-6 expression in duodenal biopsies

Interleukin 1β and IL-6 were significantly higher in supernatants from 48 pairs of duodenal biopsies cultured with *M. avium* than in controls (p = 0.002 and 0.04 respectively, Table 4-5). Although not significantly different (except IL-4), all stimulated duodenal samples expressed higher levels of cytokines than unstimulated samples. The expression of IL-6 in *M. avium* stimulated samples was higher in patients with a history of TB than those without a history of TB (p= 0.03 and 0.04, respectively, Figure 4-1). Intestinal tissue from HIV negative participants secreted more IL-6 but not other cytokines. Induction of IL-2, IL-6, IL-10 (not shown), IL-17A (Figure 4-2), and IFN-y was higher in participants with BCG vaccination, but only IL-6 was induced more in participants with no past history of TB compared with those with a history of TB (Figure 4-1). Stratifying the IL-1, IL4 and IL-12 by HIV status, being BCG vaccinated or having a history of TB did not alter the results in our patients. Similarly, the expression of the cytokines did not show any differences when stratified by age group and sex.

4.2.3 Standard curves for cytokines after flow cytometry

Using FCAP software on the FACSVERSE machine, we run standard curves for IL-17A, interferon gamma, tumour necrosis factor alpha, IL-10, IL-6, IL-4 and IL-2, based on the serial dilutions conducted during the experiment, as described in section 3.5.10. Standard curves were also run for ELISAs for MMP-1, -2, -8 and -9, as well as IL-1β and IL-12. The standard curves were used to interpolate

unknown values from the samples. The standard curves are shown in Appendix 11-23.

Table 4-5 Expression of cytokines in duodenal samples after stimulation with *Mycobacterium avium* lysate

Cytokine*	Stimulated with *M. avium* (pg/ml)[a]	Negative control (pg/ml) [a]	P value [b]
Interleukin 1β	23.60 (10.04, 83.02)	0.14 (0.00, 1.36)	**0.002**
Interleukin 2	2.18 (0.34, 4.32)	0.26 (0.00, 4.66)	0.25
Interleukin 4	0.18 (0.00, 0.70)	0.18 (0.00, 1.84)	0.31
Interleukin 6	77.08 (0.94, 408.48)	16.20 (0.00, 104.43)	**0.04**
Interleukin 10	0.08 (0.00, 0.49)	0.01 (0.00, 0.92)	0.36
Interleukin 12	7.84 (5.10, 15.82)	9.48 (4.29, 14.59)	0.97
Interleukin 17A	12.01 (2.68, 28.34)	1.15 (0.00, 19.59)	0.25
TNF - α	0.31 (0.00, 2.34)	0.24 (0.00, 2.71)	0.91
Interferon gamma	1.27 (0.28, 3.16)	0.23 (0.00, 3.39)	0.57

Notes: [a] All the figures are medians with interquartile range, in picograms per millilitre (pg/ml). [b] p-value calculated using the Wilcoxon signed-rank test. *Except for IL-1B and 12, for which ELISA was used, the other cytokines were quantified using flow cytometry via the cytometric bead array.

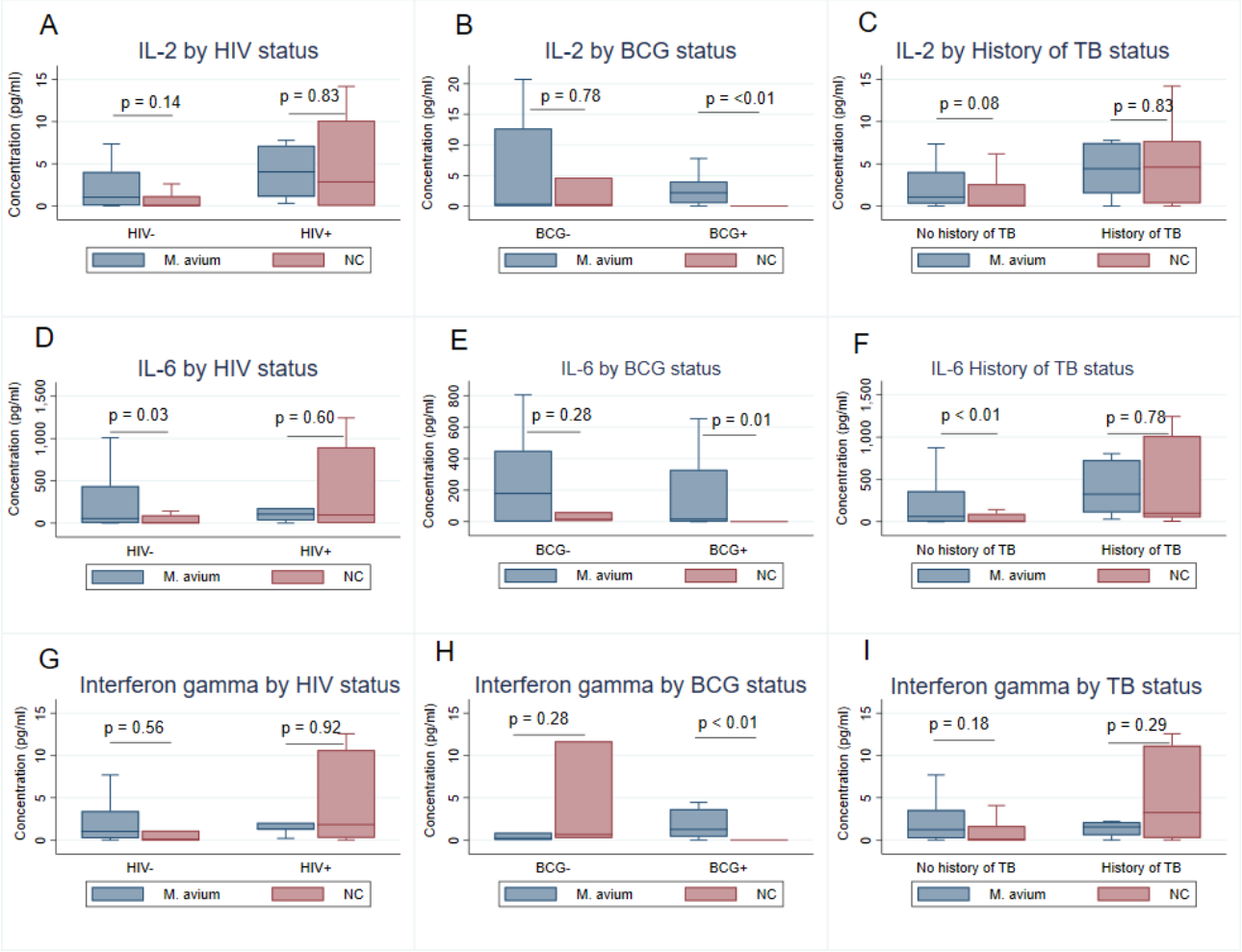


Figure 4-1 Secretion of inflammatory cytokines stratified by HIV, BCG and History of TB. Notes: p values comparing *M. avium* versus negative controls (NC) calculated using the Wilcoxon signed rank test.

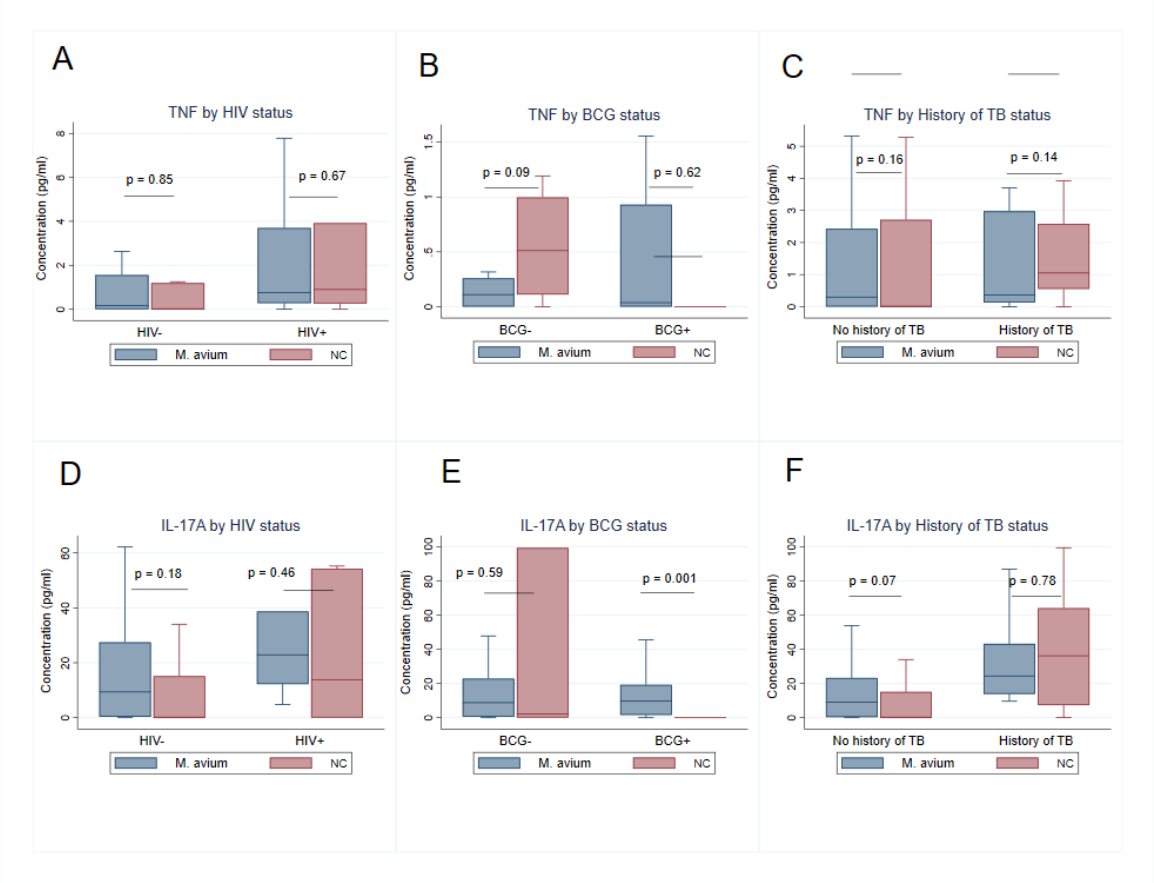


Figure 4-2 Secretion of tumour necrosis factor and interleukin 17A stratified by HIV, BCG and history of TB status. Notes: p values comparing *M. avium* versus negative controls (NC) calculated using the Wilcoxon signed rank test.

In multivariable regression analysis for duodenal samples (Table 4-6), the results showed that *M. avium*-stimulated duodenal samples expressed 156 picograms of IL-6 more than unstimulated samples (95% CI 7.6, 304; p = 0.04) after adjusting for the effect of history of TB, previous BCG vaccination, age and sex. We did not

detect any differences in expression of IL-17A, IL-10, IL-4, IL-2, IFN-γ and TNF-α in the gut between stimulated samples and unstimulated samples.

Table 4-6 Panel linear regression analysis showing the effect of *M. avium* stimulation on cytokine expression in duodenal tissue

Cytokine	Unadjusted			Adjusted		
	Mean cytokine expression (SD) [pg/ml]	Mean difference (95%CI)	p value*	Mean difference (95%CI)	p value*	Intraclass Correlation coefficient (rho)
IL-17A						
Not stimulated	19.3 (30.2)	1	0.14	1	0.07	-
Stimulated with *M. avium*	20.5 (24.5)	1.7 (-8.6 12.0)		11.8 (-1.0 24.7)[1]		
IL-10						
Not stimulated	0.60 (1.01)	1	0.64	1	0.52	0.33
Stimulated with *M. avium*	0.57 (1.24)	0.09 (-0.27, 0.44)		0.08 (-0.17, 0.35)[2]		
IL-6						
Not stimulated	202.1 (365.5)	1	0.36	1	**0.04**	0.42
Stimulated with *M. avium*	271.8 (440.1)	74.6 (-84.0 233.5)		155.8 (7.64, 304.0)[3]		
IL-4						
Not stimulated	1.31 (2.43)	1	0.46	1	0.60	0.02
Stimulated with *M. avium*	0.93 (1.70)	-0.26 (-0.93, 0.42)		-0.06 (-0.16, 0.28)[4]		
IL-2						
Not stimulated	3.46 (7.87)	1	0.82	1	0.36	0.75
Stimulated with *M. avium*	6.46 (23.6)	0.28 (-2.16, 2.71)		3.84 (-4.47, 12.2)[5]		
IFNG						
Not stimulated	3.52 (8.33)	1	0.74	1	0.45	0.64
Stimulated with *M. avium*	3.68 (9.02)	-0.41 (-2.80, 1.99)		1.41 (-2.23, 5006)[6]		
TNF						
Not stimulated	4.03 (10.9)	1	0.40	1	0.97	0.74
Stimulated with *M. avium*	2.53 (5.61)	-1.32 (-4.43, 1.79)		-0.01 (-0.39, 0.41)[7]		

Notes: * p-value was calculated using panel random effects linear regression with the maximum likelihood estimation. Each row represents a multiple linear regression model, with each cytokine as an outcome variable. 1: Adjusted for BCG vaccination, age and history of TB; 2: adjusted for sex, age, history of TB, HIV and previous BCG vaccination; 3 adjusted

for sex, age, history of TB and previous BCG vaccination; 4 adjusted for sex, age, history of TB, HIV and previous BCG vaccination; 5 Adjusted for sex, age, history of TB, previous BCG vaccination; 6 adjusted for sex, age and BCG vaccination; 7 adjusted for sex and BCG vaccination.

4.2.4 *M. avium* induces the secretion of Th1 and Th2 response but not Th17 response in whole blood

Results from the whole blood samples showed that *M. avium* induced the secretion of much higher levels of cytokines than those produced by the duodenal cells. Unlike in the gut, *M. avium* significantly increased whole blood secretion of the regulatory cytokine IL-10 ($p < 0.001$, panel B) compared to unstimulated samples. The pro-inflammatory cytokines IL-2, IL-6, TNF-α and IFN-γ were all significantly higher in stimulated samples compared to unstimulated samples as shown in Figure 4-3. Multiple linear regression (Table 4-7) showed that *M. avium* induced the secretion of a broader range of Th1 and Th2 cytokines but not Th17 cytokines in whole blood in this experiment.

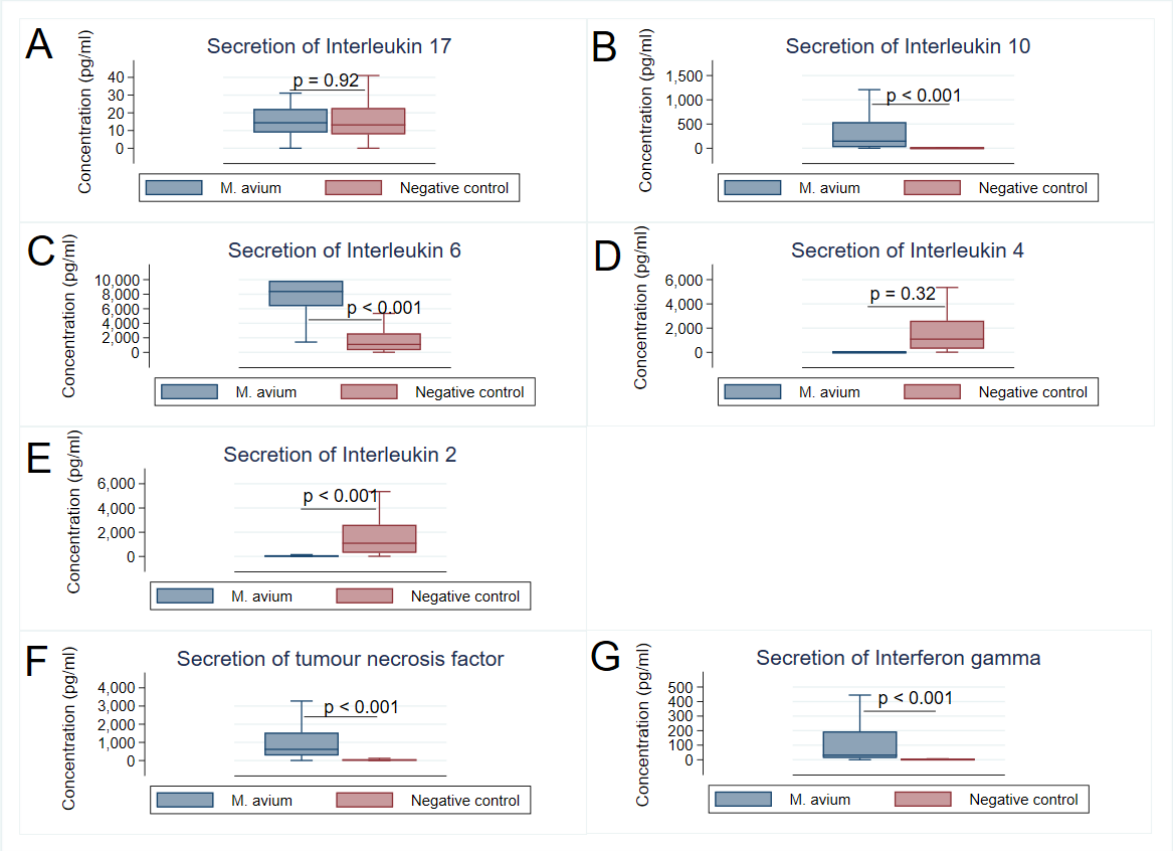


Figure 4-3 Secretion of cytokines in whole blood after stimulation with Mycobacterium avium complex. Notes: p values comparing *M. avium* versus negative controls (NC) calculated using the Wilcoxon matched pairs signed rank test

Table 4-7 Panel linear regression analysis showing the effect of M. avium stimulation on cytokine expression in whole blood

Cytokine	Unadjusted			Adjusted		
	Mean **cytokine** expression (SD) [pg/ml]	**Mean difference (95%CI)**	**p value§**	**Mean difference (95%CI)**	**P value§**	**ICC**
IL-17A						
Not stimulated	17.3 (14.4)	1	0.63	1	0.91	0.002
Stimulated with *M. avium*	19.4 (24.2)	2.19 (-6.76, 11.2)		0.52 (-8.60, 9.64)[1]		
IL-10						
Not stimulated	9.59 (17.5)	1	**<0.01**	1	**<0.01**	0.00
Stimulated with *M. avium*	434.2 (641.0)	422.3 (203.1, 641.6)		432.3 (213.2, 651.4)[2]		
IL-6						
Not stimulated	2028.6 (2666.8)	1	**<0.01**	1	**<0.01**	0.19
Stimulated with *M. avium*	7511.3 (2513.6)	5447.2 (4370.8, 6523.6)		5423.3 (4315.8, 6530.7)[3]		
IL-4						
Not stimulated	0.43 (1.22)	1	**0.05**	1	**0.04**	0.00
Stimulated with *M. avium*	1.58 (3.59)	1.17 (-0.01, 2.34)		1.27 (0.07, 2.46)[4]		
IL-2						
Not stimulated	11.14 (51.5)	1	**0.03**	1	**0.02**	0.00
Stimulated with *M. avium*	271.88 (669.9)	260.7 (31.4, 490.0)		277.0 (54.2, 500.0)[5]		
IFNG						
Not stimulated	5.26 (17.87)	1	**<0.01**	1	**<0.01**	0.00

Stimulated with *M. avium*	241.9 (424.3)	236.6 (91.6, 381.5)		216.5 (82.6, 350.3)[6]		
TNF						
Not stimulated	196.9 (594.6)	1	**<0.01**	1	**<0.01**	0.42
Stimulated with *M. avium*	1366.9 (1673.8)	1098.2 (663.9, 1532.4)		1083.2 (634.1, 1532.3)[7]		

Notes: **§** p-value was calculated using panel random effects linear regression with the maximum likelihood estimation. Each row represents a multiple linear regression model, with each cytokine as an outcome variable. 1: Adjusted for history of TB and sex; 2: adjusted for sex, age, history of TB, HIV and previous BCG vaccination; 3 history of TB; 4 adjusted for sex, history of TB and previous BCG vaccination; 5 Adjusted for sex and history of TB; 6 adjusted for sex, and History of TB; 7 adjusted for sex, age, history of TB, HIV and previous BCG vaccination.

4.2.5 *M. avium* induces the expression of Matrix Metalloproteinase-1 in duodenal tissues and whole blood

From among the 48 participants, we took a random sub-sample of 13 participants, five males and eight females. The mean age (standard deviation) for this group was 32.6 (8.3) years, with males having a mean age of 37 (11.1) years and females with 41.6 (20.5) years.

The expression of MMP-1 in supernatants from duodenal tissue stimulated with *M. avium* was increased compared to the negative control (p = 0.004). Similarly, it was found that *M. avium* induced the expression of more MMP-1 than the negative controls (p = 0.004) in whole blood. Furthermore, when the expression in the intestine was compared to that of whole blood, the difference was found to be unremarkable (p = 0.063) (Figure 4-4). Expression of MMP-2, -8 and -9 in *M. avium* stimulated tissues was not significantly higher than in negative controls as shown in Table 4-8.

Table 4-8 Expression of MMPs in duodenal tissue in response to *M. avium* stimulation.

Cytokine	Stimulated with *M. avium* (ng/ml)[a]	Negative control (ng/ml)[a]	p value[b]
MMP-1	0.18 (0.14, 2.28)	0.08 (0.05, 0.10)	0.004
MMP-8	0.23 (0.00, 0.49)	0.00 (0.00, 0.23)	0.060
MMP 2	0.36 (0.07, 0.85)	0.23 (0.02, 0.26)	0.124
MMP-9	0.98 (0.00, 2.25)	0.56 (0.00, 1.38)	0.498

Notes: [a] All the figures are medians with interquartile range, in nanograms per millilitre (ng/ml) [b] p-value calculated using the Wilcoxon signed-rank test.

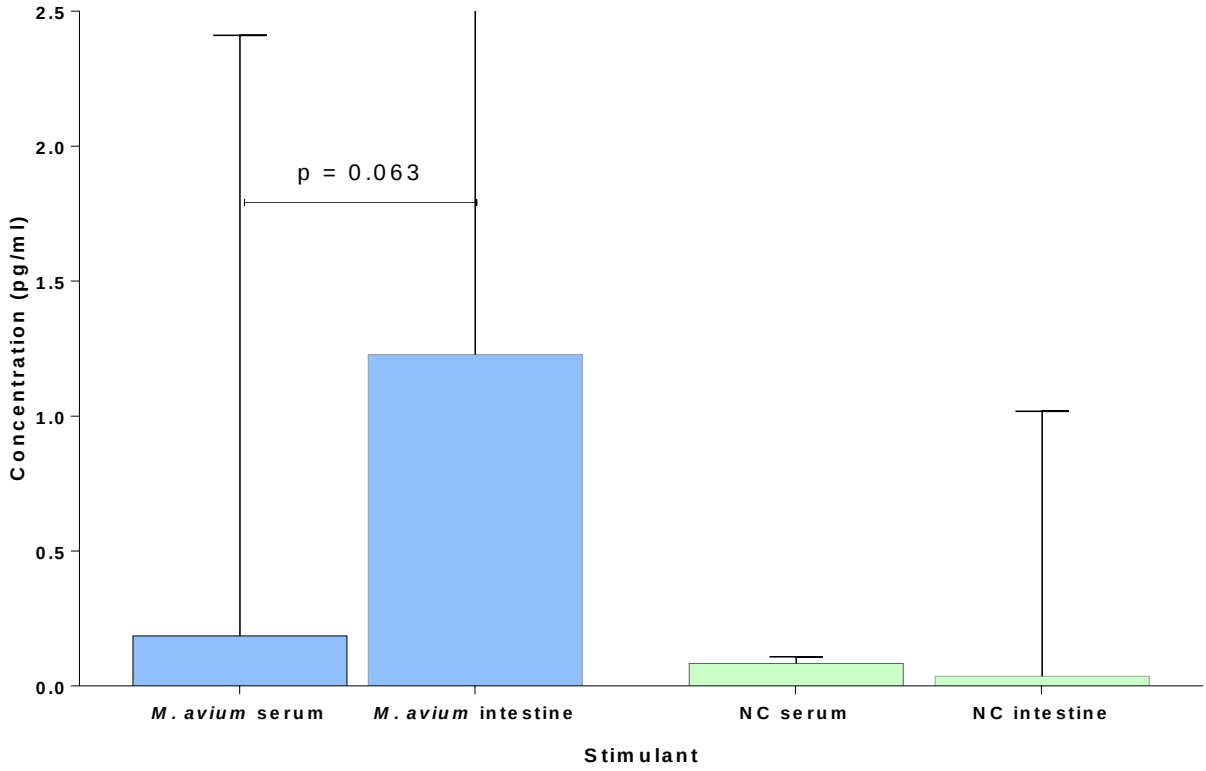


Figure 4-4 Expression of MMP-1 in *M. avium*-stimulated serum and duodenal samples.

Notes: NC = Negative Control.

We stratified the expression of all the MMPs against HIV status, having a BCG scar, gender and age group as shown in Figure 4-5. *M avium* did not induce secretion of MMP-1 among HIV positive patients (p = 0.18, panel A), but induced secretion among those who were HIV negative (p = 0.003). Similarly, those without previous BCG vaccination (panel B) showed no difference in expression (p = 0.18), while those with BCG vaccination showed a difference in expression of MMP-1 (p = 0.003). There were also differences in the expression of MMP-1 in males,

females and those aged below and above 35 years. However, stratified analysis for MMP-2, -8 and -9 did not show any differences.

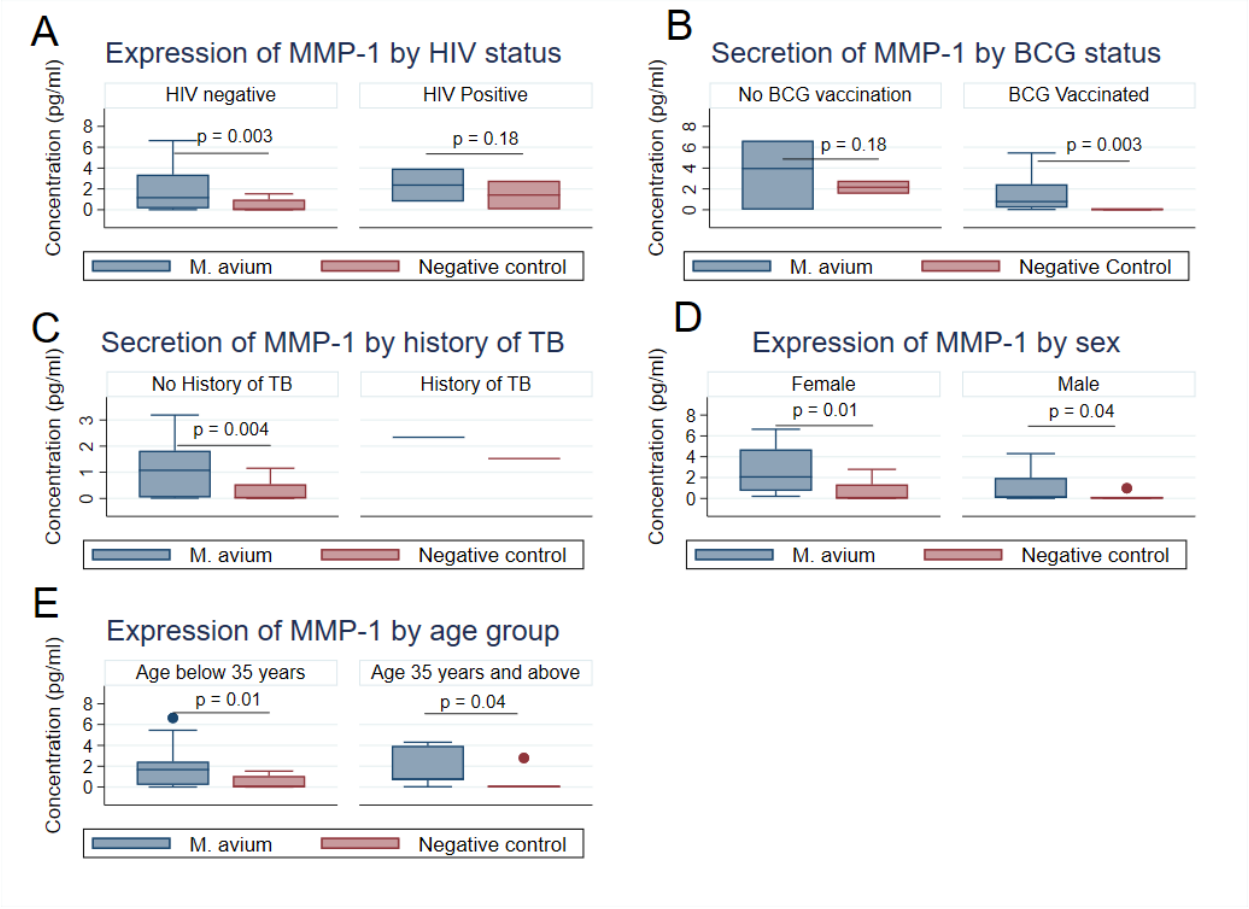


Figure 4-5 Stratified analysis of MMP-1 against HIV, BCG vaccination status, sex and age, n = 13

4.3 Correlation analysis of cytokines and MMPs

Using Spearman's correlation analysis, it was found that duodenal IL-17A expression was correlated with that of IL-12 ($\rho = 0.68$, $p = 0.01$), IL-10 ($\rho = 0.73$, $p < 0.001$), IL-4 ($\rho = 0.62$, $p < 0.001$) and IL-2 ($\rho = 0.58$, $p < 0.001$) . The expression of IL-12 was correlated with the expression of MMP-8 ($\rho = 0.60$, $p = 0.03$).

Interleukin 10 expression was correlated with the expression of IL-4 ($\rho = 0.83$, $p < 0.001$) and that of IL-2 ($\rho = 0.35$, $p = 0.01$). Interleukin 6 was found to be correlated with MMP-1 ($\rho = 0.55$, $p = 0.04$) and MMP-8 ($\rho = 0.62$, $p = 0.02$). MMP-1 and MMP-8 expression were found to be correlated ($\rho = 0.76$, $p = 0.046$), but this was not statistically significant. MMP-9 and MMP-8 ($\rho = 0.59$, $p = 0.03$) were correlated. This is shown in Table 4-9.

4.4 Examination of biopsy samples for signs of enteropathy

In order to demonstrate the link between duodenal immune expression and enteropathic changes, biopsy samples were kept for histological examination. The aim was characterise the degree of enteropathy and to link it to the immune responses that were shown after the stimulation of the tissue samples. Unfortunately, this was unsuccessful as the samples were not in a good enough state for further analysis and appeared to have been corrupted during storage. We were therefore unable to directly link the immune changes that were found with level of enteropathy in our participants.

4.5 Cell viability results

In order to ensure that the results of the cytokine expression were not due to cellular stress and death, we conducted a cytotoxicity assay using the MTT kit (Sigma Aldrich, Dorset, UK). The test is based on (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) or MTT and measures the activity of living cells via mitochondrial dehydrogenases. The results (Figure 4-6) showed that tissues treated with *M. avium*, SEB or those that were not treated showed no difference in

their cleavage of MTT, suggesting that the cell viability was similar in all the biopsy samples.

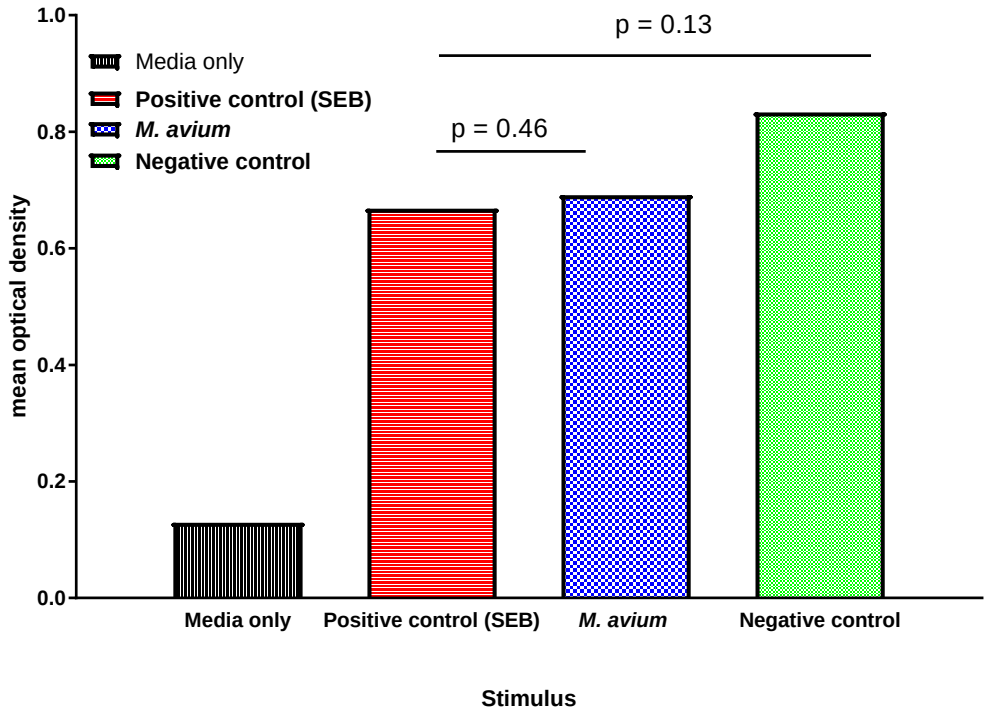


Figure 4-6 MTT cytotoxicity assay for gut tissue treated with Mycobacterium avium complex and Staphylococcus enterotoxin B antigen.

Notes: SEB = Staphylococcus Enterotoxin B.

Table 4-9 Spearman's Correlation matrix showing cytokines expressed in the intestines after stimulation with *Mycobacterium avium*

	IL-17A											
IL-17A	1	**IL-12**										
IL-12	0.68	1	**IL-10**									
IL-10	0.73	0.21	1	**IL-6**								
IL-6	0.27	-0.03	0.06	1	**IL-4**							
IL-4	0.62	0.35	0.83	-0.04	1	**IL-2**						
IL-2	0.58	0.42	0.35	0.26	0.04	1	**IL-1B**					
IL-1β	-0.25	-0.2	0.09	0.15	-0.1	0.02	1	**IFN-γ**				
IFN-γ	0.69	0.06	0.51	0.24	0.22	0.95	-0.16	1	**TNF-A**			
TNF-A	0.22	-	0.34	0.18	0.35	-0.02	-	0.09	1	**MMP-1**		
MMP-1	0.22	0.16	0.09	0.55	-0.26	0.04	0.27	0	-0.3	1	**MMP-2**	
MMP-2	-0.08	-0.03	-0.24	0.17	0.02	0.04	0.23	-0.1	-	0.72	1	**MMP-8**
MMP-8	0.56	0.6	-0.02	0.2	0.62	0.52	0.23	0.12	-	0.76	0.38	1
MMP-9	0.29	0.32	-0.01	0.48	0.28	0.53	0.24	0.23	-	0.72	0.42	0.59

Note: All numbers represent Spearman's correlation coefficient (Rho).

Key

P < 0.001	P = 0.01	P = 0.02	P = 0.03	P = 0.04	P ≥ 0.05

4.6 Summary of Results

This study has found that 7.2% (95% CI 1.9–12.4) of patients attending the endoscopy clinic carry nontuberculous mycobacteria, while 6.2% (95% CI 2.3–13.0) of them were found to have *Mycobacterium tuberculosis*. Carriage of mycobacteria was not associated with presenting symptoms. No *Mycobacterium avium* species were isolated. Further, we found that duodenal mucosal tissue stimulated with a lysate of *M. avium* expressed MMP-1, IL-1β and IL-6, compared to unstimulated tissues, p = 0.004, p = 002 and p = 0.04 respectively. It was also found that *M. avium* stimulates a wider range of cytokines in blood than in duodenal tissue, with IL-10, Il-6, IL-4, IL-2, TNF-α and IFN-gamma all significantly higher in stimulated whole blood compared to unstimulated blood (see Table 4-5 and Figure 4-3).

Cytokine expression was found to be correlated with expression of MMPs, as shown by IL-6 which was found to be correlated with MMP-1 ($\rho = 0.55$, $p = 0.04$) and MMP-8 ($\rho = 0.62$, $p = 0.02$). The expression of IL-12 was correlated with the expression of MMP-8 ($\rho = 0.60$, $p = 0.03$). Interleukin 10 expression was correlated with the expression of IL-4 ($\rho = 0.83$, $p < 0.001$) and that of IL-2 ($\rho = 0.35$, $p = 0.01$). MMP-1 and MMP-8 expression were found to be correlated ($\rho = 0.76$, $p = 0.049$), though this did not reach statistical significance. MMP-9 and MMP-8 were also correlated ($\rho = 0.59$, $p = 0.03$).

CHAPTER FIVE: DISCUSSION

5.1 Background

We set out to demonstrate the prevalence of non-tuberculous mycobacteria in intestinal specimens and in addition, evaluated the risk factors for carriage of intestinal mycobacteria. We then carried out an *in-vitro* study to test the hypothesis that NTMs could contribute to environmental enteropathy in routine endoscopy patients in Lusaka, Zambia, by measuring the expression of cytokines and matrix metalloproteinases secreted in response to NTM stimulation of small intestinal biopsies. In this chapter, we aim to summarise the main findings and interrogate the dynamics associated with the relationship between NTMs and enteropathy. We will first discuss the burden of NTMs in our patients and examine potential factors that could explain their distribution. Thereafter we will examine the core biological determinants (in the form of cytokines and matrix metalloproteinases) and other risk factors for the burden with a view to deciphering potential prognostic and diagnostic considerations. This chapter will end by examining potential limitations for the study.

5.2 Core findings

We found evidence that carriage of NTMs among patients booked for a routine colonoscopy and flexible sigmoidoscopy at UTH is quite common, but this was not associated with specific abdominal symptoms. The study also showed that descending colon biopsies gave the highest yield for detection of NTMs. Given the known biological characteristics of these organisms, this suggests environmental contamination. The *in vitro* experimental results revealed that a lysate of *Mycobacterium avium* complex bacteria induced the expression MMP-1, IL-1β and lL-6 in duodenal biopsies of healthy

patients undergoing endoscopy, i.e. patients with functional dyspepsia. Although we did not directly demonstrate a link with enteropathy because histological samples were damaged as explained in section 4.4, these findings suggest that under specific conditions, *M. avium* complex could, through the induction of MMP-1 lead to the kind of morphological changes seen in environmental enteropathy and is, therefore, a candidate for the link between environment and enteropathy. In the next section, we discuss the burden of NTMs, factors associated with intestinal carriage and diagnostic yield.

5.3 NTM Burden

The NTM burden observed in abdominal samples in this study was more than twice the prevalence that was previously found in the stools of patients with chronic diarrhoea (Kelly et al., 1996) in the same hospital. The reasons for this difference could be due to a number of factors. Firstly, it is plausible that the burden has increased given the increasing burden of opportunistic infections prevalent in HIV endemic areas such as this one. The association with HIV examined does not support this linkage, suggesting that if the burden has increased, it is not due to HIV infection. This leads to a second hypothesis. There has been the emergence of the liquid media detection methods such as the mycobacterium growth indicator tube (MGIT) system in the intervening period. Such improved diagnostic approaches could largely explain the increased burden. Notwithstanding the various reasons we could advance to explain the possible reasons for changing burden levels, the remaining fact that is there is a burden of NTMs in this population which may even be an underestimate. This is because in contrast to the findings in this study, much higher proportions of NTMs have been isolated from other Zambian patient populations using sputum samples, where the prevalence of NTMs was as high as 56% in a survey of

hospital patients in the northern and eastern parts of Zambia (Buijtels et al., 2010, Buijtels et al., 2009). A nationwide study, done as part of the first national TB prevalence survey, estimated the prevalence of NTMs among 15-49-year-old volunteers to be 15.1 (Chanda-Kapata et al., 2015). This may be an indicator of a bigger burden of NTMs in this population, suggesting the need for further vigilance and increased index of suspicion to look out for disease-causing NTMs in this population. However, given the known biological characteristics of these microorganisms, their presence in this population also suggests an environmental contamination.

Nontuberculous mycobacteria have been known to cause a number of diseases, including skin and soft-tissue diseases, pulmonary diseases, lymphadenitis, keratitis, and abdominal diseases including peritonitis (Griffith et al., 2007). Disease is more common in patients with impaired immunity such as those patients whose CD4 count has fallen to less than 50 cells/µL (Varley et al., 2017, Griffith et al., 2007). It is well known that these patients are more likely to suffer from disease caused by NTMs than the general population. However, carriage rates of NTMs were not associated with levels of haemoglobin and HIV status in our patients. The HIV prevalence among our patients was 19.3%, though most of the patients were generally healthy and without any debilitating conditions. Some of the patients were on antiretroviral drugs. According to the Zambia Demographic and Health Survey (ZDHS) (Central Statistical Office (Cso) [Zambia] et al., 2014) the HIV prevalence in the adult population was 13.3 % in 2014. In HIV uninfected patients, genetic defects in the interleukin-12/interferon-γ synthesis and response pathways, treatment with tumour necrosis factor-α inhibitors and structural lung disease from chronic obstructive pulmonary disease (COPD), cystic fibrosis and bronchiectasis

have been shown to increase the risk of NTM disease (Rosser et al., 2014, Sartor and Mazmanian, 2012, Griffith et al., 2007, Autschbach et al., 2005).

Although infrequent, NTMs are also considered an important cause of disease in solid organ transplant patients, mostly because the disease presents serious diagnostic and therapeutic difficulties for clinicians. The incidence of disease in transplant patients ranges from 0.16-0.55% in renal transplants, 0.24-2.8% in heart transplants and 0.46-8% in lung transplants (Kuhn et al., 2014, Rosser et al., 2014). Similarly, NTMs are a rare but important cause of disease among patients undergoing peritoneal dialysis (Pierce et al., 2011, Selby et al., 2007).

It was not surprising to find that the carriage of NTMs was unrelated to the patients' gender, age and employment status, given the ubiquitous nature of these organisms. However, previous studies elsewhere have shown that NTM disease is associated with being female and older than 50 years old, as well as having a slender body habitus, pectus excavatum and scoliosis (Mirsaeidi et al., 2014, Bodle et al., 2008, Autschbach et al., 2005). Having NTMs has also been associated with occupational exposure, including fishing, recreational water activities in spas and hot tubs, agricultural activities and exposure to metalworking fluid used in metal grinding in the automobile industry (Timms et al., 2016, Giuffrida et al., 2014, Falkinham, 2003). These results illustrate the abundant nature of NTMs, which are found in soil, dust and water systems (Falkinham, 2002). The results could be explained by the general environmental exposure that exists irrespective of the age, sex and other patient characteristics. In terms of occupation, it is plausible that very few people in this study may have been exposed to those occupational environments that confer an increased risk of developing the disease.

Nontuberculous mycobacteria have been isolated from a variety of sources, including hospital water supplies and diagnostic laboratories (Cooksey et al., 2008, Galassi et al., 2003, Chang et al., 2002). Tests for NTMs done on the hospital water systems from the laboratory and endoscopy unit during the period of this study were found to be negative. Given that the water samples and the clinical samples were processed in the same way, this suggests that the NTMs that were found in our patients might not have been due to contamination.

Although these organisms are frequently considered mere contaminants, it has been suggested that NTMs such as the *Mycobacterium avium* may be responsible for some non-specific abdominal symptoms such as anorexia, vomiting, weight loss, diarrhoea and abdominal pain (Giuffrida et al., 2014, Bhaijee et al., 2011). While abdominal pain was the most common presenting symptom in our patients, there was no relationship between the carriage of any NTMs and any of the presenting symptoms. While we did not isolate *Mycobacterium avium* complex (MAC) species in our patients, it is notable that most of these symptoms have been reported in patients with disseminated MAC disease, which occurs in patients with profound immune suppression.

Carriage of nontuberculous mycobacteria, as well as MTB, was more common in specimens from the descending colon than in intestinal lavage fluid, stool or even the caecal area, though this was not statistically significant. *Mycobacterium avium* is known to be the most frequently isolated NTMs from the gastrointestinal tract among immunocompromised patients (Huh et al., 2008). However, this was not the case in this study because *Mycobacterium gordonae* was the most frequently isolated species in our patients, followed by *M. Kansasii* and *M. genavense* (Chongwe et al., 2017). All the

isolated species were potentially pathogenic (Jarzembowski and Young, 2008). *Mycobacterium gordonae*, though considered a contaminant in many cases, is a low virulence organism known to cause disease in the lungs, peritoneal cavity, cornea and in soft tissue. Disseminated disease has also been reported in rare cases (Biancheri et al., 2013, Steck et al., 2012, Griffith et al., 2007). *M. kansasii* is a common cause of pulmonary and disseminated disease, while it is also known to rarely cause bone, skin and soft tissue infections. *M. genavense* is a rare cause of lymphadenitis and disseminated disease (Griffith et al., 2007), but it has also been reported to cause respiratory disease (Santos et al., 2014, Trehan et al., 2009).

Reports of increasing incidence of nontuberculous mycobacteria suggest the need for further attention to these organisms (Pedro et al., 2011, Henry et al., 2004). Due to the difficulty of differentiating NTMs from MTB in resource poor-settings, there is an obvious need to introduce mechanisms for hospital-based surveillance and as well as provide such as GenoType Mycobacterium (CM/AS) assay and other assays for laboratory diagnosis.

In addition to NTMs, some of our patients had *Mycobacterium tuberculosis* isolates in the intestinal samples. Stool has been reported to be a useful specimen for diagnosing pulmonary tuberculosis especially in HIV seropositive patients (Abaye et al., 2017, Oramasionwu et al., 2013) and in children (Moussa et al., 2016, Nicol et al., 2013) due to their inability to produce sputum specimens. In present study, there was no association between the isolation of MTB in the gut and the patient's HIV status, although the majority of the participants who had MTB in stool were HIV negative. Incidentally, one patient (who was not suspected to have TB at the time) was found with signs that were consistent with

abdominal tuberculosis in the ileocaecal area during endoscopy. His caecal biopsy was positive for *M. tuberculosis* on culture, and the patient was consequently treated for abdominal TB. Other patients with MTB isolates were evaluated for TB, although most of them did not show the typical symptoms of abdominal or pulmonary disease.

Having demonstrated the presence of NTMs in the intestines in our population (Chongwe et al., 2017), we wanted to investigate the hypothesis that NTMs, particularly *Mycobacterium avium* may cause some of the changes associated with environmental enteropathy through the activation of specific cytokines that are associated with the condition. We thus set out to conduct *in-vitro* experiments in which we exposed intestinal tissue and whole blood to a lysate of *M. avium* in order to elicit cytokine expression, as described in section 3.5.8. In the next section, we describe the expression of these cytokines in these tissues.

5.4 Cytokine expression.

In this part of the study, we showed that a lysate of *Mycobacterium avium* bacteria induced the expression MMP-1, IL-1β and IL-6 in duodenal biopsies of patients undergoing endoscopy. MMPs have been identified as the predominant proteases involved in the pathogenesis of inflammatory bowel disease, as well as other diseases that are characterized by destruction of the extracellular matrix such as rheumatoid arthritis, periodontal disease, celiac disease, COPD and tuberculosis (De Bruyn et al., 2016, Ciccocioppo et al., 2005, Quiding-Järbrink et al., 2001, Naito and Yoshikawa, 2005 , Elkington and Friedland, 2006). Similarly, patients with gluten-sensitive enteropathy have been shown to express more MMPs than normal controls (Mohamed et al., 2006).

Although we did not directly investigate a link between environmental enteropathy and MMPs in this study, these findings suggest a mechanism through which *M. avium*, as a consequence of its induction of MMP-1 (Giuffrida et al., 2014, Biancheri et al., 2013, Steck et al., 2012), could at least be partially responsible for some of the morphological changes seen in environmental enteropathy. Elkington and others have previously shown that MMP-1 plays an important role in driving the immunopathological process in *Mycobacterium tuberculosis* infections in the lung (Elkington et al., 2011, Elkington and Friedland, 2006, Elkington et al., 2005). Lamina propria mononuclear cells in the duodenum have been shown to express multiple MMPs after cytokine stimulation (Ciccocioppo et al., 2005).

Kelly and others have previously shown that environmental enteropathy is virtually ubiquitous in communities of low socio-economic status in Africa (Prendergast and Kelly, 2012, Kelly et al., 2004, Lindenbaum et al., 1972), so we postulate that non-tuberculous mycobacteria, which are also virtually ubiquitous, could contribute to intestinal mucosal remodeling. *M. avium* is just one of the NTMs which could be implicated, and further work is required to ascertain if the effects that are being reported in this thesis might be observed with other species. It is known that environmental enteropathy shows some seasonal variation (Kelly et al., 2004), but data in this study do not permit a seasonality analysis; greater numbers would be required for this.

We were unable to demonstrate the effect of *M. avium* on the expression of MMPs 2, 8 or 9 in duodenal tissue. However, other studies have shown that MMP-1,-3 and -9 are involved in the pathogenesis of gluten-sensitive enteropathy through tissue remodelling, while MMP-1 and 3 are highly expressed in chronic small and large intestinal ulcers.

(Mohamed et al., 2006, Saarialho-Kere, 1998). MMP-2 has previously been implicated in delayed healing of duodenal ulcers (Deng et al., 2011). In the lungs, it is well known that MMP-1 and MMP-9 are involved in the pathogenesis of TB. For MMP-9, this occurs through its effect on recruitment of macrophages and role in the formation of granulomas (Taylor et al., 2006) in addition to its effect on extracellular matrix.

We found evidence that *M. avium* induced the expression of IL-1β in intestinal tissue. IL-1 is a pro-inflammatory cytokine that is activated via a variety of microbial and non-microbial mechanisms including by *M. avium* (Elkington et al., 2011, Weber et al., 2010). IL-1β is produced by tissue macrophages, blood monocytes and dendritic cells and is known to be a potent stimulator of the extracellular tissue to produce MMPs including MMP-1, leading to tissue remodelling (Garlanda et al., 2013, Dinarello et al., 2012). It has been postulated that the tissue destruction seen in environmental enteropathy could be as a result of constant activation of T-cells caused by the presence of intestinal pathogens in the lumen (Korpe and Petri, 2012, Prendergast and Kelly, 2012). While IL-1β may lead to the production of MMP-1, it has been shown that MMP-1, in turn, is involved in the degradation of IL-1β (Ito et al., 1996).

This study showed that *M. avium*-stimulated duodenal tissue secreted IL-6 more than unstimulated controls. IL-6 is a potent pleiotropic inflammatory cytokine known to be involved in epithelial proliferation and wound repair (Kuhn et al., 2014), and has been shown to induce the expression of MMP-1, -2 and -9 (Kothari et al., 2014, Sengupta and Macdonald, 2007). Its expression is regulated by a number of inflammatory pathways including the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), the CCAAT [cytosine-cytosine-adenosine-adenosine-thymidine]/enhancer-binding protein

beta (C/EBPβ) and the activator protein 1 (AP-1). IL-6 activation takes place in macrophages, fibroblasts, monocytes, endothelial cells, lymphocytes and various cancer cells (Kishimoto et al., 1992, Naugler and Karin, 2008, Waldner and Neurath, 2014). It has been found to be involved in the pathogenesis of inflammatory bowel disease (Wang et al., 2003). In the present study, there was a strong correlation between IL-6 and MMP-1, suggesting that this could be one mechanism through which NTMs could lead to tissue remodelling in enteropathy. Despite years of searching, there is no conclusive evidence for a role of *Mycobacterium paratuberculosis* (MAP) in Crohn's disease (Selby et al., 2007).

This experiment did not demonstrate the production of IL-10 or IL-4 in duodenal samples, although we showed significant amounts of IL-10 but not IL-4 cytokines in blood (Figure 4-3). Gut microbiota have been implicated in the induction of regulatory B cells in the spleen and mesenteric lymph nodes through the production of IL-1β and IL-6. This inflammatory response, in turn, leads to the production of the anti-inflammatory IL-10 (Rosser et al., 2014). It is generally accepted that gut microbiota provide continuous antigenic stimulation that leads to activation of T-cells leading to intestinal injury (Sartor and Mazmanian, 2012). It is plausible that *M. avium*, which induced inflammatory cytokines but not anti-inflammatory cytokines in duodenal tissue, could contribute to this intestinal T-cell activation that could lead to enteropathy.

These findings have important implications on future strategies for not only diagnosing enteropathy but also for a surveillance system targeting the detection of these cytokines as biomarkers (Guerrant et al., 2016, Gilmartin and Petri, 2015). Strategies for

ameliorating the effects of environmental enteropathy could be directly anchored on managing the effects of specific cytokines through the use of anti-inflammatory agents.

5.5 Study limitations

The first part of the study was a hospital-based survey investigating the prevalence of NTMs among endoscopy patients; we cannot, therefore, generalise these findings to the population of Zambia as this study was restricted to a highly selected population scheduled for endoscopy. It may be argued that generalisability can only be guaranteed if such a study had been done in the general population in Lusaka. However, we posit that the great majority of our participants were generally healthy, apart from having sought endoscopy services for their abdominal symptoms. In addition, and in order to get appropriate diagnostic yield from internal abdominal tissue, one cannot escape this level of selection. Furthermore, it would not have been ethical to justify colonoscopy in unselected patients in the general population, simply for the purpose of obtaining biopsies. Notwithstanding these limitations, we suspect that these findings may even be an under-estimate of the burden of NTMs. This could have arisen because our methods did not include special incubation temperatures or additional nutritional supplements to culture more fastidious NTMs (Hillemann et al., 2006) from both the clinical and water samples. Furthermore, our decontamination procedures using N-Acetyl L-Cysteine – 6% NaOH (NALC-NaOH) may have been overly harsh for some samples with low levels of organisms (Ferroni et al., 2006). In addition, measuring the CD4 count would have helped to characterise the effect of HIV status on carriage of NTM, but this was not possible. Despite the foregoing, and in addition to a low non-participation rate of below five percent,

we consider it likely that this population has a measurable carriage rate of NTMs in the intestine.

The *in-vitro* part of this study also has potential limitations. Firstly, it is quite possible that the observed expression of cytokines and MMPs in response to stimulation by *M. avium* could have turned out to be different in a more complex *in-vitro* system. Secondly, not using different strengths of *M. avium* to elicit cytokine expression may have led to under-stimulation of duodenal tissue in some cases, leading to low or no expression of some cytokines, where some expression would normally be expected. Thirdly, it may be argued that cytokine secretion in our experimental model may take place even without stimulation simply because of tissue stress and/or death. However, as described in section 3.5.8, tissues from the same individual were subjected to different stimuli, namely nothing (as a negative control), SEB (as a positive control) and *M. avium* as the main experimental stimuli. This implies that any differences in the expression of cytokines between the negative control and *M. avium* stimulated tissues would be solely due to the *M. avium*. Furthermore, the results of the cytotoxicity assay done on the intestinal samples were within normal range. It is, therefore, reasonable to assume that the expression of the different cytokines seen in this study was due to the stimulation by *M. avium*.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Our results have demonstrated a prevalence of seven percent for NTMs in this population and suggests that there is environmental contamination of the gut by potentially pathogenic nontuberculous mycobacteria that was not associated with any symptoms or demographic status. However, this study did not isolate any *M. avium* species, contrary to findings elsewhere that have found this organism to be highly prevalent. We also found that descending colon biopsies gave the highest yield for detection of NTMs. The extent to which these organisms are responsible for some of the morbidity in our patients remains to be determined. Our observations suggest that in the past our diagnostic and consequently our treatment efforts have lacked this vital information, thereby contributing to misclassification of disease and the associated disease burden that has been reported over time. As the techniques for isolation of these organisms improve, coupled with a deeper understanding of the scope of disease caused by the organisms, the case can be made for an increase in the index of suspicion for NTMs disease during diagnostic procedures, as well as mechanisms to improve both surveillance and diagnostic capacity. This study also found that MTB was the most frequently isolated mycobacterium species from stool specimens, suggesting the need to consider abdominal samples for diagnosis of not only abdominal but pulmonary TB as well.

This study demonstrated that *M. avium* induced the expression of MMP-1 in duodenal tissue and in peripheral blood. *M. avium* also induced the expression of a restricted set of cytokines in duodenal tissue, namely IL-1β and IL-6 as well as eliciting a Th1 and Th2 response in the blood. We speculate that the induction of these cytokines by *M. avium*

suggests a possible pathway through which NTMs, and *M. avium* in particular, could remodel the intestinal mucosa and lead to environmental enteropathy. These outcomes give indirect support to the work that is currently going on to find biomarkers for detecting environmental enteropathy through non-invasive means. Further work will be required to demonstrate if MMP-mediated mucosal remodelling actually operates *in vivo*. These results also call for further investment in research in order to continually refine this evidence as a necessary element that has the potential to contribute to creating a healthy people and a healthy nation.

6.2 Recommendations

The findings presented in this thesis raise important public health policy and research related issues. Environmental enteropathy affects millions of people, especially children in the developing world and is linked to significant public health problems including stunting, malnutrition and poor oral vaccine efficacy (Prendergast and Kelly, 2012).

Implications for policy

Findings from this study suggest that gut microbiota such as *M. avium* may at least be partially responsible for triggering the immune response that is seen in the condition through the secretion of cytokines leading to tissue disruption. Therefore, public health interventions aimed at reducing environmental contamination by *M. avium* and other environmental mycobacteria may help in reducing this condition. Furthermore, hospital-based surveillance systems of water and other environmental sources may help to reduce contamination and infection of patients who are susceptible to disease.

Implications for research

The cytokines identified in this thesis may be used as markers of inflammation in the gut, and research to explore this for practice is recommended. Innovations to create cost-efficient diagnostic tools targeting biological markers are urgently needed. In addition, cohorts of patients with these conditions could be followed up to generate better information, examine and understand what kind of environments and contaminations could act as triggers of disease as well as conducting research to explore the anti-inflammatory agents and other drug products that can be used specifically to target these cytokines to reduce the morbidity seen in this condition. In addition, research on whether antimycobacterial treatment of individuals carrying NTMs and having environmental enteropathy can lead to reduction in enteropathic changes would be informative, though the duration of treatment and side effect profile of the drugs involved would render such work untenable.

Further areas of research include understanding how *in-vivo* MMP activity can actually lead to enteropathic changes, how the cytokine activation seen in enteropathy affects vaccine response, whether wealth status is associated with degree of enteropathic changes and whether different NTM species produce different immune responses in gut tissue.

REFERENCES

Abaye, G.E., Abebe, T., Worku, A., Tolessa, D., Ameni, G. & Mihret, A. 2017. Detection of Mycobacterium tuberculosis from the stool of HIV sero-positive individuals suspected of pulmonary tuberculosis. *PLoS One,* 12**,** e0177529.

Adjemian, J., Frankland, T.B., Daida, Y.G., Honda, J.R., Olivier, K.N., Zelazny, A., Honda, S. & Prevots, D.R. 2017. Epidemiology of Nontuberculous Mycobacterial Lung Disease and Tuberculosis, Hawaii, USA. *Emerg Infect Dis,* 23**,** 439-447.

Adjemian, J., Olivier, K.N., Seitz, A.E., Holland, S.M. & Prevots, D.R. 2012. Prevalence of nontuberculous mycobacterial lung disease in U.S. Medicare beneficiaries. *Am J Respir Crit Care Med,* 185**,** 881-6.

Aliyu, G., El-Kamary, S.S., Abimiku, A., Brown, C., Tracy, K., Hungerford, L. & Blattner, W. 2013. Prevalence of non-tuberculous mycobacterial infections among tuberculosis suspects in Nigeria. *PLoS One,* 8**,** e63170.

Amato, B., Coretti, G., Compagna, R., Amato, M., Buffone, G., Gigliotti, D., Grande, R., Serra, R. & De Franciscis, S. 2015. Role of matrix metalloproteinases in non-healing venous ulcers. *Int Wound J,* 12**,** 641-5.

Amofah, G., Bonsu, F., Tetteh, C., Okrah, J., Asamoa, K., Asiedu, K. & Addy, J. 2002. Buruli ulcer in Ghana: results of a national case search. *Emerg Infect Dis,* 8**,** 167-70.

Antony, M.A., Brandt, L.J., Klein, R.S. & Bernstein, L.H. 1988. Infectious Diarrhoea in patients with AIDS. *Digestive Diseases and Sciences.,* 33**,** 1141-6.

Appelberg, R., Castro, A.G., Pedrosa, J., Silva, R.A., Orme, I.M. & Minoprio, P. 1994. Role of gamma interferon and tumor necrosis factor alpha during T-cell-independent and -dependent phases of Mycobacterium avium infection. *Infect Immun,* 62**,** 3962-71.

Armah, G.E., Sow, S.O., Breiman, R.F., Dallas, M.J., Tapia, M.D., Feikin, D.R., Binka, F.N., Steele, A.D., Laserson, K.F., Ansah, N.A., Levine, M.M., Lewis, K., Coia, M.L., Attah-Poku, M., Ojwando, J., Rivers, S.B., Victor, J.C., Nyambane, G., Hodgson, A., Schodel, F., Ciarlet, M. & Neuzil, K.M. 2010. Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in sub-Saharan Africa: a randomised, double-blind, placebo-controlled trial. *Lancet,* 376**,** 606-14.

Atkinson, J.J. & Senior, R.M. 2003. Matrix metalloproteinase-9 in lung remodeling. *Am J Respir Cell Mol Biol,* 28**,** 12-24.

Autschbach, F., Eisold, S., Hinz, U., Zinser, S., Linnebacher, M., Giese, T., Löffler, T., Büchler, M.W. & Schmidt, J. 2005. High prevalence of Mycobacterium avium subspecies paratuberculosis IS900 DNA in gut tissues from individuals with Crohn's disease. *Gut,* 54**,** 944-949.

Beauchamp, T.L. 2008. The Belmont Report. *The Oxford textbook of clinical research ethics***,** 21-28.

Becton Dickinson & Company 2012. BD Cytometric Bead Array (CBA) human Th1/Th2/Th17 cytokine kit. San Jose, California, USA: Becton, Dickinson & Company

Becton Dickinson & Company 2013. Mycobacteria Growth Indicator Tube 7 mL with BACTEC MGIT 960 Supplement Kit. *In:* BECTON, D. C. (ed.). Sparks, Maryland, USA: Becton, Dickinson & Company.

Benjamini, Y. & Hochberg, Y. 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological),* 57**,** 289-300.

Benson, C.A. 1994. Disease due to the Mycobacterium avium complex in patients with AIDS: epidemiology and clinical syndrome. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America,* Suppl 3**,** S218-22.

Beres, L.K., Tate, J.E., Njobvu, L., Chibwe, B., Rudd, C., Guffey, M.B., Stringer, J.S., Parashar, U.D. & Chilengi, R. 2016. A Preliminary Assessment of Rotavirus Vaccine Effectiveness in Zambia. *Clin Infect Dis,* 62 Suppl 2**,** S175-82.

Bermudez, L. & Young, L. 1988. Tumor necrosis factor, alone or in combination with IL-2, but not IFN- gamma, is associated with macrophage killing of Mycobacterium avium complex. *The Journal of Immunology,* 140**,** 3006-3013.

Bermudez, L.E., Wu, M., Petrofsky, M. & Young, L.S. 1992. Interleukin-6 antagonizes tumor necrosis factor-mediated mycobacteriostatic and mycobactericidal activities in macrophages. *Infect Immun,* 60**,** 4245-52.

Bhaijee, F., Subramony, C., Tang, S.J. & Pepper, D.J. 2011. Human immunodeficiency virus-associated gastrointestinal disease: common endoscopic biopsy diagnoses. *Patholog Res Int,* 2011**,** 247923.

Bhandari, N., Rongsen-Chandola, T., Bavdekar, A., John, J., Antony, K., Taneja, S., Goyal, N., Kawade, A., Kang, G., Rathore, S.S., Juvekar, S., Muliyil, J., Arya, A., Shaikh, H., Abraham, V., Vrati, S., Proschan, M., Kohberger, R., Thiry, G., Glass, R., Greenberg, H.B., Curlin, G., Mohan, K., Harshavardhan, G.V., Prasad, S., Rao, T.S., Boslego, J., Bhan, M.K. & India Rotavirus Vaccine, G. 2014. Efficacy of a monovalent human-bovine (116E) rotavirus vaccine in Indian infants: a randomised, double-blind, placebo-controlled trial. *Lancet,* 383**,** 2136-43.

Bhutta, Z.A., Ahmed, T., Black, R.E., Cousens, S., Dewey, K., Giugliani, E., Haider, B.A., Kirkwood, B., Morris, S.S., Sachdev, H.P., Shekar, M., Maternal & Child Undernutrition Study, G. 2008. What works? Interventions for maternal and child undernutrition and survival. *Lancet,* 371**,** 417-40.

Biancheri, P., Di Sabatino, A., Corazza, G.R. & Macdonald, T.T. 2013. Proteases and the gut barrier. *Cell and Tissue Research,* 351**,** 269-280.

Birkedal-Hansen, H., Moore, W.G., Bodden, M.K., Windsor, L.J., Birkedal-Hansen, B., Decarlo, A. & Engler, J.A. 1993. Matrix metalloproteinases: a review. *Crit Rev Oral Biol Med,* 4**,** 197-250.

Black, R.E., Victora, C.G., Walker, S.P., Bhutta, Z.A., Christian, P., De Onis, M., Ezzati, M., Grantham-Mcgregor, S., Katz, J., Martorell, R. & Uauy, R. 2013. Maternal and

child undernutrition and overweight in low-income and middle-income countries. *The Lancet,* 382**,** 427-451.

Blanchard, D.K., Michelini-Norris, M.B., Pearson, C.A., Freitag, C.S. & Djeu, J.Y. 1991. Mycobacterium avium-intracellulare induces interleukin-6 from human monocytes and large granular lymphocytes. *Blood,* 77**,** 2218-24.

Blanshard, C. & Gazzard, B.G. 1995. Natural history and prognosis of diarrhoea of unknown cause in patients with acquired immunodeficiency syndrome (AIDS). *Gut,* 36**,** 283-6.

Bodle, E.E., Cunningham, J.A., Della-Latta, P., Schluger, N.W. & Saiman, L. 2008. Epidemiology of nontuberculous mycobacteria in patients without HIV infection, New York City. *Emerging Infectious Diseases,* 14**,** 390-6.

Buijtels, P.C., Van-Der-Sande, M.A., De-Graaff, C.S., Parkinson, S., Verbrugh, H.A., Petit, P.L. & Van-Soolingen, D. 2009. Nontuberculous mycobacteria, zambia. *Emerg Infect Dis,* 15**,** 242-9.

Buijtels, P.C., Van Der Sande, M.A., Parkinson, S., Verbrugh, H.A., Petit, P.L. & Van Soolingen, D. 2010. Isolation of non-tuberculous mycobacteria at three rural settings in Zambia; a pilot study. *Clin Microbiol Infect,* 16**,** 1142-8.

Campbell, D.I., Elia, M. & Lunn, P.G. 2003a. Growth Faltering in Rural Gambian Infants Is Associated with Impaired Small Intestinal Barrier Function, Leading to Endotoxemia and Systemic Inflammation. *The Journal of Nutrition,* 133**,** 1332-1338.

Campbell, D.I., Murch, S.H., Elia, M., Sullivan, P.B., Sanyang, M.S., Jobarteh, B. & Lunn, P.G. 2003b. Chronic T cell-mediated enteropathy in rural west African children: relationship with nutritional status and small bowel function. *Pediatr Res,* 54**,** 306-11.

Central Statistical Office 2006. Living Conditions Monitoring Survey.

Central Statistical Office (Cso) [Zambia]. 2011. *Population and Demographic Projections* [Online]. Lusaka: Central Statistical Office Available: http://zambia.opendataforafrica.org/ZMPHC2015/population-and-demographic-projections-2011-2035 [Accessed 26.06.2017.

Central Statistical Office (Cso) [Zambia], Ministry of Health (Moh) [Zambia] & Icf International 2014. Zambia Demographic and Health Survey 2013–2014. Rockville: Central Statistical Office, Ministry of Health, ICF International.

Chaisson, R.E., Moore, R.D., Richman, D.D., Keruly, J. & Creagh, T. 1992. Incidence and natural history of Mycobacterium avium-complex infections in patients with advanced human immunodeficiency virus disease treated with zidovudine. The Zidovudine Epidemiology Study Group. *Am Rev Respir Dis,* 146**,** 285-9.

Chanda-Kapata, P., Kapata, N., Klinkenberg, E., Mulenga, L., Tembo, M., Katemangwe, P., Sunkutu, V., Mwaba, P. & Grobusch, M.P. 2015. Non-tuberculous mycobacteria (NTM) in Zambia: prevalence, clinical, radiological and microbiological characteristics. *BMC Infect Dis,* 15**,** 500.

Chang, C.-T., Wang, L.-Y., Liao, C.-Y. & Huang, S.-P. 2002. Identification of Nontuberculous Mycobacteria Existing in Tap Water by PCR-Restriction Fragment Length Polymorphism. *Applied And Environmental Microbiology,* 68**,** 3159-3161.

Chiodini, R.J. 1989. Crohn's disease and the mycobacterioses: a review and comparison of two disease entities. *Clin Microbiol Rev,* 2**,** 90-117.

Chongwe, G., Michelo, C. & Kelly, P. 2017. Diagnostic yield of nontuberculous mycobacteria in patients booked for endoscopy at the University Teaching Hospital, Lusaka. *BMC Res Notes,* 10**,** 27.

Ciccocioppo, R., Di Sabatino, A., Bauer, M., Della Riccia, D.N., Bizzini, F., Biagi, F., Cifone, M.G., Corazza, G.R. & Schuppan, D. 2005. Matrix metalloproteinase pattern in celiac duodenal mucosa. *Lab Invest,* 85**,** 397-407.

Clayburgh, D.R., Barrett, T.A., Tang, Y., Meddings, J.B., Van Eldik, L.J., Watterson, D.M., Clarke, L.L., Mrsny, R.J. & Turner, J.R. 2005. Epithelial myosin light chain kinase-dependent barrier dysfunction mediates T cell activation-induced diarrhea in vivo. *J Clin Invest,* 115**,** 2702-15.

Cook, G. 1980. *Tropical gastroenterology.,* Oxford, Oxford University Press.

Cooksey, R.C., Jhung, M.A., Yakrus, M.A., Butler, W.R., Adekambi, T., Morlock, G.P., Williams, M., Shams, A.M., Jensen, B.J., Morey, R.E., Charles, N., Toney, S.R., Jost, K.C., Jr., Dunbar, D.F., Bennett, V., Kuan, M. & Srinivasan, A. 2008. Multiphasic approach reveals genetic diversity of environmental and patient isolates of Mycobacterium mucogenicum and Mycobacterium phocaicum

associated with an outbreak of bacteremias at a Texas hospital. *Appl Environ Microbiol,* 74**,** 2480-7.

Cooper, A.M. 2009. Cell mediated immune responses in Tuberculosis. *Annual review of immunology,* 27**,** 393-422.

Covert, T.C., Rodgers, M.R., Reyes, A.L. & Stelma, G.N., Jr. 1999. Occurrence of nontuberculous mycobacteria in environmental samples. *Appl Environ Microbiol,* 65**,** 2492-6.

Cunliffe, N.A., Witte, D., Ngwira, B.M., Todd, S., Bostock, N.J., Turner, A.M., Chimpeni, P., Victor, J.C., Steele, A.D., Bouckenooghe, A. & Neuzil, K.M. 2012. Efficacy of human rotavirus vaccine against severe gastroenteritis in Malawian children in the first two years of life: A randomized, double-blind, placebo controlled trial. *Vaccine,* 30**,** A36-A43.

Da Costa, A.R., Lopes, M.L., Furlaneto, I.P., De Sousa, M.S. & Lima, K.V. 2010. Molecular identification of nontuberculous mycobacteria isolates in a Brazilian mycobacteria reference laboratory. *Diagn Microbiol Infect Dis,* 68**,** 390-4.

De Bruyn, M., Vandooren, J., Ugarte-Berzal, E., Arijs, I., Vermeire, S. & Opdenakker, G. 2016. The molecular biology of matrix metalloproteinases and tissue inhibitors of metalloproteinases in inflammatory bowel diseases. *Crit Rev Biochem Mol Biol,* 51**,** 295-358.

Deng, X., Xiong, X., Khomenko, T., Sandor, Z., Osapay, K., Tolstanova, G., Shiloach, J., Chen, L., Folkman, J. & Szabo, S. 2011. Inappropriate angiogenic response as a

novel mechanism of duodenal ulceration and impaired healing. *Digestive diseases and sciences,* 56**,** 2792-2801.

Dhaliwal, W., Bajaj-Elliott, M. & Kelly, P. 2003. Intestinal defensin gene expression in human populations. *Molecular Immunology,* 40**,** 469-75.

Dhaliwal, W., Kelly, P. & Bajaj-Elliott, M. 2009. Differential effects of Staphylococcal enterotoxin B-mediated immune activation on intestinal defensins. *Clinical & Experimental Immunology,* 156**,** 263-270.

Dinarello, C.A., Simon, A. & Van Der Meer, J.W.M. 2012. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nature reviews. Drug discovery,* 11**,** 633-652.

Donohue, M.J. & Wymer, L. 2016. Increasing Prevalence Rate of Nontuberculous Mycobacteria Infections in Five States, 2008-2013. *Ann Am Thorac Soc,* 13**,** 2143-2150.

Eaton, T., Falkinham, J.O., Aisu, T.O. & Daniel, T.M. 1995 Isolation and characteristics of Mycobacterium avium complex from water and soil samples in Uganda. *Tuberculosis and Lung Diseases,* 76**,** 570-4.

Edirisinghe, E.A., Dissanayake, D.R., Abayasekera, C.L. & Arulkanthan, A. 2014. Occurrence of nontuberculous mycobacteria in aquatic sources of Sri Lanka. *Int J Mycobacteriol,* 3**,** 242-6.

Elkington, P., Shiomi, T., Breen, R., Nuttall, R.K., Ugarte-Gil, C.A., Walker, N.F., Saraiva, L., Pedersen, B., Mauri, F., Lipman, M., Edwards, D.R., Robertson, B.D., D'armiento, J. & Friedland, J.S. 2011. MMP-1 drives immunopathology in human tuberculosis and transgenic mice. *J Clin Invest,* 121**,** 1827-33.

Elkington, P.T., Nuttall, R.K., Boyle, J.J., O'kane, C.M., Horncastle, D.E., Edwards, D.R. & Friedland, J.S. 2005. Mycobacterium tuberculosis, but not vaccine BCG, specifically upregulates matrix metalloproteinase-1. *Am J Respir Crit Care Med,* 172**,** 1596-604.

Elkington, P.T.G. & Friedland, J.S. 2006. Matrix metalloproteinases in destructive pulmonary pathology. *Thorax,* 61**,** 259-266.

Falkinham, J.O. 1996. Epidemiology of Infection by Nontuberculous Mycobacteria *Clinical Microbiology Reviews***,** 177-215.

Falkinham, J.O. 2002. Nontuberculous mycobacteria in the environment. *Clinics in chest medicine,* 23**,** 529-551.

Falkinham, J.O. 2003. The changing pattern of nontuberculous mycobacterial disease. *The Canadian Journal of Infectious Diseases,* 14**,** 281-286.

Ferreira, R., Antunes, L. & Finlay, B. 2010. Should the human microbiome be considered when developing vaccines? *PLoS Pathology,* 6**,** e1001190.

Ferroni, A., Vu-Thien, H., Lanotte, P., Le Bourgeois, M., Sermet-Gaudelus, I., Fauroux, B., Marchand, S., Varaigne, F., Berche, P., Gaillard, J.-L. & Offredo, C. 2006.

Value of the Chlorhexidine Decontamination Method for Recovery of Nontuberculous Mycobacteria from Sputum Samples of Patients with Cystic Fibrosis. *Journal of Clinical Microbiology,* 44**,** 2237-2239.

Galassi, L., Donato, R., Tortoli, E., Burrini, D., Santianni, D. & Dei, R. 2003. Nontuberculous mycobacteria in hospital water systems: application of HPLC for identification of environmental mycobacteria. *J Water Health,* 1**,** 133-139.

Garlanda, C., Dinarello, C.A. & Mantovani, A. 2013. The Interleukin 1 family: Back to the future. *Immunity,* 39**,** 1003-1018.

Gerson, C.D., Kent, T.H., Saha, J.R., Siddiqi, N. & Lindenbaum, J. 1971. Recovery of small-intestinal structure and function after residence in the tropics. II. Studies in Indians and Pakistanis living in New York City. *Ann Intern Med,* 75**,** 41-8.

Gilmartin, A.A. & Petri, W.A. 2015. Exploring the role of environmental enteropathy in malnutrition, infant development and oral vaccine response. *Phil. Trans. R. Soc. B,* 370**,** 20140143.

Giuffrida, P., Biancheri, P. & Macdonald, T.T. 2014. Proteases and small intestinal barrier function in health and disease. *Curr Opin Gastroenterol,* 30**,** 147-53.

Global Laboratory Initiative 2014. Mycobacteriology Laboratory Manual. Geneva: WHO Stop TB Partnership.

Gopinath, K. & Singh, S. 2010. Non-tuberculous mycobacteria in TB-endemic countries: are we neglecting the danger? *PLoS neglected tropical diseases,* 4**,** e615.

Griffith, D.E., Aksamit, T., Brown-Elliott, B.A., Catanzaro, A., Daley, C., Gordin, F., Holland, S.M., Horsburgh, R., Huitt, G., Iademarco, M.F., Iseman, M., Olivier, K., Ruoss, S., Von Reyn, C.F., Wallace, R.J., Jr. & Winthrop, K. 2007. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *American Journal of Respiratory and Critical Care Medicine,* 175**,** 367-416.

Guerrant, R.L., Leite, A.M., Pinkerton, R., Medeiros, P.H., Cavalcante, P.A., Deboer, M., Kosek, M., Duggan, C., Gewirtz, A. & Kagan, J.C. 2016. Biomarkers of environmental enteropathy, inflammation, stunting, and impaired growth in children in northeast Brazil. *PLoS One,* 11**,** e0158772.

Haghighi, P. & Wolf, P.L. 1997. Tropical sprue and subclinical enteropathy: a vision for the nineties. *Crit Rev Clin Lab Sci,* 34**,** 313-41.

Hain Life Science. 2014. *GenoType Mycobacterium CM VER 1.0* [Online]. Hain Life Science GmbH. Available: https://www.hain-lifescience.de/include_datei/kundenmodule/packungsbeilage/download.php?id=776 [Accessed 16.08.18.

Hellyer, T.J., Brown, I.N., Dale, J.W. & Easmon, C.S. 1991. Plasmid analysis of Mycobacterium avium-intracellulare (MAI) isolated in the United Kingdom from patients with and without AIDS. *Journal of Medical Microbiology,* 34**,** 225-31.

Henry, M., Inamdar, L., O'riordain, D., Schweiger, M. & Watson, J. 2004. Nontuberculous mycobacteria in non-HIV patients: epidemiology, treatment and response. *European Respiratory Journal,* 23**,** 741-746.

Hillemann, D., Richter, E. & Rüsch-Gerdes, S. 2006. Use of the BACTEC Mycobacteria Growth Indicator Tube 960 Automated System for Recovery of Mycobacteria from 9,558 Extrapulmonary Specimens, Including Urine Samples. *Journal of Clinical Microbiology,* 44**,** 4014-4017.

Hoza, A.S., Mfinanga, S.G.M., Rodloff, A.C., Moser, I. & König, B. 2016. Increased isolation of nontuberculous mycobacteria among TB suspects in Northeastern, Tanzania: public health and diagnostic implications for control programmes. *BMC Research Notes,* 9**,** 109.

Huh, J.-G., Kim, Y.-S., Lee, J.-S., Jeong, T.-Y., Ryu, S.-H., Lee, J.-H., Moon, J.-S., Kang, Y.-K., Shim, M.-S. & Oh, M.-D. 2008. Mycobacterium ulcerans infection as a cause of chronic diarrhea in an AIDS patient: A case report. *World Journal of Gastroenterology : WJG,* 14**,** 808-811.

Humphrey, J.H. 2009. Child undernutrition, tropical enteropathy, toilets, and handwashing. *Lancet,* 374**,** 1032-5.

Inderlied, C.B., Kemper, C.A. & Bermudez, L.E. 1993. The Mycobacterium avium complex. *Clinical Microbiology Reviews,* 6**,** 266-310.

Ito, A., Mukaiyama, A., Itoh, Y., Nagase, H., Thogersen, I.B., Enghild, J.J., Sasaguri, Y. & Mori, Y. 1996. Degradation of interleukin 1beta by matrix metalloproteinases. *J Biol Chem,* 271**,** 14657-60.

Jarzembowski, J.A. & Young, M.B. 2008. Nontuberculous Mycobacterial Infections. *Archives of Pathology & Laboratory Medicine,* 132**,** 1333-1341.

Katoch, V.M. 2004. Infections due to non-tuberculous mycobacteria. *Indian Journal of Medical Research* 120**,** 290-304.

Kau, A.L., Ahern, P.P., Griffin, N.W., Goodman, A.L. & Gordon, J.I. 2011. Human nutrition, the gut microbiome and the immune system. *Nature,* 474**,** 327-36.

Kelly, P., Baboo, K.S., Wolff, M., Ngwenya, B., Luo, N. & Farthing, M.J. 1996. The prevalence and aetiology of persistent diarrhoea in adults in urban Zambia. *Acta Tropica.,* 61**,** 183-90.

Kelly, P., Menzies, I., Crane, R., Zulu, I., Nickols, C., Feakins, R., Mwansa, J., Mudenda, V., Katubulushi, M., Greenwald, S. & Farthing, M. 2004. Responses of Small Intestinal Architecture and Function over Time to Environmental Factors in a Tropical Population. *American Journal of Tropical Medicine And Hygiene,* 70**,** 412-419.

Kirkegaard, T., Hansen, A., Bruun, E. & Brynskov, J. 2004. Expression and localisation of matrix metalloproteinases and their natural inhibitors in fistulae of patients with Crohn's disease. *Gut,* 53**,** 701-9.

Kishimoto, T., Akira, S. & Taga, T. 1992. Interleukin-6 and its receptor: A paradigm for cytokines. *Science,* 258**,** 593-597.

Kleeberg, H.H. 1981. Epidemiology of Mycobacteria Other than Tubercle Bacilli in South Africa. *Review of Infectious Diseases,* 3**,** 1008-1012.

Korpe, P.S. & Petri, W.A. 2012. Environmental Enteropathy: Critical implications of a poorly understood condition. *Trends in Molecular Medicine,* 18**,** 328-336.

Kothari, P., Pestana, R., Mesraoua, R., Elchaki, R., Khan, K.M., Dannenberg, A.J. & Falcone, D.J. 2014. IL-6-mediated induction of matrix metalloproteinase-9 is modulated by JAK-dependent IL-10 expression in macrophages. *J Immunol,* 192**,** 349-57.

Kuhn, K.A., Manieri, N.A., Liu, T.-C. & Stappenbeck, T.S. 2014. IL-6 Stimulates Intestinal Epithelial Proliferation and Repair after Injury. *PLoS ONE,* 9**,** e114195.

Lagos, R., Fasano, A., Wasserman, S., Prado, V., San Martin, O., Abrego, P., Losonsky, G., Alegria, S. & Levine, M. 1999. Effect of small bowel bacterial overgrowth on the immunogenicity of single-dose live oral cholera vaccine CVD 103-HgR. *Journal of Infectious Diseases,* 180**,** 1709-12.

Lake, M.A., Ambrose, L.R., Lipman, M.C. & Lowe, D.M. 2016. ‘" Why me, why now?" Using clinical immunology and epidemiology to explain who gets nontuberculous mycobacterial infection. *BMC medicine,* 14**,** 54.

Lee, E.-J., Han, J.E., Woo, M.-S., Shin, J.A., Park, E.-M., Kang, J.L., Moon, P.G., Baek, M.-C., Son, W.-S., Ko, Y.T., Choi, J.W. & Kim, H.-S. 2014. Matrix Metalloproteinase-8 Plays a Pivotal Role in Neuroinflammation by Modulating TNF-α Activation. *The Journal of Immunology,* 193**,** 2384-2393.

Lee, H., Park, H.J., Cho, S.N., Bai, G.H. & J., K.S. 2000. Species identification of Mycobacteria by PCR-Restriction Fragment Length Polymorphism of the Rpob Gene. *Journal of Clinical Microbiology,* 38**,** 2966-71.

Lee, K.Y., Kim, E.H., Yang, W.S., Ryu, H., Cho, S.-N., Lee, B.I. & Heo, J.H. 2004. Persistent increase of matrix metalloproteinases in cerebrospinal fluid of tuberculous meningitis. *Journal of the Neurological Sciences,* 220**,** 73-78.

Levine, M. 2010. Immunogenicity and efficacy of oral vaccines in developing countries: lessons from a live cholera vaccine. *BMC Biology,* 8**,** 129.

Lindenbaum, J., Harmon, J.W. & Gerson, C.D. 1972. Subclinical malabsorption in developing countries. *The American journal of clinical nutrition,* 25**,** 1056-61.

Linhares, A.C., Velázquez, F.R., Pérez-Schael, I., Sáez-Llorens, X., Abate, H., Espinoza, F., López, P., Macías-Parra, M., Ortega-Barría, E., Rivera-Medina, D.M., Rivera, L., Pavía-Ruz, N., Nuñez, E., Damaso, S., Ruiz-Palacios, G., De Vos, B., O'ryan, M., Gillard, P. & Bouckenooghe, A. 2008. Efficacy and safety of an oral live attenuated human rotavirus vaccine against rotavirus gastroenteritis during the first 2 years of life in Latin American infants: a randomised, double-blind, Placebo-controlled phase III study. *Lancet,* 371**,** 1181-89.

Lionetti, P., Breese, E., Braegger, C.P., Murch, S.H., Taylor, J. & Macdonald, T.T. 1993. T-cell activation can induce either mucosal destruction or adaptation in cultured human fetal small intestine. *Gastroenterology,* 105**,** 373-81.

Louis-Auguste, J. & Kelly, P. 2017. Tropical Enteropathies. *Current Gastroenterology Reports,* 19**,** 29.

Macdonald, T. & Spencer, J. 1988. Evidence that activated mucosal T-cells play a role in the pathogenesis of enteropathy in human small intestine. *Journal of Experimental Medicine,* 167 1341-1349.

Madhi, S.A., Cunliffe, N.A., Steele, D., Witte, D., Kirsten, M., Louw, C., Ngwira, B., Victor, J.C., Gillard, P.H., Cheuvart, B.B., Han, H.H. & Neuzil, K.M. 2010. Effect of human rotavirus vaccine on severe diarrhea in African infants. *N Engl J Med,* 362**,** 289-98.

Manicone, A.M. & Mcguire, J.K. 2008. Matrix Metalloproteinases as Modulators of Inflammation. *Seminars in cell & developmental biology,* 19**,** 34-41.

Marras, T.K., Chedore, P., Ying, A.M. & Jamieson, F. 2007. Isolation prevalence of pulmonary non-tuberculous mycobacteria in Ontario, 1997-2003. *Thorax,* 62**,** 661-6.

Marras, T.K. & Daley, C.L. 2002. Epidemiology of Human Pulmonary Infection with nontuberculous mycobacteria. *Clinics in Chest Medicine,* 23**,** 553-67.

Marras, T.K., Mendelson, D., Marchand-Austin, A., May, K. & Jamieson, F.B. 2013. Pulmonary Nontuberculous Mycobacterial Disease, Ontario, Canada, 1998–2010. *Emerging Infectious Diseases,* 19**,** 1889-1891.

Mcdonald, J.H. 2009. *Handbook of biological statistics, 2nd Edition,* Baltimore, MD, Sparky House Publishing.

Medina, C. & Radomski, M.W. 2006. Role of Matrix Metalloproteinases in Intestinal Inflammation. *Journal of Pharmacology and Experimental Therapeutics,* 318**,** 933-938.

Menzies, I.S., Zuckerman, M.J., Nukajam, W.S., Somasundaram, S.G., Murphy, B., Jenkins, A.P., Crane, R.S. & Gregory, G.G. 1999. Geography of intestinal permeability and absorption. *Gut,* 44**,** 483-9.

Ministry of Health [Zambia] 2010. Adult and adolescent antiretroviral therapy protocols. Lusaka: Government of the Republic of Zambia.

Mirsaeidi, M., Farshidpour, M., Ebrahimi, G., Aliberti, S. & Falkinham, J.O. 2014. Management of nontuberculous mycobacterial infection in the elderly. *European journal of internal medicine,* 25**,** 356-363.

Mohamed, B.M., Feighery, C., Kelly, J., Coates, C., O'shea, U., Barnes, L. & Abuzakouk, M. 2006. Increased protein expression of matrix metalloproteinases -1, -3, and -9 and TIMP-1 in patients with gluten-sensitive enteropathy. *Dig Dis Sci,* 51**,** 1862-8.

Mondal, D., Minak, J., Alam, M., Liu, Y., Dai, J., Korpe, P., Liu, L., Haque, R. & Petri, W.A., Jr. 2012. Contribution of enteric infection, altered intestinal barrier function, and maternal malnutrition to infant malnutrition in Bangladesh. *Clin Infect Dis,* 54**,** 185-92.

Moore, J.E., Kruijshaar, M., Ormerod, L., Drobniewski, F. & Abubakar, I. 2010. Increasing reports of non-tuberculous mycobacteria in England, Wales and Northern Ireland, 1995-2006. *BMC Public Health,* 10**,** 612.

Morrissey, A.B., Aisu, T.O., Falkinham, J.O., Eriki, P.P., Ellner, J.J. & Daniel, T.M. 1992. Absence of Mycobacterium avium complex disease in patients with AIDS in Uganda. *Journal of Acquired Immune Deficiency Syndrome,* 5**,** 477-8.

Moussa, H.S., Bayoumi, F.S. & Mohamed, A.M.A. 2016. Gene Xpert for Direct Detection of Mycobacterium Tuberculosis in Stool Specimens from Children with Presumptive Pulmonary Tuberculosis. *Annals of Clinical & Laboratory Science,* 46**,** 198-203.

Muchwa, C., Akol, J., Etwom, A., Morgan, K., Orikiriza, P., Mumbowa, F., Odong, P.R., Kateete, D.P., Eisenach, K.D. & Joloba, M.L. 2012. Evaluation of Capilia TB assay for rapid identification of Mycobacterium tuberculosis complex in BACTEC MGIT 960 and BACTEC 9120 blood cultures. *BMC Res Notes,* 5**,** 44.

Musch, M.W. 2002. T cell activation causes diarrhea by increasing intestinal permeability and inhibiting epithelial Na+/K+-ATPase. *Journal of Clinical Investigation,* 110**,** 1739-1747.

Muyoyeta, M., Schaap, J.A., De Haas, P., Mwanza, W., Muvwimi, M.W., Godfrey-Faussett, P. & Ayles, H. 2009. Comparison of four culture systems for Mycobacterium tuberculosis in the Zambian National Reference Laboratory. *International Journal Tuberculosis and Lung Disease,* 13**,** 460-465.

Naito, Y. & Yoshikawa, T. 2005 Role of matrix metalloproteinases in inflammatory bowel disease. *Molecular aspects of medicine,* 26**,** 379-90.

Nasiri, M.J., Dabiri, H., Darban-Sarokhalil, D. & Shahraki, A.H. 2015. Prevalence of non-tuberculosis mycobacterial infections among tuberculosis suspects in Iran: systematic review and meta-analysis. *PLoS One,* 10**,** e0129073.

Naugler, W.E. & Karin, M. 2008. The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. *Trends in Molecular Medicine,* 14**,** 109-119.

Nicol, M.P., Spiers, K., Workman, L., Isaacs, W., Munro, J., Black, F., Zemanay, W. & Zar, H.J. 2013. Xpert MTB/RIF Testing of Stool Samples for the Diagnosis of Pulmonary Tuberculosis in Children. *Clinical Infectious Diseases,* 57**,** e18-e21.

Nightingale, S.D., Byrd, L.T., Southern, P.M., Jockusch, J.D., Cal, S.X. & Wynne, B.A. 1992. Incidence of Mycobacterium avium-intracellulare complex bacteremia in human immunodeficiency virus-positive patients. *Journal of infectious diseases,* 165**,** 1082-1085.

Okello, D.O., Sewankambo, N., Goodgame, R., Aisu, T.O., Kwezi, M., Morrissey, A. & Ellner, J.J. 1990. Absence of bacteremia with Mycobacterium avium-intracellulare in Ugandan patients with AIDS. *Journal of Infectious Diseases,* 162**,** 208-10.

Ong, C.W., Elkington, P.T., Brilha, S., Ugarte-Gil, C., Tome-Esteban, M.T., Tezera, L.B., Pabisiak, P.J., Moores, R.C., Sathyamoorthy, T., Patel, V., Gilman, R.H., Porter, J.C. & Friedland, J.S. 2015. Neutrophil-Derived MMP-8 Drives AMPK-Dependent Matrix Destruction in Human Pulmonary Tuberculosis. *PLoS Pathog,* 11**,** e1004917.

Opdenakker, G., Van Den Steen, P.E. & Van Damme, J. 2001. Gelatinase B: a tuner and amplifier of immune functions. *Trends in Immunology,* 22**,** 571-579.

Oramasionwu, G.E., Heilig, C.M., Udomsantisuk, N., Kimerling, M.E., Eng, B., Nguyen, H.D., Thai, S., Keo, C., Mccarthy, K.D., Varma, J.K. & Cain, K.P. 2013. The utility of stool cultures for diagnosing tuberculosis in people living with the human immunodeficiency virus. *The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease,* 17**,** 1023-1028.

Patriarca, P., Wright, P. & John, T. 1991. Factors affecting the immunogenicity of oral poliovirus vaccine in developing countries: review. *Review of Infectious Diseases,* 13**,** 926-39.

Pedro, H.S.P., Pereira, M.I.F., Goloni, M.R.A., Pires, F.C., Oliveira, R.S., Da Rocha, M.a.B., Conceição, L.M., Fraga, V.D., Fenley, J.C., Cordeschi, T., Machado, R.L.D., Franco, C. & Rossit, A.R.B. 2011. Mycobacterium tuberculosis in a HIV-1-infected population from Southeastern Brazil in the HAART era. *Tropical Medicine & International Health,* 16**,** 67-73.

Pender, S.L., Lionetti, P., Murch, S.H., Wathan, N. & Macdonald, T.T. 1996. Proteolytic degradation of intestinal mucosal extracellular matrix after lamina propria T cell activation. *Gut,* 39**,** 284-90.

Pender, S.L., Tickle, S.P., Docherty, A.J., Howie, D., Wathen, N.C. & Macdonald, T.T. 1997. A major role for matrix metalloproteinases in T cell injury in the gut. *Journal of Immunology,* 158**,** 1582-90.

Pettipher, C.A., Karstaedt, A.S. & Hopley, M. 2001. Prevalence and Clinical Manifestations of Disseminated Mycobacterium avium Complex Infection in South Africans with Acquired Immunodeficiency Syndrome. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America,* 33**,** 2068-2071.

Pham, C.T.N. 2006. Neutrophil serine proteases: specific regulators of inflammation. *Nat Rev Immunol,* 6**,** 541-550.

Phillips, P., Bonner, S., Gataric, N., Bai, T., Wilcox, P., Hogg, R., O'shaughnessy, M. & Montaner, J. 2005. Nontuberculous Mycobacterial Immune Reconstitution Syndrome in HIV-Infected Patients: Spectrum of Disease and Long-Term Follow-Up. *Clinical Infectious Diseases,* 41**,** 1483-1497.

Phua, K.B., Lim, F.S., Lau, Y.L., Nelson, E.A., Huang, L.M., Quak, S.H., Lee, B.W., Teoh, Y.L., Tang, H., Boudville, I., Oostvogels, L.C., Suryakiran, P.V., Smolenov, I.V., Han, H.H. & Bock, H.L. 2009. Safety and efficacy of human rotavirus vaccine

during the first 2 years of life in Asian infants: randomised, double-blind, controlled study. *Vaccine,* 27**,** 5936-41.

Pierce, E.S. 2010. Ulcerative colitis and Crohn's disease: is Mycobacterium avium subspecies paratuberculosis the common villain? *Gut Pathogens,* 2**,** 21.

Pierce, E.S., Borowitz, S.M. & Naser, S.A. 2011. The Broad Street pump revisited: dairy farms and an ongoing outbreak of inflammatory bowel disease in Forest, Virginia. *Gut Pathogens,* 3**,** 20-20.

Prendergast, A. & Kelly, P. 2012. Enteropathies in the developing world: neglected effects on global health. *Am J Trop Med Hyg,* 86**,** 756-63.

Puthanakit, T., Oberdorfer, P., Ukarapol, N., Akarathum, N., Punjaisee, S., Sirisanthana, T. & Sirisanthana, V. 2006. Immune reconstitution syndrome from nontuberculous mycobacterial infection after initiation of antiretroviral therapy in children with HIV infection. *Pediatr Infect Dis J,* 25**,** 645-8.

Quiding-Järbrink, M., Smith, D.A. & Bancroft, G.J. 2001. Production of matrix metalloproteinases in response to mycobacterial infection. *Infection and Immunity,* 69**,** 5661-5670.

R&D Systems 2011a. DuoSet ELISA Development System, Human Total MMP-1. *In:* SYSTEMS, R. D. (ed.). Minneapolis, USA: R&D Systems Inc.

R&D Systems 2011b. Quantikine ELISA Human Total MMP-8. *In:* SYSTEMS, R. D. (ed.). Minneapolis, USA: R&D Systems Inc.

R&D Systems 2013a. Quantikine ELISA Human IL-1β/IL-1F2 *In:* R&D SYSTEMS (ed.). Minneapolis, USA: R&D Systems Inc.

R&D Systems 2013b. Quantikine ELISA Human IL-12/IL23 p40. *In:* SYSTEMS, R. D. (ed.). Minneapolis, USA: R&D Systems.

R&D Systems 2013c. Quantikine ELISA Total MMP-2. . *In:* R&D SYSTEMS (ed.). Minneapolis, USA R&D Systems.

R&D Systems 2014. Quantikine ELISA Human MMP-9. *In:* R&D SYSTEMS, I. (ed.). Minneapolis, USA: R&D Systems, Inc.

Ravi, A., Garg, P. & Sitaraman, S. 2007. Matrix Metalloproteinases in Inflammatory Bowel Disease: Boon or a Bane? *Inflammatory Bowel Disease,* 13**,** 97-107.

Rosser, E.C., Oleinika, K., Tonon, S., Doyle, R., Bosma, A., Carter, N.A., Harris, K.A., Jones, S.A., Klein, N. & Mauri, C. 2014. Regulatory B cells are induced by gut microbiota-driven interleukin-1[beta] and interleukin-6 production. *Nat Med,* 20**,** 1334-1339.

Ruiz-Palacios, G.M., Pérez-Schael, I., Velázquez, F.R., Abate, H., Breuer, T., Clemens, S.C., Cheuvart, B., Espinoza, F., Gillard, P., Innis, B.L., Cervantes, Y., Linhares, A.C., López, P., Macías-Parra, M., Ortega-Barría, E., Richardson, V., Rivera-Medina, D.M., Rivera, L., Salinas, B., Pavía-Ruz, N., Salmerón, J., Rüttimann, R., Tinoco, J.C., Rubio, P., Nuñez, E., Guerrero, M.L., Yarzábal, J.P., Damaso, S., Tornieporth, N., Sáez-Llorens, X., Vergara, R.F., Vesikari, T., Bouckenooghe, A., Clemens, R., De Vos, B. & O'ryan, M. 2006. Safety and Efficacy of an Attenuated

Vaccine against Severe Rotavirus Gastroenteritis. *New England Journal of Medicine,* 354**,** 11-22.

Saarialho-Kere, U. 1998. Patterns of matrix metalloproteinase and TIMP expression in chronic ulcers. *Archives of Dermatological Research,* 290**,** S47-S54.

Santos, M., Gil-Brusola, A., Escandell, A., Blanes, M. & Gobernado, M. 2014. Mycobacterium genavense Infections in a Tertiary Hospital and Reviewed Cases in Non-HIV Patients. *Pathology Research International,* 2014**,** 8.

Sartor, R.B. & Mazmanian, S.K. 2012. Intestinal Microbes in Inflammatory Bowel Diseases. *Am J Gastroenterol Suppl,* 1**,** 15-21.

Schorey, J.S. & Sweet, L. 2008. The mycobacterial glycopeptidolipids: structure, function, and their role in pathogenesis. *Glycobiology,* 18**,** 832-41.

Selby, W., Pavli, P., Crotty, B., Florin, T., Radford-Smith, G., Gibson, P., Mitchell, B., Connell, W., Read, R., Merrett, M., Ee, H. & Hetzel, D. 2007. Two-year combination antibiotic therapy with clarithromycin, rifabutin, and clofazimine for Crohn's disease. *Gastroenterology,* 132**,** 2313-9.

Sengupta, N. & Macdonald, T.T. 2007. The Role of Matrix Metalloproteinases in Stromal/Epithelial Interactions in the Gut. *Physiology,* 22**,** 401-409.

Sharma, S.K., Mohan, A. & Kadhiravan, T. 2005. HIV-TB co-infection: epidemiology, diagnosis, & management. *Indian Journal of Medical Research* 121**,** 550-567.

Shin, D., Yang, C., Yuk, J., Lee, J., Kim, K., Shin, S., Takahara, K., Lee, S. & Jo, E. 2008. Mycobacterium abscessus activates the macrophage innate immune response via a physical and functional interaction between TLR2 and dectin-1. *Cellular Microbiology,* 10**,** 1608-21.

Singh, A.K., Maurya, A.K., Umrao, J., Kant, S., Kushwaha, R.a.S., Nag, V.L. & Dhole, T.N. 2013. Role of GenoType(®) Mycobacterium Common Mycobacteria/Additional Species Assay for Rapid Differentiation Between Mycobacterium tuberculosis Complex and Different Species of Non-Tuberculous Mycobacteria. *Journal of Laboratory Physicians,* 5**,** 83-89.

Sinkala, E., Grey, S., Zulu, I., Mudenda, V., Zimba, L., Vermund, S.H., Drobniewski, F. & Kelly, P. 2009. Clinical and Ultrasonographic features of abdominal tuberculosis in HIV positive adults in Zambia. *BioMed Central Infectious Diseases,* 9**,** 44.

Steck, N., Mueller, K., Schemann, M. & Haller, D. 2012. Bacterial proteases in IBD and IBS. *Gut,* 61**,** 1610-1618.

Taylor, J.L., Hattle, J.M., Dreitz, S.A., Troudt, J.M., Izzo, L.S., Basaraba, R.J., Orme, I.M., Matrisian, L.M. & Izzo, A.A. 2006. Role for matrix metalloproteinase 9 in granuloma formation during pulmonary Mycobacterium tuberculosis infection. *Infection and Immunity,* 74**,** 6135-44.

Timms, V.J., Daskalopoulos, G., Mitchell, H.M. & Neilan, B.A. 2016. The Association of <italic>Mycobacterium avium</italic> subsp. <italic>paratuberculosis</italic> with Inflammatory Bowel Disease. *PLoS ONE,* 11**,** e0148731.

Torriani, F.J., Behling, C.A., Mccutchan, J.A., Haubrich, R.H. & Havlir, D.V. 1996. Disseminated Mycobacterium avium Complex: Correlation between Blood and Tissue Burden. *Journal of Infectious Diseases,* 173**,** 942-949.

Trehan, I., Shulman, R.J., Ou, C.N., Maleta, K. & Manary, M.J. 2009. A randomized, double-blind, placebo-controlled trial of rifaximin, a nonabsorbable antibiotic, in the treatment of tropical enteropathy. *Am J Gastroenterol,* 104**,** 2326-33.

United Nations Children's Fund (Unicef) 2009. Tracking progress on child and maternal nutrition, A survival and development priority. New York: UNICEF

Uzoigwe, J.C., Khaitsa, M.L. & Gibbs, P.S. 2007. Epidemiological evidence for Mycobacterium avium subspecies paratuberculosis as a cause of Crohn's disease. *Epidemiology and Infection,* 135**,** 1057-68.

Van Crevel, R., Ottenhoff, T.H.M. & Van Der Meer, J.W.M. 2002. Innate Immunity to Mycobacterium tuberculosis. *Clinical Microbiology Reviews,* 15**,** 294-309.

Varley, C.D., Ku, J.H., Henkle, E., Schafer, S.D. & Winthrop, K.L. 2017. Disseminated Nontuberculous Mycobacteria in HIV-Infected Patients, Oregon, USA, 2007–2012. *Emerging Infectious Diseases,* 23**,** 533-535.

Veitch, A.M., Kelly, P., Zulu, I.S., Segal, I. & Farthing, M.J. 2001. Tropical enteropathy: a T-cell-mediated crypt hyperplastic enteropathy. *European Journal of Gastroenterology & Hepatology,* 13**,** 1175-81.

Vesikari, T., Matson, D., Dennehy, P., Van Damme, P., Santosham, M., Rodriguez, Z., Dallas, M., Heyse, J., Goveia, M., Black, S., Shinefield, H., Christie, C., Ylitalo, S., Itzler, R., Coia, M., Onorato, M., Adeyi, B., Marshall, G., Gothefors, L., Campens, D., Karvonen, A., Watt, J., Kl, O.B., Dinubile, M., Clark, H., Boslego, J., Offit, P. & Heaton, P. 2006. Safety and efficacy of a pentavalent human-bovine (WC3) reassortant rotavirus vaccine. *New England Journal of Medicine,* 354**,** 23-33.

Waldner, M.J. & Neurath, M.F. 2014. Master regulator of intestinal disease: IL-6 in chronic inflammation and cancer development. *Seminars in Immunology,* 26**,** 75-79.

Wang, H. & Keiser, J.A. 1998. Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: role of flt-1. *Circ Res,* 83**,** 832-40.

Wang, L., Walia, B., Evans, J., Gewirtz, A.T., Merlin, D. & Sitaraman, S.V. 2003. IL-6 induces NF-kappa B activation in the intestinal epithelia. *J Immunol,* 171**,** 3194-201.

Weber, A., Wasiliew, P. & Kracht, M. 2010. Interleukin-1beta (IL-1beta) processing pathway. *Sci Signal,* 3**,** cm2.

Winthrop, K.L., Mcnelley, E., Kendall, B., Marshall-Olson, A., Morris, C., Cassidy, M., Saulson, A. & Hedberg, K. 2010. Pulmonary nontuberculous mycobacterial disease prevalence and clinical features: an emerging public health disease. *Am J Respir Crit Care Med,* 182**,** 977-82.

Wood, G.M., Gearty, J.C. & Cooper, B.T. 1991. Small bowel morphology in British Indian and Afro-Caribbean subjects: evidence of tropical enteropathy. *Gut,* 32**,** 256-9.

World Health Organisation 2009. Weekly Epidemiological Record. 84**,** 213–236.

Wu, T.-S., Lu, C.-C. & Lai, H.-C. 2009. Current Situations on Identification of Nontuberculous Mycobacteria. *Journal of Biomedical Laboratory Sciences* 21**,** 1-6.

Zaman, K., Dang, D.A., Victor, J.C., Shin, S., Yunus, M., Dallas, M.J., Podder, G., Vu, D.T., Le, T.P., Luby, S.P., Le, H.T., Coia, M.L., Lewis, K., Rivers, S.B., Sack, D.A., Schodel, F., Steele, A.D., Neuzil, K.M. & Ciarlet, M. 2010. Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in Asia: a randomised, double-blind, placebo-controlled trial. *Lancet,* 376**,** 615-23.

APPENDICES

Appendix 1: Questionnaire – Prevalence study

QUESTIONNAIRE FOR THE NTM PREVALENCE SURVEY

SCREENING AND ELIGIBILITY

1. Consent Form Signed [Yes] [No]
2. Assign Id No. to Patient ___ ___ ___ ___ ___
3. Date _____/_____/_____ (dd/mm/yyyy)

DO NOT PROCEED IF THE ANSWER TO SCREENING AND ELLIGIBILITY [1.] ABOVE IS 'NO'

PERSONAL DETAILS

4. Age _____years
5. Sex 0[F] 1[M.]
6. **Highest Level of education attained**

    0. None

    1. Primary
    2. Secondary
    3. Tertiary
7. **What is your Occupation**

    0. Unemployed

    1. Self employed
    2. Casual work
    3. Employed full time
8. **Where do you live (Residential Area)**

    0. Low Cost

    1. Medium Cost
    2. High Cost

SIGNS AND SYMPTOMS

9. Please state what symptoms are applicable to the patient

    Abdominal Pain 1. [Yes] 0. [No]

Diarrhoea 1. [Yes] 0. [No]

Weight loss 1. [Yes] 0. [No]

Vomiting 1. [Yes] 0. [No]

Nausea 1. [Yes] 0. [No]

Dizziness 1. [Yes] 0. [No]

Anorexia 1. [Yes] 0. [No]

Fever 1. [Yes] 0. [No]

Other (Specify)_____

10. Working Diagnosis _____

CONCOMITANT DRUGS

11. Please list the Drugs that the patient has been taking in the last two weeks.
    1. _____
    2. _____
    3. _____
    4. _____
    5. _____

LABORATORY RESULTS CHECKLIST

1. Please indicate which of the tests below have been conducted on this patient.

Done/not done	Test	Results
	Haemoglobin	
	Heamatocrit	
	HIV Test	
	CD4 count	
	Alkaline Phosphatase	
	Stool Culture MGIT	

	HAIN	
	Intestinal Lavage MGIT	
	HAIN	
	Caecal Biopsy MGIT	
	HAIN	
	Descend Colon MGIT	
	HAIN	

Appendix 2: Questionnaire – *in-vitro* study

QUESTIONNAIRE FOR THE IN-VITRO CYTOKINE ASSESSMENT STUDY

In-vitro assessment of enterocyte cytokine response to mycobacteria avium complex

Assign Id No. to Patient ___ ___ ___ ___ ___

12. Date _____/_____/_____ (dd/mm/yyyy)
13. Age _____years
14. Sex 0[F] 1[M.]
15. Reason for endoscopy_____
16. History of TB _____
17. Taking any drugs _____
18. HIV Test Result 1. [R] 0. [NR] 2[I/NA/ND]
19. BCG Scar present 1. [Yes] 0. [No]
20. Please state what symptoms are applicable to the patient

Abdominal Pain	1. [Yes]	0. [No]
Diarrhoea	1. [Yes]	0. [No]
Weight loss	1. [Yes]	0. [No]
Vomiting	1. [Yes]	0. [No]
Nausea	1. [Yes]	0. [No]
Night sweats	1. [Yes]	0. [No]
Anorexia	1. [Yes]	0. [No]
Fever	1. [Yes]	0. [No]
Cough	1. [Yes]	0. [No]
Other	(Specify)_____	

Appendix 3: Information Sheet and Consent form

INFORMATION SHEET AND CONSENT FORM

Title: Intestinal Carriage of Nontuberculous Mycobacteria and its association Environmental Enteropathy

Introduction

Hello. My name is Gershom Chongwe from the University of Zambia. I am a student and as part of my studies, I need to conduct some research.

Purpose of Study

We are carrying out research in order for us to understand the contribution of a type of tuberculosis germ (NTM) to some changes that are found in the intestines of people living in poor countries (a condition called environmental enteropathy).

Study Procedures

If you agree to take part in this study you will be required to sign a consent form. Once we establish your eligibility, we will ask you questions about yourself and your signs and symptoms. We will collect biopsy samples from the endoscopy that you are about to have in order for us to understand how the abdominal lining works in the presence of the TB germs. We will also collect blood for HIV and CD4 tests.

Confidentiality

The results of your tests and your answers to the questions will be kept confidential and will only be used for research purposes. Only members of the study team will have access

to the information and the samples that will be collected. The samples will only be used for the stated purposes.

Study Benefits

There may not be any direct benefit to you for participating in this study but many people may benefit in future if we are able to find the answers to our questions.

Study Risks

The endoscopy procedure, which lasts a few minutes may also be a little uncomfortable and carries with it some risks such as reaction to sedation, risk of bleeding and aspiration of stomach contents. The team will take every precaution to ensure that the risks are minimized and that the procedure is as comfortable for you as possible.

Voluntariness

Your participation in this study is completely voluntary. Should you choose not to participate, no penalty or injury shall occur to you and you will continue to receive the same health care that you otherwise enjoy. You have the right to withdraw your participation any time you wish to do so.

If you have any doubts or you wish to seek clarification on the research please feel free to contact the main researcher on the address below:

Dr Gershom Chongwe

UNZA School of Medicine

Ridgeway Campus

SACORE Building

Box 50110, **Lusaka**

Email: gchongwe@yahoo.co.uk

Tel: +260966769144

If you have any complaints about the study, please contact the Chairperson of the UNZA Biomedical Ethics Review Committee at the following address:

The Chairman

UNZA Biomedical Ethics Review Committee

Ridgeway Campus

Box 50110

Lusaka

Tel: 0211-256067

I understand the information given to me and that my participation in this research is completely voluntary and its purpose has been fully explained to me. I also understand that my rights and privacy will be respected.

Name of participant:……

Signature or thumb print of participant:……..

Name and signature of person obtaining consent: ……

Date: …….

Name of Witness .....

Signature of Witness.....

Phone_____

Appendix 4. Materials Required for Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences)

- A dual-laser flow cytometer equipped with a 488-nm or 532-nm and a 633-nm or 635-nm laser capable of distinguishing 576-nm, 660-nm, and >680-nm fluorescence.
- Falcon® 12 × 75-mm sample acquisition tubes (Catalogue No. 352008), or equivalent
- 15-mL conical, polypropylene tubes (BD Falcon, Catalogue No. 352097), or equivalent
- FCAP Array software (Catalogue No. 641488 [PC] or 645447 [Mac®])

Appendix 5. Materials Required for IL-1β Quantikine ELISA (R&D Systems)

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Pipettes and pipette tips
- Deionised or distilled water
- Squirt bottle, maniford dispenser, or automated microplate washer
- 500 mL graduated cylinder
- Human IL-1β Controls (optional).

Appendix 6. Materials Required for IL-12 Quantikine ELISA (R&D Systems)

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Pipettes and pipette tips
- 100 mL and 500 mL graduated cylinders
- Deionised or distilled water
- Squirt bottle, maniford dispenser, or automated microplate washer
- Polypropylene test tubes for dilution of standards
- Human IL-12/IL-23 p40 Controls (Optional)

Appendix 7. Materials Required for Duoset Human Total MMP-1 ELISA (R&D Systems)

- PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 µm filtered (R&D Systems, Catalogue # DY006).
- Wash Buffer: 0.05% Tween® 20 in PBS, pH 7.2-7.4 (R&D Systems, Catalogue # WA126).
- Reagent Diluent: 1% Bovine Serum Albumin in PBS, pH 7.2-7.4, 0.2 µm filtered (R&D Systems, Catalogue # DY995).
- Substrate Solution: 1:1 mixture of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine) (R&D Systems, Catalogue # DY999).
- Stop Solution: 2 N H_2SO_4 (R&D Systems, Catalogue # DY994).

Appendix 8. Materials Required for Total MMP-2 ELISA (R&D Systems)

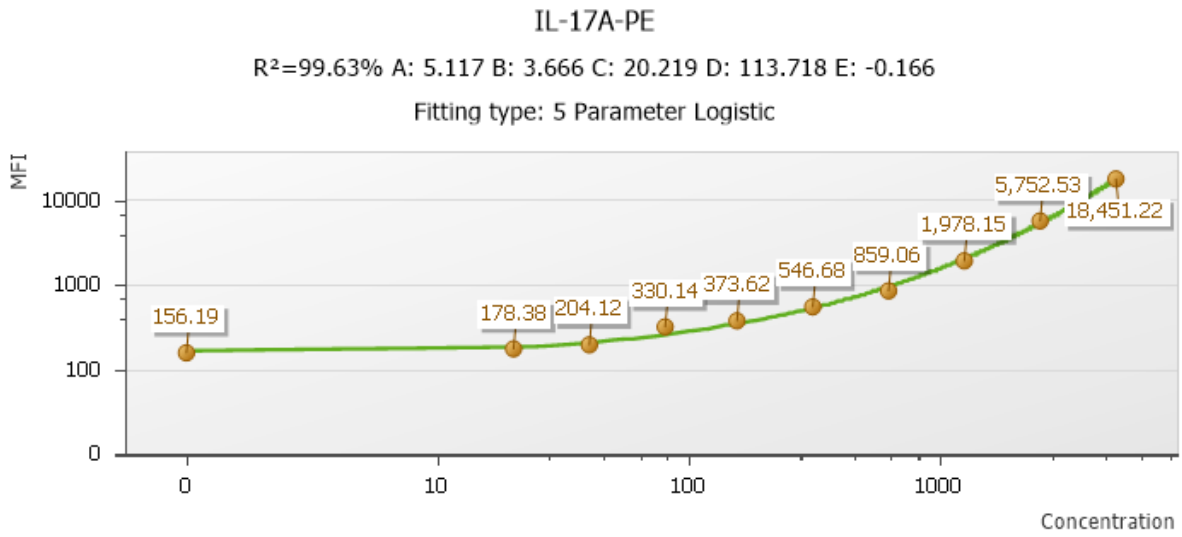
- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Pipettes and pipette tips
- 100 mL and 500 mL graduated cylinders
- Deionised or distilled water
- Squirt bottle, maniford dispenser, or automated microplate washer
- Horizontal orbital microplate shaker (0.12” orbit) capable of maintaining a speed if 500 ± 50 rpm
- Test tubes for the dilution of standards and samples
- MMP-2 Controls (Optional)

Appendix 9. Materials Required for Human Total MMP-8 ELISA (R&D Systems)

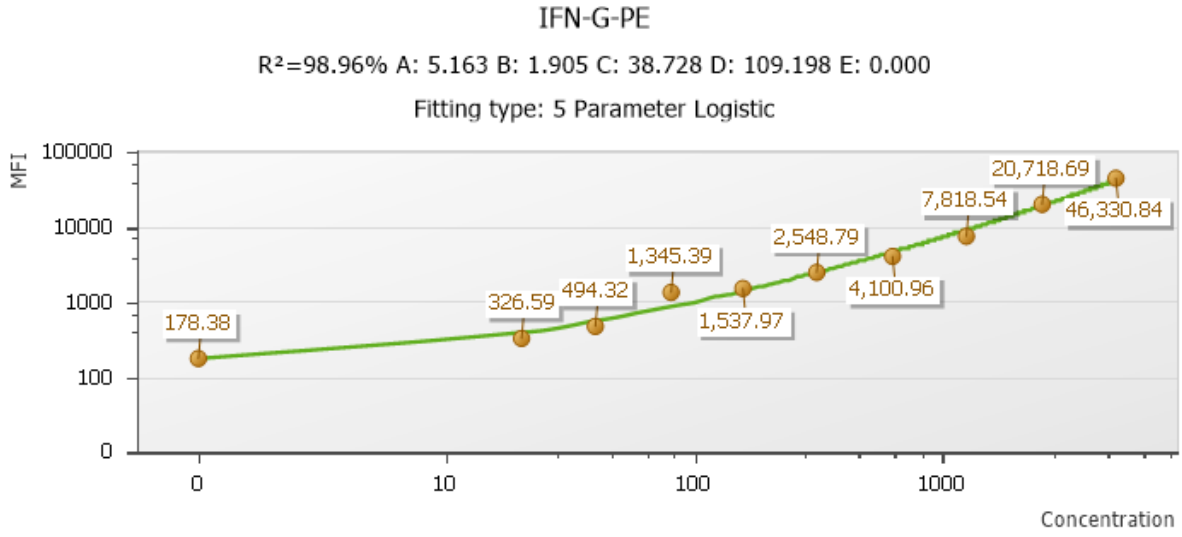
- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Pipettes and pipette tips
- 500 mL graduated cylinder
- Deionised or distilled water
- Squirt bottle, maniford dispenser, or automated microplate washer
- Horizontal orbital microplate shaker (0.12” orbit) capable of maintaining a speed if 500 ± 50 rpm
- Polypropylene test tubes for serial dilution
- MMP-8 Controls (Optional)

Appendix 10. Materials Required for Human MMP-9 ELISA (R&D Systems)

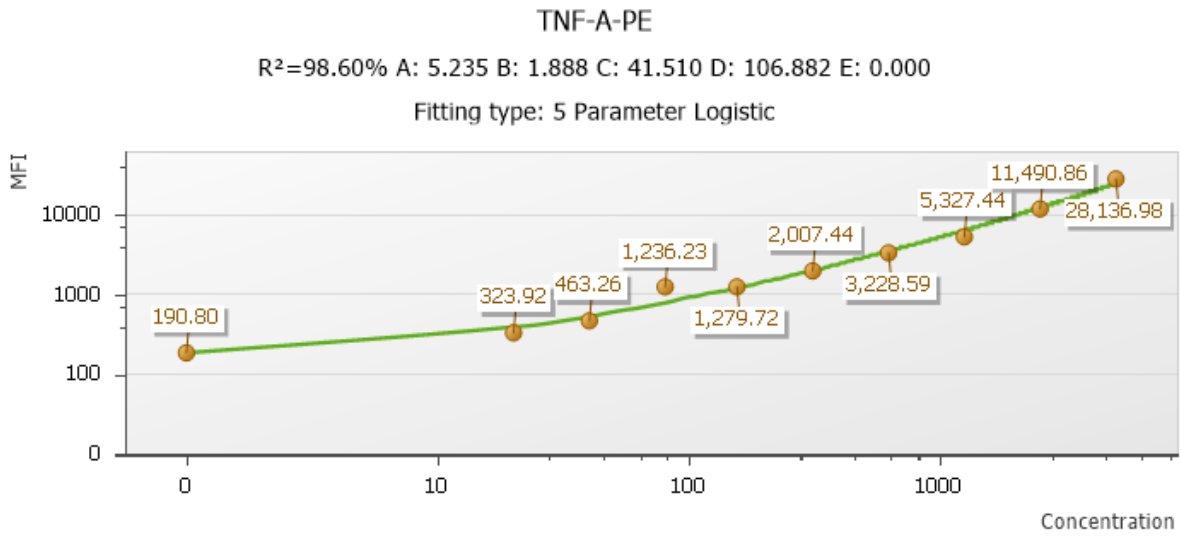
- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Pipettes and pipette tips
- 500 mL graduated cylinder
- Deionised or distilled water
- Squirt bottle, maniford dispenser, or automated microplate washer
- Horizontal orbital microplate shaker (0.12” orbit) capable of maintaining a speed if 500 ± 50 rpm
- Polypropylene test tubes for dilution of standards and samples
- MMP-9 Controls (Optional)



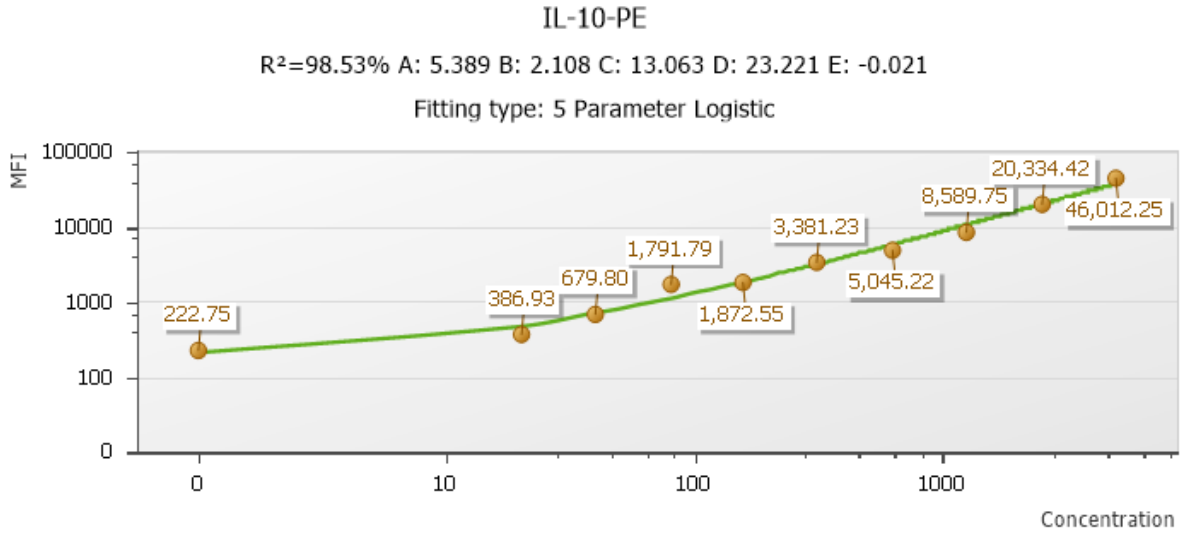
Appendix 11. Standard curve for interleukin 17A using FCAP software



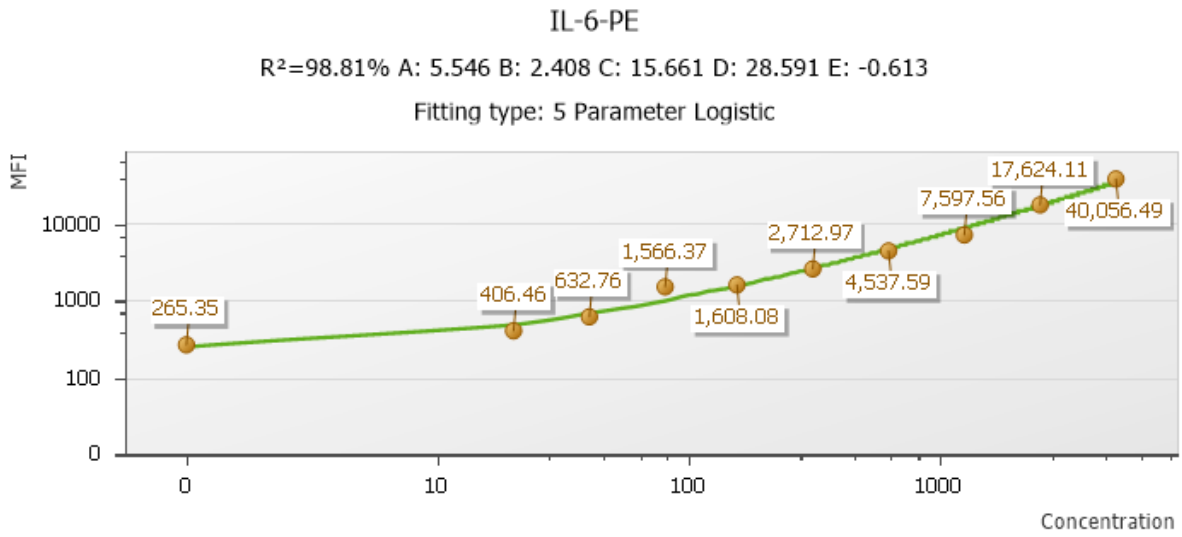
Appendix 12. Standard curve for interferon gamma using FCAP software.



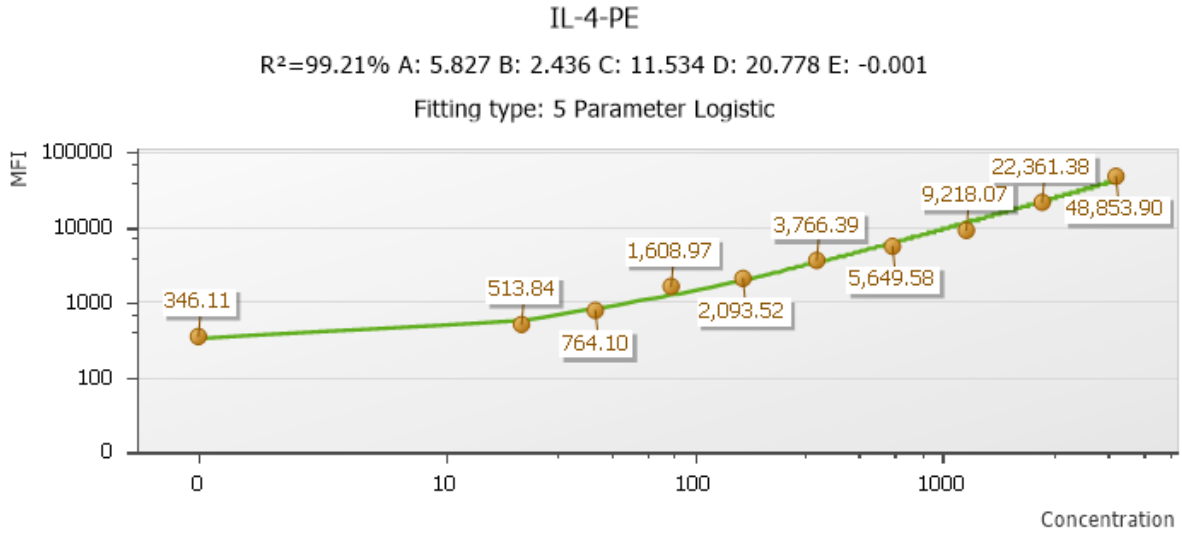
Appendix 13. Standard curve for tumour necrosis factor A using FCAP software.



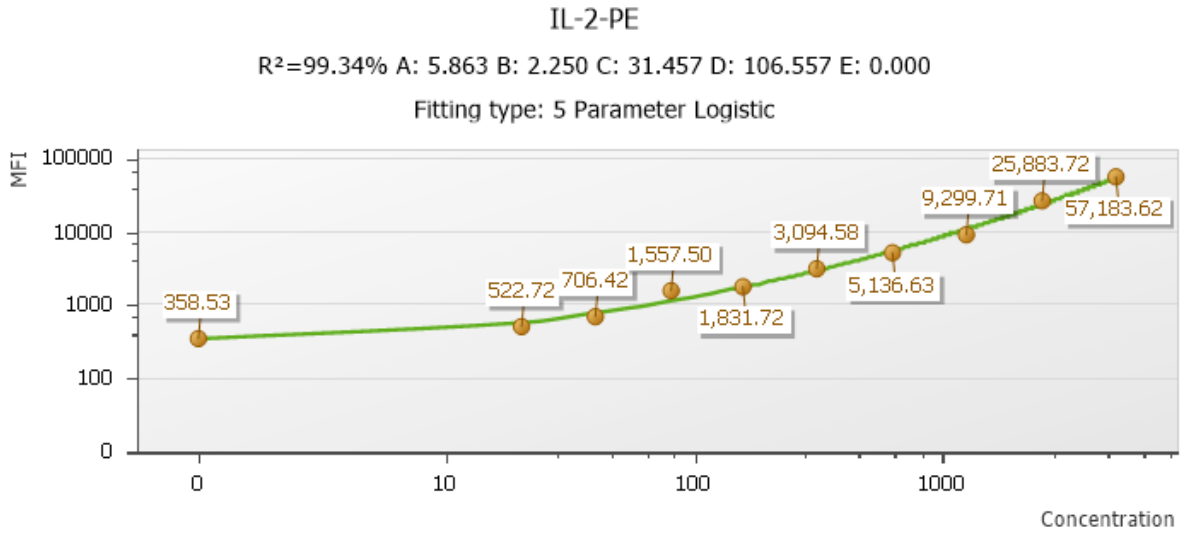
Appendix 14. Standard curve for interleukin 10 using FCAP software.



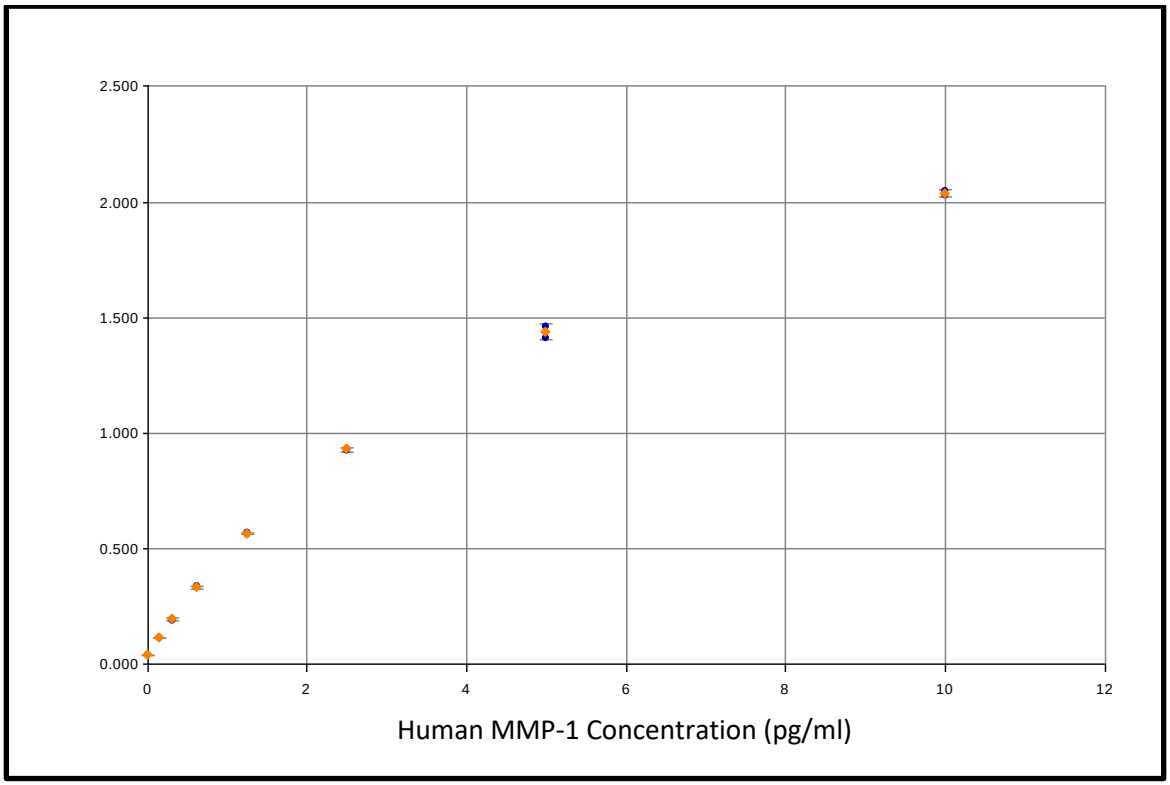
Appendix 15. Standard curve for interleukin 6 using FCAP software



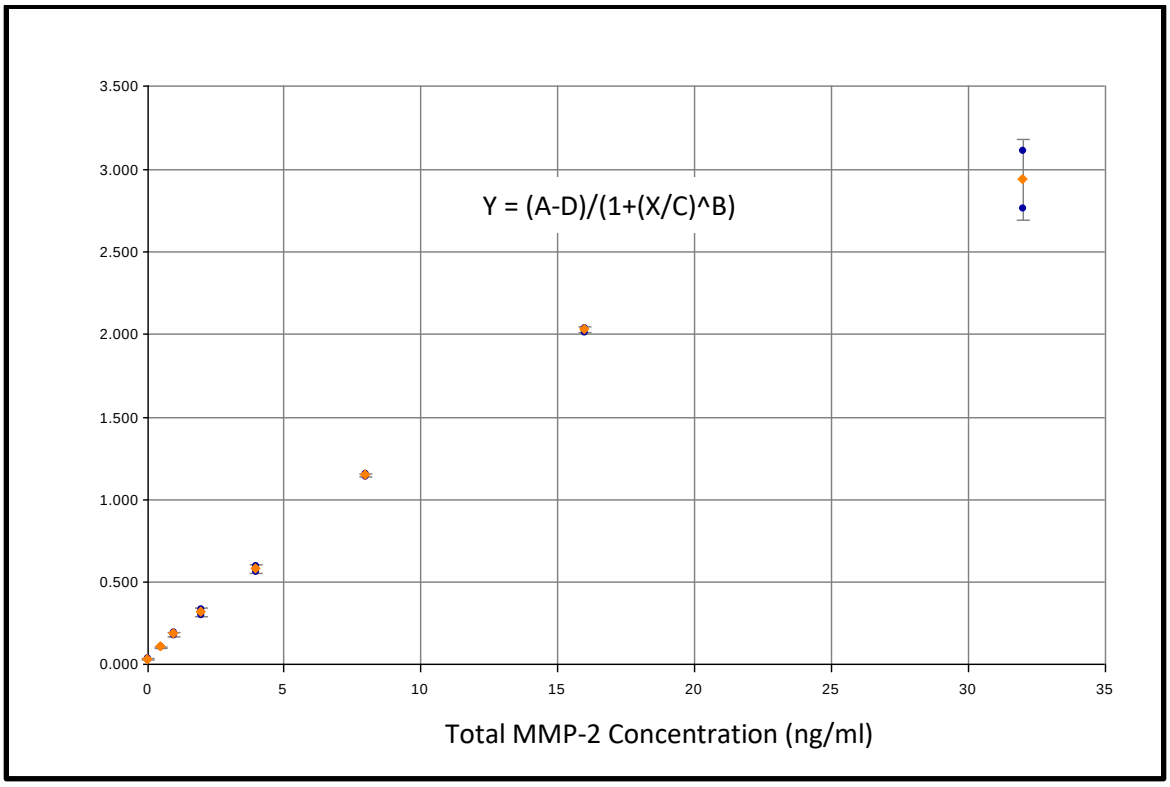
Appendix 16 Standard curve for interleukin 4 using FCAP software.



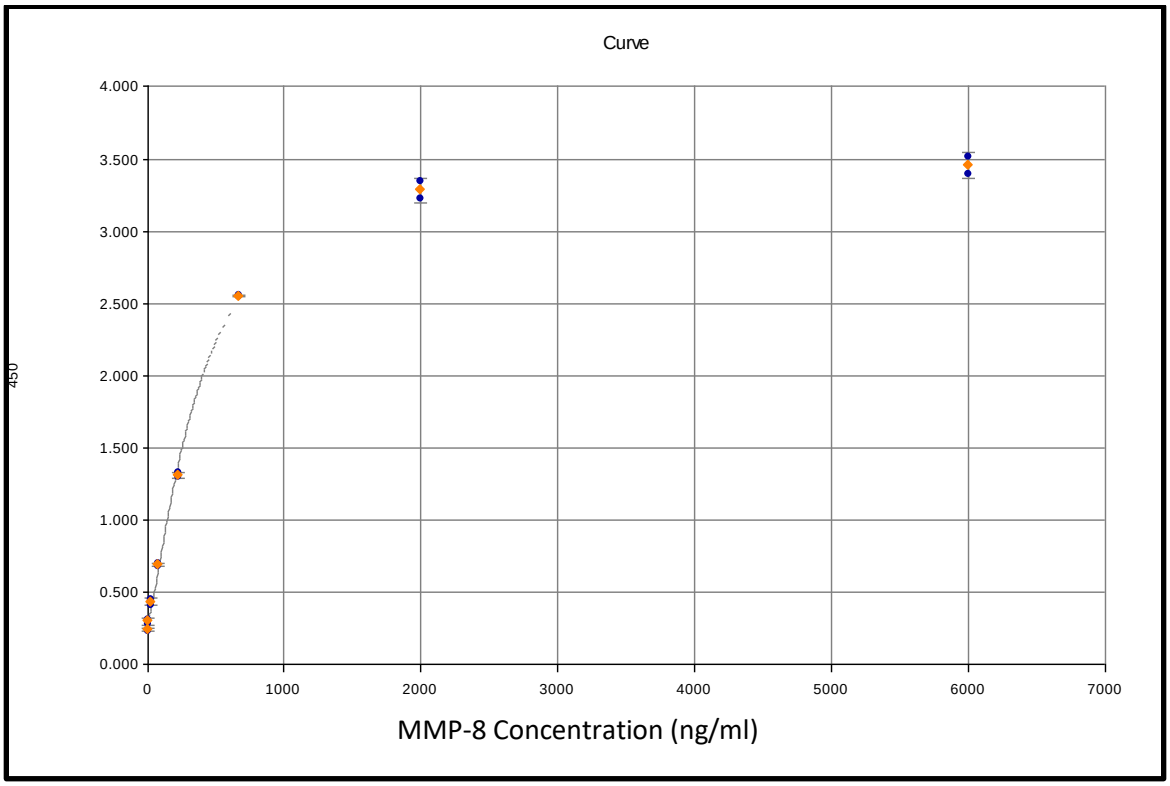
Appendix 17. Standard curve for interleukin 2 using FCAP software.



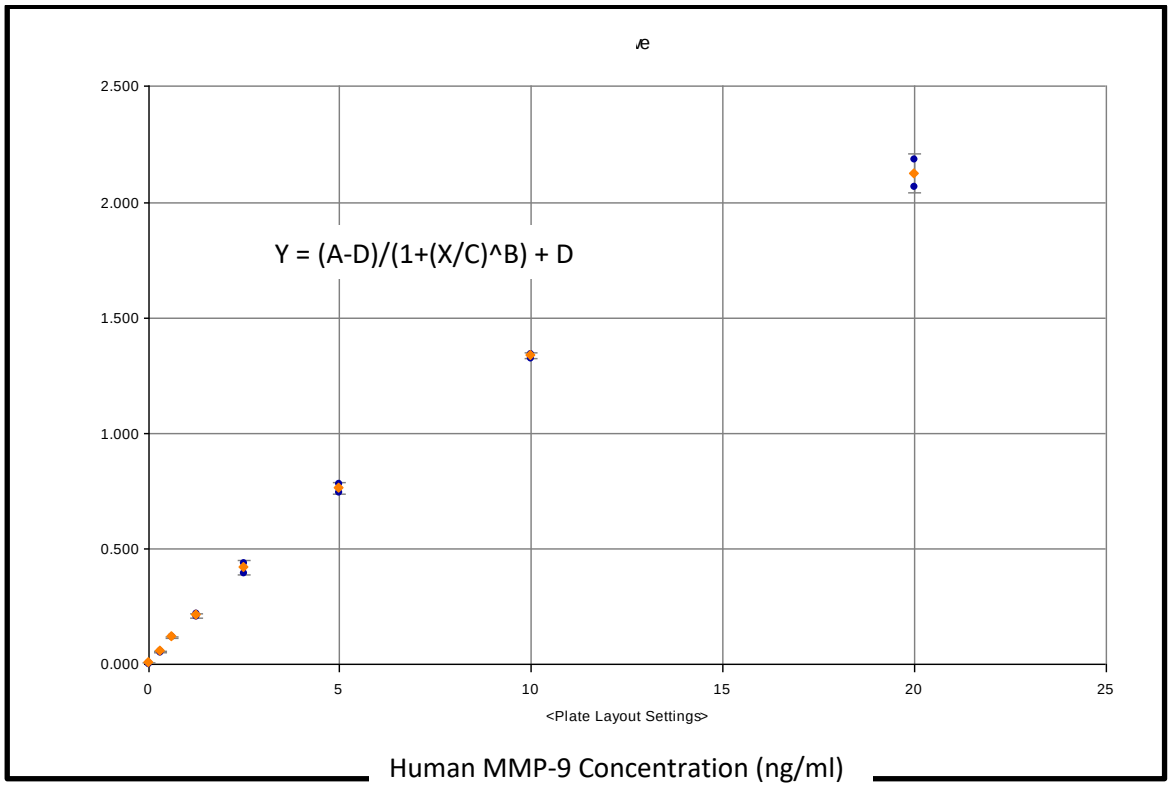
Appendix 18. Standard curve for MMP-1 ELISA



Appendix 19. Standard curve for MMP-2 ELISA



Appendix 20. Standard Curve for MMP-8 ELISA



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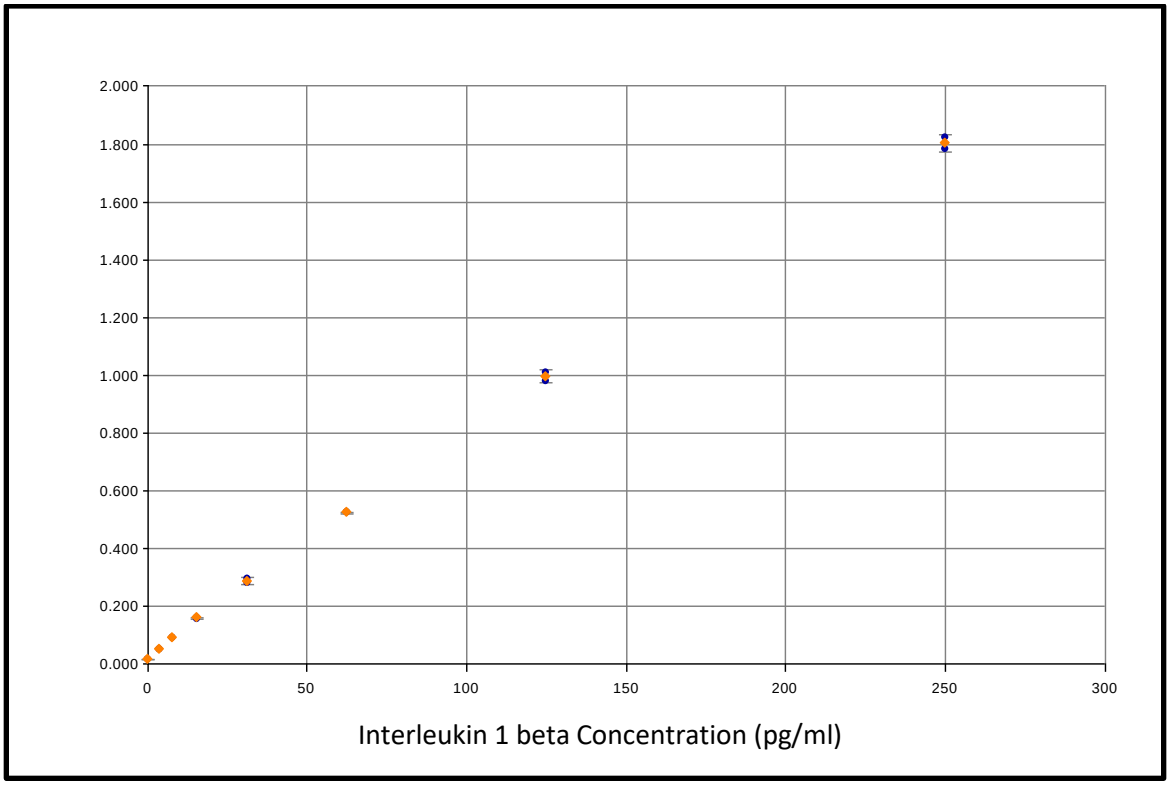
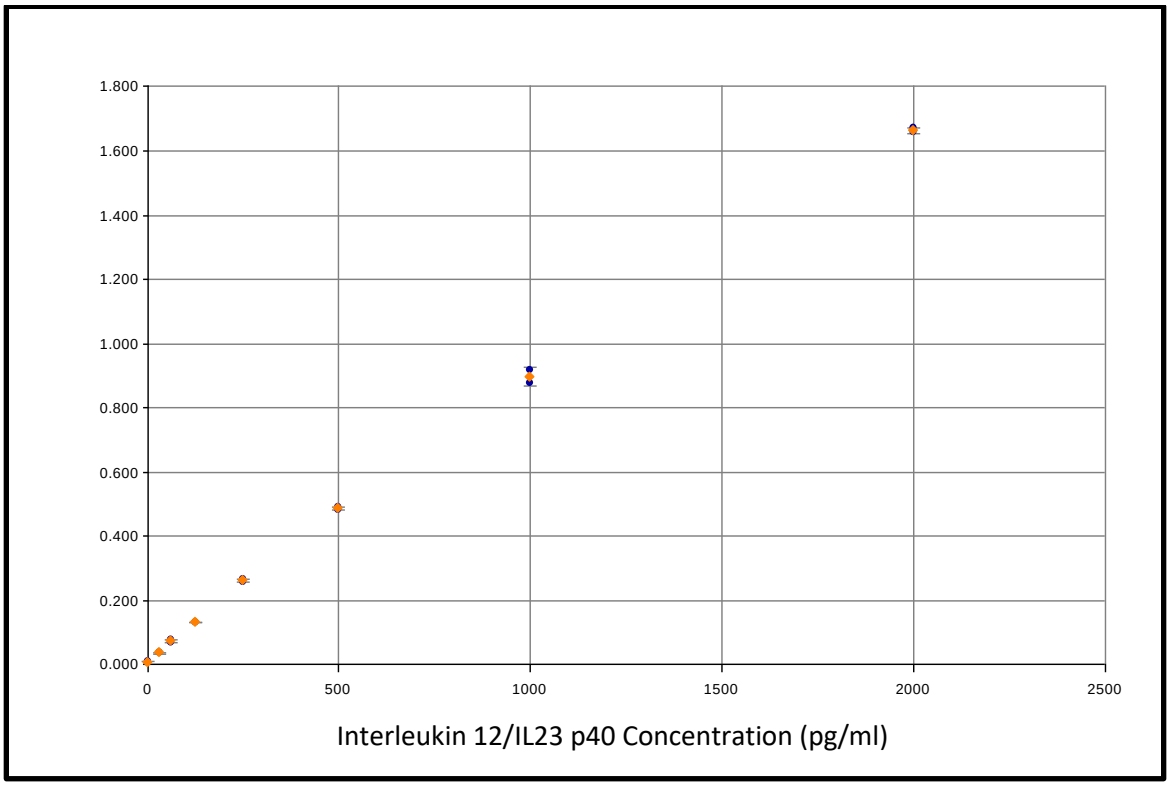
Appendix 21. Standard curve for MMP-9 ELISA.



Appendix 22. Standard curve for Interleukin 1 beta ELISA



Appendix 23. Standard curve for IL-12 ELISA