THE KINETICS OF MYCOLACTONE IN RELATION TO THE MICROBIOLOGICAL, CLINICAL AND IMMUNOLOGICAL RESPONSES TO ANTIBIOTIC THERAPY FOR *MYCOBACTERIUM ULCERANS* DISEASE.

By

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Certification

I hereby declare that this submission is my own work towards the PhD and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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ABSTRACT

Background: *Mycobacterium ulcerans* is the causative agent for a chronic necrotising skin infection called Buruli ulcer. Pathology of the disease is closely linked with the elaboration of a unique lipid toxin, mycolactone, which has potent cytotoxic and immunomodulatory properties. In this study, assays were developed to detect and quantify mycolactone concentrations in tissues during curative antibiotic therapy in mice and in humans to understand its dynamics in pathogenesis and to explore its potential as a biomarker for diagnosis and monitoring of patients with Buruli ulcer disease on antibiotic therapy. The currently recommended antibiotic regimen for the management of Buruli ulcer is a combination of daily intramuscular injections of streptomycin and oral rifampicin for 8 weeks (RS8). This regimen was compared with streptomycin/rifampicin for 2 weeks followed by clarithromycin/rifampicin (RS2RC6) for 6 weeks in patients to determine the clinical and bacteriological effectiveness in a pilot study.

Methods: Biopsies were obtained from infected human skin tissues and BALB/c mouse hind footpads before, during and after 8-weeks of rifampicin-containing combination antibiotic therapy. Lipids were extracted from tissues using organic solvents, mycolactone concentrations were measured using a cytotoxicity assay and mass spectrometry. Trends in mycolactone concentrations and clinical, bacteriological and immuno-histopathological responses were determined. Concentrations of cytokines in supernates of whole blood assays in humans or murine splenocytes after stimulation with mycobacterial antigens/T-cell mitogens were measured using ELISA.

Results: Eighty-three patients with confirmed Buruli ulcer were randomized to RS8 or RS2RC6 and monitored for recurrence free-healing. Bacterial load in tissue samples before and after treatment for 6 and 12 weeks was monitored in samples obtained by 4mm punch biopsy by semi-quantitative culture. There was no difference in using RS8 or RS2RC6 with respect to healing rate or the proportion healed in each group after 4, 8, 12, 16, 20, 24 and up to 52 weeks. The success rate was 93% in each group and there was no recurrence after 12-month follow-up. There was no difference in the number of bacteria cultured at the different time points for the two regimens. Mycolactone was detectable in 92% and 77% of human samples (n=80) using cytotoxicity assays and mass spectrometry respectively. Antibiotic therapy was associated with a decline in tissue concentration of mycolactone in both human and murine-infected tissues which was paralleled by resolution of clinical lesions, reductions in bacteriological counts and restoration of local and systemic immune responses.

Discussions/Conclusions: This study shows that mycolactone concentrations in tissues is closely associated with the presence of *M. ulcerans* and provides useful proof-of-concept data that mycolactone detection could potentially be used to monitor response to antibiotic therapy as well as for diagnosis of Buruli ulcer disease. The findings from the pilot study indicate that rifampicin combined with clarithromycin can replace rifampicin and streptomycin for the continuation phase after rifampicin-streptomycin treatment for 2 weeks without any apparent loss of efficacy. The implication is that a controlled trial of fully oral therapy using rifampicin and clarithromycin for 8 weeks (RC8) is justified.

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List of Abbreviations

AFB = Acid fast bacilli

AIDS= Acquired Immunodeficiency syndrome

APC= Antigen Presenting Cell

BCG= Bacillus Calmette-Guérin

BU= Buruli ulcer

CD= Cluster of Differentiation

CFU= Colony forming units

CFP-10= Culture Filtrate Protein-10

CTLA-4= Cytotoxic T-lymphocyte antigen 4

DNA= Deoxyribonucleic acid

ESAT-6= Early Secreted Antigen Target-6

H&E= hematoxylin and eosin

HIV= Human Immunodeficiency virus

HspX= Heat Shock Protein X

IFN-γ= gamma interferon

IL-2= Interleukin-2

IL-5= Interleukin-5

IS2404= Insertion sequence 2404

ITAMs= Immunoreceptor signaling motifs

LAMP= Loop Mediated Isothermal Amplification

LAT= Linker of activated T-cell

LC-MRM= Liquid chromatography multiple reaction monitoring mass spectrometry

LJ= Löwenstein Jensen

LPS= lipopolysaccharide

MAPK= Mitogen Activated Protein Kinase

MCP= Monocyte Chemoattractant Protein

MHC= Major histocompatibilty complex

MIP= Macrophage Inflammatory Protein

MPM= Mycolactone-producing mycobacteria

m-RNA= messenger- ribonucleic acid

MS= mass spectrometry

Mu= *Mycobacterium ulcerans*

MWJ= Mark Wansbrough-Jones

m/z = mass to charge ratio

N-WASP= Neural Wiskott-Aldrich syndrome protein

NF-κB= Nuclear Factor kappa-light-chain-enhancer of activated B cells

PBMC= Peripheral Blood Mononuclear Cells

PBS= Phosphate Buffered Saline

PCR= Polymerase chain reaction

PHA= Phytohaemagglutinin

PKS= Polyketide synthase

PLC-γ1= Phospholipase C-gamma 1

PPD= Purified protein derivative

RANTES= Regulated upon activation normal T cell expressed and secreted

RC8= rifampicin-clarithromycin for 8 weeks

Rf= Retention factor

RNA= ribonucleic acid

RP= Richard Phillips

RS2CR6=rifampicin-streptomycin for two weeks followed by rifampicin-clarithromycin for 6 weeks

RS8= rifampicin-streptomycin for 8 weeks

TB= tuberculosis

TCR= T-cell receptors

Th-1= T-cell helper-1

Th-2= T-cell helper-2

TLC= Thin layer chromatography

TLR= Toll-like receptors

TNF-α= Tumour Necrosis Factor- alpha

WHO= World Health Organisation

WASP= Wiskott-Aldrich syndrome protein

ZAP-70= Zeta-chain associated protein kinase-70

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Dedications

This is dedicated to my lovely wife, Maame and to my son “superhero” Jason. Your support and encouragement have been great!

CHAPTER 1

INTRODUCTION

1.1. Background

Mycobacterium ulcerans infection causes Buruli ulcer, a chronic necrotising skin infection with high prevalence among children in rural West Africa (WHO, 2000). *M. ulcerans* infection may manifest initially as a pre-ulcerative nodule, a plaque or as a rapidly progressing oedema which breaks down to form characteristic ulcers with undermined edges (WHO, 2000). The virulence of *M. ulcerans* is dependent on mycolactone, a lipid toxin with cytotoxic or immunosuppressive properties depending on its concentration (Pahlevan et al., 1999, Adusumilli et al., 2005, Coutanceau et al., 2005, Coutanceau et al., 2007, Torrado et al., 2007, Hong et al., 2008). Histopathology of Buruli ulcer shows clumps of extracellular bacteria surrounded by necrotic subcutaneous fat with little inflammation around the organisms (Connor and Lunn, 1965). Mycolactone, which is responsible for the necrosis, is synthesised by giant polyketide synthases and polyketide-modifying enzymes whose genes are carried on a 174-kb plasmid known as pMUM001 (Stinear et al., 2004). The resulting molecule consists of a 12-membered lactone ring with two polyketide-derived side chains. Variations in the side chains can give rise to small differences among the family of mycolactones.

Mycolactone causes a cytopathic effect on mouse fibroblasts (George et al., 1999), adipocytes (Dobos et al., 2001) and primary human keratinocytes (Bozzo et al., 2010). At lower concentrations *in-vivo* mycolactone suppresses dendritic cell, macrophage and T-cell functions by abrogating cytokine and chemokine secretion in

response to mitogens (Coutanceau et al., 2007), an effect which has been shown to be post-transcriptional (Simmonds et al., 2010). It also impairs T-cell homing by suppressing microRNA let-7b control of L-selectin (CD62-L) expression (Guenin-Macé et al., 2011) in a murine model. Data from these experiments posits mycolactone as central to the immunology and pathology of *M. ulcerans* disease. This study therefore seeks to further our understanding of pathogenesis of *M. ulcerans* disease by studying the kinetics of mycolactone in a murine model of *M. ulcerans* infection as well as in humans.

The currently available laboratory methods for confirmation of Buruli ulcer, namely microscopy for acid-fast bacilli (AFB), culture for *M. ulcerans*, polymerase chain reaction (PCR) for *IS2404* and histopathology, all have drawbacks including low sensitivity or specificity, a requirement for prolonged culture at 32°C, and a need for relatively sophisticated laboratory facilities and expertise, respectively. *M. ulcerans* is the only human pathogen known to be associated with mycolactone secretion and therefore it may be a useful diagnostic marker as well as having potential as an indicator of the presence of viable organisms in clinical samples.

The WHO recommended treatment for *M. ulcerans* disease is combination antibiotic therapy of daily oral rifampicin and injection streptomycin for 8 weeks with surgery limited to debridement and / or grafting for large ulcers where necessary (WHO, 2004). This treatment regimen is highly effective and practical in endemic communities with a low recurrence rate after treatment (Sarfo et al., 2010). However, profound differences have been demonstrated in time to healing of similar sizes of Buruli ulcers on standard antibiotic therapy with daily oral rifampicin and

intramuscular streptomycin for 8 weeks (Sarfo et al., 2010) and persistence of viable organisms or mycolactone in wounds may be important contributors to this observation. It would be useful to study in a murine model and indeed in humans, whether and how mycolactone concentrations decline during antibiotic therapy in relation to reduction in colony forming units of *M. ulcerans* and with resolution of lesions.

In spite of the promising efficacy of the current standard therapy of the combination of rifampicin and streptomycin, there is a growing desire for an all-oral antibiotic treatment regimen. This would obviate the need for the daily painful injections of streptomycin as well as reduce the risk of transmission of parenteral infections such as HIV/AIDS, hepatitis B and C, and others. A randomised clinical trial carried out in Ghana compared 8 weeks of rifampicin plus streptomycin with 4 weeks of rifampicin plus streptomycin followed by 4 weeks of rifampicin and clarithromycin and reported by an intent-to-treat analysis that 96% of patients on the standard treatment and 91% on the other arm had healed lesions at 1 year with no recurrences. (Neinhuis et al., 2010). This provided encouraging data for a potential role of an all-oral regimen. It would be useful to determine if shortening the duration of streptomycin further and replacing it with clarithromycin would maintain the efficacy of treatment. This will provide data that would justify whether an all-oral regimen would be feasible.

1.2 Research questions

This thesis addresses the following research questions:

1. Can mycolactone in *M. ulcerans* infected tissue be detected and its concentration measured?

2. What is the kinetics of mycolactone in a mouse model of *M. ulcerans* infection with and without antibiotic therapy?

3. What is the kinetics of mycolactone in human skin during curative antibiotic therapy?

4. How do the secretions of gamma interferon and interleukin-5 in the systemic circulation change during and after antibiotic therapy in humans?

1.3. Main objective

To study the kinetics of mycolactone during curative antibiotic therapy and its relationship with treatment response and the immune response to *M. ulcerans* infection.

1.4. Specific objectives

1. To develop and optimise a technique for extraction, detection and quantification of mycolactone from *M. ulcerans* infected tissue.

2. To study the kinetics of mycolactone secretion and colony forming units of *M. ulcerans* with and without antibiotic therapy in a mouse footpad model.

3. To characterise the histopathological and immunological changes in a mouse footpad model of *M. ulcerans* infection with and without antibiotic therapy.

4. To study the kinetics of mycolactone in human skin tissue during antibiotic therapy.

5. To characterise the evolution of the systemic Th-1 and Th-2 cytokine kinetics during antibiotic therapy in humans and in a murine model of *M. ulcerans* infection.

6. To compare the clinical and bacteriological efficacy of daily treatment with rifampicin-streptomycin for two weeks followed by rifampicin-clarithromycin for 6 weeks with daily treatment with rifampicin-streptomycin for 8 weeks in humans.

7. To evaluate mycolactone as a potential biomarker for *M. ulcerans* disease.

1.5. Why is the study important?

This thesis addresses key questions on the role of mycolactone in the pathogenesis of *M. ulcerans* disease, provides proof-of-concept data on the potential for mycolactone detection for diagnosing Buruli ulcer disease and assesses whether mycolactone kinetics could be used to optimise the duration of antibiotic therapy for *M. ulcerans* disease. The impact of mycolactone on evolution of immune response is also studied at the systemic level in both murine and human hosts with and without antibiotic therapy. This is important in furthering our understanding of how a protective immunity develops in *M. ulcerans* disease with implications for future vaccine development. A pilot trial comparing the bacteriological and clinical efficacy of the WHO recommended antibiotic regimen with rifampicin-streptomycin for two weeks followed by rifampicin-clarithromycin for 6 weeks was conducted in this study. By evaluating the efficacy of this regimen, this study also provides data

towards the development of a fully oral regimen for the clinical management of Buruli ulcer disease.

1.6. Justification for this study and methods employed

(A) Why develop assays for mycolactone detection and quantification? Mycolactone is a polyketide lipid with poor immunogenicity, hence approaches to its detection and quantification from infectious foci have been hampered to date. In the present study, mycolactone detection was approached using two chemical methods namely thin layer chromatography and mass spectrometry. Thin layer chromatography using organic solvents allowed lipids to be separated on the column according to their hydrophilic-hydrophobic interactions with synthetic mycolactone as a ladder for detection of mycolactone in lipid extracts from human and murine tissues. Mass spectrometry relied on the identification of a mass/charge (m/z) ratio of mycolactone after separation in a high performance liquid chromatography column. The m/z of mycolactone A/B of 765 was already known, however, specificity was improved by fragmentation on mass spectrometry to identify daughter ions which were specific for mycolactone.

Quantification of mycolactone was approached with a cytotoxicity assay which was optimised for this study and also by mass spectrometry. The cytotoxicity assay was based on the known cytotoxic property of mycolactone on cultured cell lines such as fibroblasts. This cytotoxic effect followed a dose-response relationship which was exploited to determine the quantity of mycolactone in a lipid extract from infected tissue. The mass spectrometry technique employed was the multiple reaction monitoring method. Here, the area under the curve of the m/z peaks

corresponding to the parent mycolactone A/B molecule and that of the core lactone ring were used to construct calibration curves from serial dilution of synthetic mycolactone. The lower limits of detection by mass spectrometry and cytotoxicity assays were 10ng/ml and 1.25ng/ml, respectively, making these 2 assays sensitive for both detection and quantification of mycolactone. To put it in context, 100μg of purified mycolactone was required to cause overt skin ulcers in guinea pigs but not doses below 10μg (Kathleen et al. 1999). This suggests that if mycolactone is present at concentrations high enough to cause disease, the techniques described above should be able to detect and quantify it in infected tissues.

(B) Why study the kinetics of mycolactone in-vivo? Various *in-vitro* and *in-vivo* murine studies have shown that mycolactone is central to tissue destruction and immune suppression that is characteristic of *M. ulcerans* disease. Many of these studies have extrapolated their findings to human subjects to explain the pathogenesis of *M. ulcerans* disease. However, direct evidence of the presence of mycolactone in human tissues and, indeed, the concentrations required for virulence in humans is largely lacking due to the challenges of measuring mycolactone concentration. Even though, it would be ideal to study mycolactone kinetics in humans, there is a limitation to the number and extent of biopsies one could harvest from human tissue given that surgical excision of Buruli lesions is not the preferred standard modality of treatment as at now. Hence, in human participants, only a limited tissue using a 4mm punch biopsy was obtained to study mycolactone concentrations. Furthermore, human subjects present with *M. ulcerans* disease at various stages and forms of the disease giving rise to a heterogeneity in clinical presentations.

Thus to better characterise the evolution of mycolactone secretion during the course of *M. ulcerans* infection, a murine footpad model of *M. ulcerans* infection was employed where serially-timed samples was obtained to answer these questions. Furthermore, the mouse model was suitable to monitor the impact of antibiotic therapy on mycolactone production, since antibiotics are thought to block mycolactone production and reverse local immunosuppression leading to the restoration of an active inflammatory process (Peduzzi, et al. 2007). These studies are important particularly to evaluate whether mycolactone could serve as potential biomarker for *M. ulcerans* disease.

(C) Why compare the bacteriologic and clinical efficacy of standard antibiotic combination of rifampicin-streptomycin for 8 weeks (RS8) with rifampicin-streptomycin for two weeks followed by rifampicin-clarithromycin for 6 weeks (RS2CR6)? Clarithromycin is bacteriostatic whereas streptomycin is bactericidal for *M. ulcerans* in mouse footpad models (Stanford et al., 1972, Dega et al., 2000, Bentoucha et al., 2001, Sizaire et al., 2006). An all-oral regimen of clarithromycin-rifampicin for 8-weeks (RC8) has been proposed by the WHO as a replacement for the currently recommended RS8 subject to evidence from a randomised controlled trial. However because the safety and efficacy of RC8 has not been fully assessed in comparison with RS8, it was thought that a pilot study of RS8 vs RS2RC6 would provide useful clinical and bacteriologic data to help inform a decision to go ahead with a comparison of RC8 with RS8 in a randomised controlled trial. Thus, the 2 weeks lead-in period of treatment of the bactericidal combination of streptomycin-rifampicin would help achieve bacteriologic killing which would be maintained by a 6 weeks continuation with an all-oral regimen of clarithromycin-rifampicin

combination. Although a pilot study, the patients were randomised to receive either the standard treatment or the alternative regimen to minimize bias. The bacteriological end-point was chosen to assess the bacteriologic efficacy of the alternative regimen compared with the standard regimen given that clarithromycin is considered bacteriostatic.

1.7. Structure of thesis

This dissertation is made up of five chapters. The introductory chapter (chapter 1) states the background, research questions, objectives, importance, and justification for conducting this study. In chapter two, literature on historical perspectives, microbiology, transmission, and immunology of *M. ulcerans* is reviewed with greater emphasis on the role and mechanisms of action of mycolactone in pathogenesis and concludes by evaluating the evidence in support of efficacy of antibiotic treatment for *M. ulcerans* disease, which is a paradigm shift from surgical management. Chapter three describes the methodology and materials used in this study as well as statistical considerations in analysis of results. This is followed by chapter four, where the results of this study are presented under three major themes: mycolactone detection and quantification; the kinetics of mycolactone in relation to clinical, microbiological, histological and immunological responses with and without antibiotic therapy in mouse model of *M. ulcerans* infection; and the kinetics of mycolactone detection in humans in relation to clinical, microbiological and immunological responses with antibiotic therapy. In chapter five, a discussion of the

results is done as well as recommendations and conclusions on the basis of these results.

CHAPTER 2

LITERATURE REVIEW

2.1 Historical perspectives

Mycobacterium ulcerans infection produces progressive skin ulceration in man and other mammals. *Mycobacterium ulcerans* disease is also commonly known as Buruli ulcer and was first described by Sir Albert Cook in 1897 in Uganda (Cook. 1970). However, the first detailed description was in 1948 by MacCallum and Colleagues who established the aetiology in a small group of patients in Australia, although contact with their first patient was in 1940. After initial failed attempts at cultivation, *M. ulcerans* was identified by coincidence when an incubator broke down which was normally set at a temperature of 37^{o}C (MacCallum et al. 1948). Later in 1950, the first report in Africa came from Zaire but there are suggestions that the disease had been in Zaire since 1935 (Meyers et al. 1974). The Uganda Buruli Group coined the name "Buruli ulcer" from the Buruli County, near Lake Kyoga in Uganda where the first large number of patients were described (Dodge et al. 1962, Lunn et al. 1965).

2.2 The causative organism of Buruli ulcer

M. ulcerans, the causative organism for Buruli ulcer, is a slow-growing environmental mycobacterium that falls into a group of closely related mycobacterial pathogens which comprise the *M. marinum* complex (Stinear et al., 2000). The *M. marinum* complex contains mycobacterial species pathogenic for aquatic vertebrates and include *M. marinum* (fish), *M. pseudoshottsii* (fish), and *M. liflandii* (frogs) (Rhodes et al. 2005, Mve-Obiang et al. 2005, Ranger et al. 2006, Kaser et al. 2009).

These species are commonly characterised by slow growth rates and low optimal growth temperatures (Garrity 2001). For instance, *M. ulcerans* can be isolated from primary lesions after a 5-8 week incubation period, although up to 6 months may be required (WHO 2000; Yeboah-Manu et al. 2004).

From a genomic standpoint, the species in the *M. marinum* complex can be considered a single species based on the fact that they share over 97% identity in the 16sRNA gene sequence (Stinear et al. 2007). Genomic analysis suggests that *M. ulcerans* evolved from an *M. marinum*-like ancestor (Stinear et al. 2007; Demangel et al. 2009) through the acquisition of a large virulence plasmid and accumulation of multiple copies of insertion sequences, IS*2404* and IS*2606.* The genome has undergone considerable reductive evolution through a number of mutational events leading to, for instance, the loss of a number of genes in ion transport and lipid biosynthesis compared with *M. tuberculosis* or *M. marinum*. These changes in genomics suggest that *M. ulcerans* is undergoing an adaptation to a different and narrower niche than *M. marinum* which may be relevant for the ecology and transmission of *M. ulcerans*.

A key phenotypic feature of *M. ulcerans* is the low optimal growth temperature and the extremely restricted growth temperature range of between 28-34°C (Boisvert et al. 1977). The restricted growth temperature of *M. ulcerans* is thought to play a substantial role in the pathogenesis of Buruli ulcer by limiting infection to the skin. The organism has not been isolated from internal organs of human patients or from the bone in cases of osteomyelitis, or from the internal

organs or blood of experimentally infected animals (Fenner 1956; Hong et al. 2008; Vandelannoote et al. 2010).

The generation time of *M. ulcerans* is 20 hours and is similar to that of other slow growing mycobacterial species (Wayne et al. 1992). Löwenstein Jensen (L-J) medium is the preferred solid medium for cultivation but Middlebrook 7H10 and 7H11 can also be used (Portaels et al. 2001). Cultivation has also been done in media containing albumin: Dubos albumin medium (Krieg et al. 1974; Read et al. 1974; Hockmeyer et al. 1978) and Middlebrook 7H9 medium supplemented with oleic, albumin, dextrose and catalase (OADC) (George et al. 1998; George et al. 1999). Mve-Obiang et al. in 1999 described Sauton medium and modified Reid medium for the study of secreted mycobacterial proteins and toxin studies but other workers have used Middlebrook 7H9 (M7H9) broth supplemented with tryptose and glucose for similar work (George et al. 1999; Dobos et al. 2000). The optimal pH for growth of *M. ulcerans* lies between 5.4 and 7.4 (Portaels et al., 1982) and it has been suggested that microaerophilic conditions (2.5-5.0% oxygen) could improve growth yield (Portaels et al., 1982). Temperature requirement for cultivation is 30 to 33^{o}C (MacCallum et al., 1948).

2.3 Epidemiology of *M. ulcerans* disease

M. ulcerans is now the third most common mycobacterial pathogen of humans, after *M. tuberculosis* and *M. leprae* (which cause tuberculosis and leprosy, respectively) . Buruli ulcer is classified as a neglected tropical disease that has been reported in 33 countries from Africa, the Americas, and the Western Pacific (WHO, 2000). The main burden of disease falls on children living in sub-Saharan Africa, but

healthy people of all ages, races, and socioeconomic classes are susceptible. The majority of cases occur in tropical and subtropical regions, although, cases have been reported in Australia, China, and Japan (WHO, 2000). Most cases are from West Africa notably Benin, Cote d'Ivoire and Ghana. Cote d'Ivoire is the most affected country reporting over 2500 cases per year. Globally, between 5000-6000 cases are reported every year from 15 of the 33 countries but considerable under-reporting exists within countries (WHO, 2000).

Buruli ulcer disease is characteristically focal in distribution even within endemic regions making an accurate assessment of disease burden difficult. In some highly endemic districts in Ghana, point prevalence has been estimated to be as high as 150.8/100,000 individuals (Amofa et al. 2002) with prevalence per 100,000 population in the Amansie West, Asante Akim North and Upper Denkyira districts of 151, 132, and 115 respectively (Amofa et al. 2002). In southern Benin, a study has reported detection rates of 21.5/100,000 per year, higher than for either tuberculosis or leprosy (Debacker et al. 2004). In West Africa, nearly 50% of people affected are children below 15 years old and until antibiotic therapy became available mostly were left with permanent disabilities (WHO, 2000). In Australia, the disease remains uncommon, but there have been increases in both incidence and the number of endemic areas (Johnson et al. 1996).

2.4 Transmission of *M. ulcerans* infection

Buruli ulcer has been referred to as mysterious disease because the exact mode(s) of transmission remains unclear although several hypotheses have been proposed. The disease has been widely associated with proximity to aquatic habitats.

For example, Buruli ulcer is highly associated with residence along several major river systems in Ghana and Benin (Amofa et al. 1993; WHO 2000; Amofa et al. 2002; Johnson et al. 2005; Sopoh et al. 2007), whereas the disease is essentially non-existent in communities within the dry savannah regions. Direct cultivation of *M. ulcerans* in environmental samples to provide evidence linking the ecology and distribution of the pathogen and disease has been challenged by the slow growth rate of *M. ulcerans* and the complex mix of many faster growing bacteria and fungi. A major breakthrough occurred with the development of the first PCR probes for *M. ulcerans* based on detection of IS*2404* by Ross et al. in 1997. A number of laboratories rapidly employed this method leading to the identification of *M. ulcerans* DNA in environmental samples including detritus, soil, biofilms, water filtrates, fish, frogs, snails, insects and a host of other invertebrates and mammals (Portaels et al. 1999; Stinear et al. 2000; Portaels et al. 2001; Marsollier et al. 2002; Eddyani et al. 2004; Marsollier et al. 2004a; Marsollier et al. 2004b; Kotlowski et al. 2004; Trott et al. 2004; Johnson et al. 2007; Williamson et al. 2008; Benbow et al. 2008; Portaels et al. 2008; Fyfe et al. 2007; and Fyfe et al. 2010).

In two different countries in Africa, Williamson et al. (2008) and Vandelannoote et al. (2010) found *M. ulcerans* DNA in 9.7% (8/82) and 7.7% (1/13) of water filtrant samples from endemic communities. Also in Australia, Fyfe et al. (2010) reported that 30% of selected samples including detritus, plant material, suspended solids, and soils collected from one highly-endemic area were weakly positive by quantitative PCR but in low endemicity areas only 3% (4/156) of samples were positive. The major caveat with the use of IS*2404* PCR on environmental samples is that PCR detects DNA, not intact organisms hence it does

not provide definitive proof for the presence of intact bacteria in a matrix. A major achievement was the cultivation of *M. ulcerans* from an aquatic water bug collected in Benin (Portaels et al. 2008) providing definitive evidence for the presence of *M. ulcerans* from an aquatic invertebrate.

The specific route and / or modality of transmission of *M. ulcerans* from the environment to the human host to establish an infection is being intensively investigated by several investigators. Portaels and colleagues (1999) were first to suggest that aquatic bugs (Hemiptera) might be reservoirs of *M. ulcerans* in nature and subsequently described the first isolation of a pure culture of *M. ulcerans* from a water strider (Hemiptera: Gerridae, *Gerris* sp.) (Portaels et al. 2008). Marsollier and colleagues (2002; 2003; 2004; 2005; 2006; 2007) through a series of laboratory experiments demonstrated that *M. ulcerans* could survive and show limited replication within the salivary glands of biting aquatic bugs (Naucoridae: *Naucoris cimicoides*). In their experimental model they demonstrated that *M. ulcerans* could be acquired from feeding on inoculated insect prey (a blow fly maggot), and transmitted to mice via biting; and that the mice subsequently developed clinical Buruli ulcer (Marsollier et al. 2006). Marsollier and colleagues (2007) concluded that biting water bugs belonging to the families of Naucoridae (creeping water bugs) and Belastomatidae (giant water bugs) could be considered reservoirs, and most importantly could serve as vectors in the transmission of *M. ulcerans* to humans in nature. However, on the basis of the biology and behaviour of these predaceous aquatic insects, biting humans appears to be a rare event associated with a purely defensive reaction of these bugs (Haddad et al. 2009 and Scahaefer et al. 2000).

A conceptual model of *M. ulcerans* transmission initially propounded by Portaels et al. (1999) and expanded on by Merritt et al. (Merritt et al. 2005) and Marion et al. (Marion et al. 2010) is described as follows: *M. ulcerans* present in mud, detritus, water filtrants, and plant biofilms can be picked up and concentrated in grazing or filtering aquatic insects (eg. midges and mosquito larvae) or other invertebrates (snails, crustaceans, plankton) through their feeding activities. Then, predatory aquatic vertebrates (i.e., some fish) and invertebrates (e.g., true bugs, beetles and dragonfly larvae) feed on other invertebrate prey or small fish, serving to move *M. ulcerans* from prey to biting insects. Lastly, aquatic insects capable of flight, and birds that prey on fish and/or aquatic invertebrates may potentially disseminate *M. ulcerans* to other aquatic environments (Merritt et al. 2005; Merritt et al. 2010). Within this model humans may come into contact with *M. ulcerans* via several potential vectors through their activities in endemic communities.

2.4.1 Risk factors for Buruli ulcer

A number of epidemiological studies have identified potential risk factors associated with *M. ulcerans* disease. Jacobson and Padgett (2010) systematically reviewed the risk factors associated with *M. ulcerans* infection throughout the world and identified failure to wear protective clothing, living or working near water bodies and poor wound care as common risk factors reported in most studies. A review of these potential risk factors suggests that transmission of *M. ulcerans* might occur through direct inoculation of bacteria into skin via contact with environmental sources, insect bites or trauma, however further comparative studies are needed to clarify the potential modes of transmission of *M. ulcerans*.

2.5 Clinical features of *M. ulcerans* disease

Mycobacterium ulcerans disease (Buruli ulcer) manifests as a painless skin nodule, papule, plaque or oedematous lesion prior to ulceration (Evans et al. 2003). Rapidly progressive oedematous disease is the most acute form of *M. ulcerans* disease, which previously was managed by aggressive surgery with wide excision of the lesion to include all the oedematous tissue (Figure 2.1 to Figure 2.3). Classically, ulcerative lesions may have undermined edges with subcutaneous spread producing nodules of induration.

Lesions may occur at any location but a predilection for affecting limbs have been noted. Interestingly, in spite of presence of large ulcers, patients are systemically well which may explain why sufferers seek medical help late. At advanced stages of the disease osteomyelitis associated with Buruli ulcer may develop. Severity of lesions is assessed using diameter of lesions: category I- <5cm in widest diameter, category II- between 5cm and 14.9 cm and category III- more than 15cm in widest diameter. The natural history of the disease is not well described but in a randomized controlled trial of treatment with clofazimine 30% of patients in whom treatment was withheld went on to healing without recurrence and without surgery (Revill et al. 1973). Lesions heal with scar formation and often deformity but death from the disease is rare.

Figure 2.1. Nodular form of Buruli ulcer.

A painless, subcutaneous swelling over the shoulder of 2 weeks duration in a 13-year old boy. Source: A patient from Tepa Government Hospital, Ghana (personal).

Figure 2.2 Typical oedematous form of Buruli ulcer.

This is the most acute form of Buruli ulcer characterized by non-pitting painless swelling. A 10-year-old with a lesion of 4 weeks.

Source: A patient from Tepa Government Hospital, Ghana (personal).

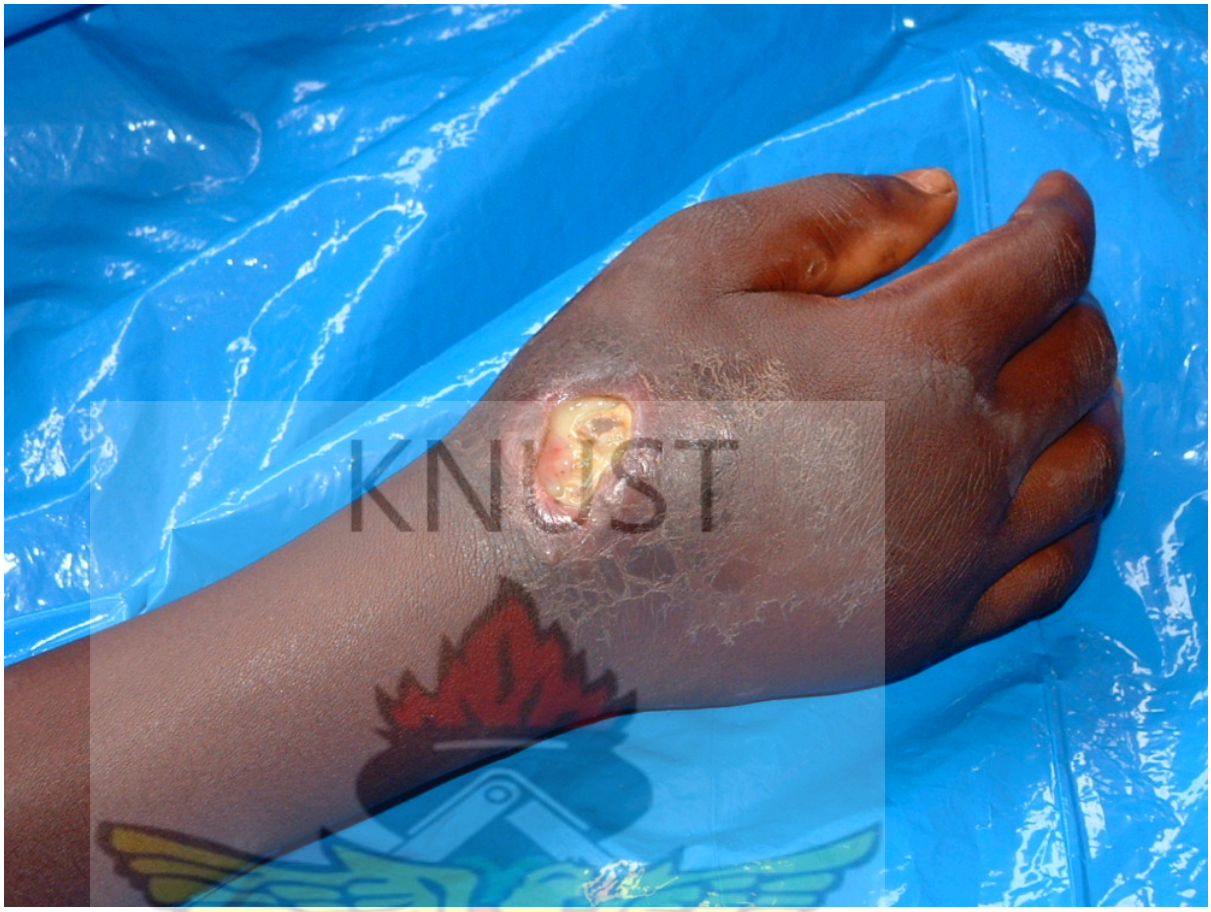


Figure 2.3. Ulcerative form of Buruli ulcer.

A typical painless ulcerative *M. ulcerans* disease with undermined edges in an 8-year old boy.

Source: A patient from Tepa Government Hospital, Ghana (personal).

2.6. Pathological features of *M. ulcerans* disease

M. ulcerans causes a subcutaneous infection leading to a panniculitis (inflammation of the subcutaneous tissue). Histological specimens typically show large clumps of extracellular acid-fast organisms surrounded by areas of necrosis and a poor or absent inflammatory response (Hayman et al. 1985). Janssens et al. (1959; 1972) described an oedematous form of the disease in Belgian Congo patients and noted that there was subcutaneous calcification but no involvement of the lymph nodes even in patients with extensive lesions.

Connor (1965) gave a description of 38 Ugandan cases and detailed three histopathological stages: a stage of necrosis with widespread tissue destruction, many micro-organisms and little or no inflammatory response; an organising stage with large numbers of macrophages, plasma cells, and lymphocytes collecting at the margin of the necrotic fat, with proliferating fibroblasts and capillaries growing into the coagulum; in the final healing stage, the reaction becomes granulomatous as free lipid and necrotic cell debris are phagocytosed with few if any mycobacteria found in sections of lesions (Connor et al. 1965 and Connor et al. 1966). Vascular changes noted around the perimeter of the necrotic area in *M. ulcerans* infected tissue include eccentric intimal thickening in arteries, which may also be infiltrated by chronic inflammatory cells. Occasionally there is intense infiltration by lymphocytes, oedema of the muscularis, and thrombotic obliteration of the lumen. A detailed descriptive account of the histopathological features of *M. ulcerans* can be found in a review by Hayman et al (1993).

2.7. Pathogenesis of *M. ulcerans* disease

The histological hallmark of *M. ulcerans* infection (shown in Figure 2.4) is that despite extensive necrosis, lesions are painless and there is minimal histological evidence of an initial acute inflammatory response. In contrast to other pathogenic mycobacteria, which are facultative intracellular parasites of macrophages, *M. ulcerans* occurs in lesions primarily as extracellular microcolonies. A peculiar feature of Buruli ulcer pathology is the observation that organisms lie a necrotic focus with the necrosis extending some distance from the site of bacterial colonisation, leading to the hypothesis that *M. ulcerans* probably secreted an exotoxin (Connor et al. 1966).

Figure 2.4. Histopathology of Buruli ulcer. Clumps of extracellular acid-fast bacilli lie in necrotic foci with scanty inflammatory cells.

Read et al. (1974) reported that a sterile filtrate of *M. ulcerans* produced a cytopathic effect on cultured murine fibroblasts. In that study sterile filtrate from other environmental mycobacteria did not elicit cytopathicity (Read et al. 1974). Also Pimsler et al. (1998) showed that sterile filtrates from *M. ulcerans* had immunosuppressive properties. However, initial efforts to isolate the putative toxin were unsuccessful (Hockmeyer et al. 1978 and Krieg et al. 1974).

In 1999, a major breakthrough occurred with the purification and isolation of a toxin among acetone soluble lipid fraction of lipid extracts from *M. ulcerans* bacterial pellets (George et al. 1999). This lipid toxin designated as mycolactone was a polyketide-derived macrolide and was shown to produce cytopathicity on cultured L929 murine fibroblasts and cell cycle arrest in the G_0/G_1 phase of the cell cycle. Furthermore, intradermal inoculation of purified toxin into guinea pigs produced a lesion similar to that of Buruli ulcer in humans. The lipid toxin was called mycolactone to reflect the mycobacterial source and chemical structure.

2.8. Mycolactone and pathogenesis of *M. ulcerans* disease.

Mycolactone is composed of an invariant 12-membered macrolactone to which two polyketide-derived highly unsaturated acyl side chains are attached as shown in Figure 2.5.

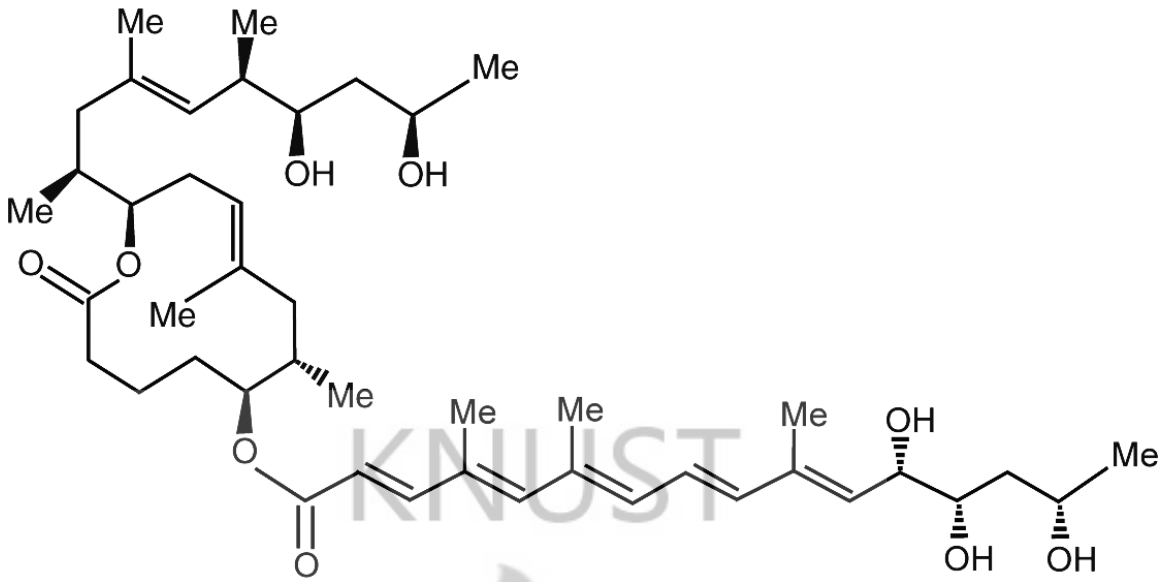


Figure 2.5. Molecular structure of mycolactone showing a core cyclic lactone ring and polyketide-derived highly unsaturated acyl side chains.

Source: Institut Pasteur

The initial molecule reported by George et al. (1999) was identified on thin layer chromatography as a light yellow, ultraviolet-active lipid band with a retention factor value of 0.23 in a solvent system containing reversed-phase high-performance liquid chromatography (as shown in Figure 2.6A). Mass spectrometric analysis of the toxin under microspray conditions showed a strong peak at *m/z* 765 (Figure 2.6B) with a formula of $C_{44}H_{70}0_9Na$ corresponding to the sodium adduct of mycolactone. Serial dilutions of mycolactone up to 25pg/ml when added to culture of L929 fibroblasts led to rounding up of cells within 24 hours and detachment from the plate by 48 hours confirming the cytopathic effect of mycolactone (Figure 2.6C). Finally, injection of 100μg of purified mycolactone intradermally into Hartely

guinea pigs resulted in the appearance of a dark, necrotic, open ulcer by day 5. Histopathological examination of the ulcerative lesion produced by injection of mycolactone revealed it to be closely identical to that produced by inoculation of *M. ulcerans*. It was observed that at the site of mycolactone injection, there was focal necrosis extending through the dermis and adipose tissue into the muscle with few polymorphonuclear neutrophils in spite of extensive tissue destruction (George et al. 1999).

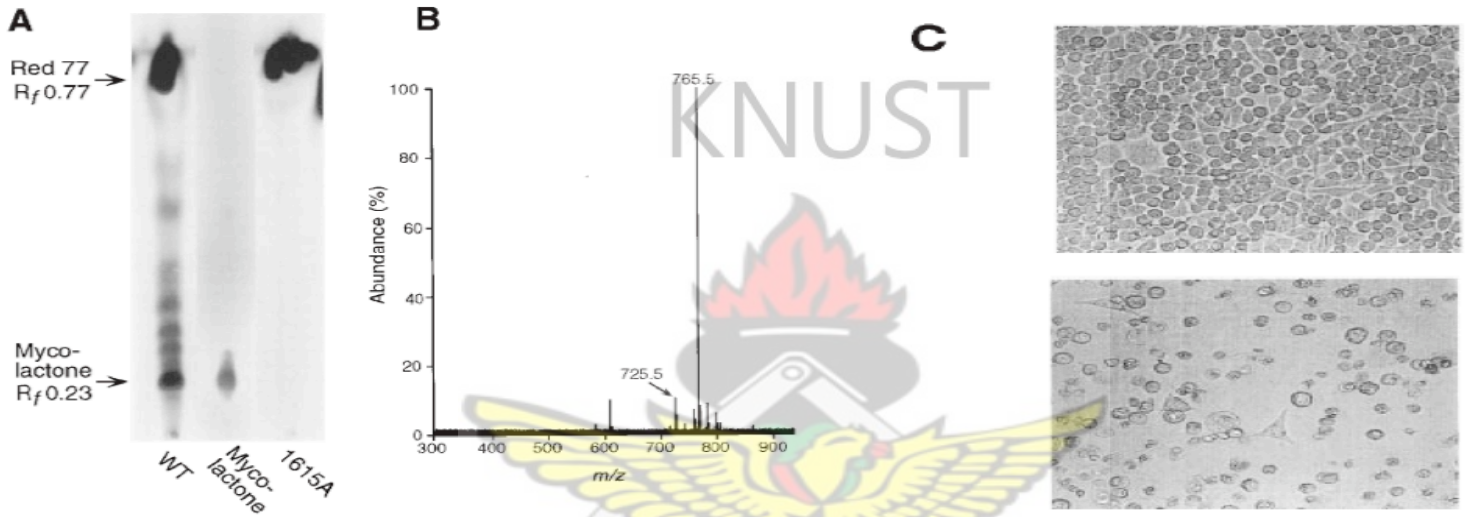


Figure 2.6. Chemical and biological properties of mycolactone. (A)Thin layer chromatography of lipid extracts from *M. ulcerans* cultures showing mycolactone with an Rf of 0.23 in a solvent phase of chloroform :methanol :water 90:10:1. (B) Mass spectrometry showing mycolactone A/B with a peak mass-to-charge ratio (m/z) of 765.5. (C) Cytopathicity of mycolactone on cultured murine fibroblast cell line showing cell rounding and detachment from tissue plates (George et al., 1999).

There are variations in the type or more appropriately congeners of mycolactone produced depending on the origin of strains of *M. ulcerans*. For instance, pathogenic human *M. ulcerans* strains from Africa, Australia and China predominantly produced mycolactone A/B (*m/z* 765), C (*m/z* 749), and D (*m/z* 779) respectively, with each strain producing minor quantities other congeners (Mve-Obiang et al. 2003). *Mycobacterium liflandii*, a pathogen of frogs produces mycolactone E (m/z 737) and the fish pathogens (*Mycobacterium pseudoshottsii* and *Mycobacterium marimum* DL240490) produce mycolactone F (m/z 723) (George et al. 1999; Fidanze et al. 2001; Song et al. 2002; Mve-Obiang et al. 2003; Judd et al. 2004; Mve-Obiang et al. 2005; Ranger et al. 2006; Kim et al. 2008). By far the most cytotoxic congener of mycolactone is the mycolactone A/B produced by the African strains (Mve-Obiang et al. 2003). Interestingly, the potency of mycolactone associated virulence appears to be determined by the presence of the polyketide side chain, since the core lactone ring has biological activity a 1,000-fold lower than intact molecule (Mve-Obiang et al. 2003). These congeners arise during the biosynthesis of mycolactone as reviewed below (section 2.8.1).

Lipids are important virulence determinants for many microorganisms. For instance, lipopolysaccharide (LPS) is a major virulence factor of gram-negative bacteria (Heumann et al. 2002). *Mycobacterium* species lack LPS, but their cell envelope is composed of several biologically active lipids, many of which are polyketide-derived. Phthiocerol dimycocerosate in *M. tuberculosis* and phenolic glycolipids PGL-1 in *M. leprae* are two examples of cell wall-associated polyketide-derived virulence determinants which may play a role in virulence (Cox et al. 1999 and Rambukkana et al. 2002). Mycolactone is a novel cell-associated or secreted

molecule which appears so far to be restricted to *M. ulcerans.* Daniel et al. (2004) extracted lipids from the cell envelope and culture filtrate from 52 representative isolates of fast- and slow-growing *Mycobacterium* species and determined that although several strains of *mycobacterium* produced cytopathic lipids, none produced a phenotype on cultured cells consistent with that of mycolactone. For instance, two mycobacterial species *M. scrofulaceum* and *M. kansasii*, and eight of the environmental mycobacterial isolates contained cell-associated lipids cytopathic to fibroblasts at concentrations of 33 to 1,000 μg/ml in contrast with mycolactone associated cytopathicity which was observed at concentrations less than 2 ng/ml (Daniel et al. 2004).

2.8.1 Synthesis of mycolactone

Mycolactone is synthesised by a 174 kb megaplasmid named pMUM001 which has three very large genes (*mlsA1*: 51 kb, *mlsA2*: 7kb and *mlsB*: 42kb) that encode the modular type I polyketide synthases (PKS) (Stinear et al. 2004) (shown in figure 2.7). The plasmid also has three putative accessory genes (MUP038, encoding a type II thioesterase; MUP045 encoding a beta-ketoacyl synthase and *cyp140A7* [MUP053] encoding a cytochrome P450 hydroxylase. MlsA1 and MlsA2 form a nine-extension module complex that synthesises the mycolactone core, whilst the MlsB is a single polypeptide, comprising seven extension modules that are required for the synthesis of the side chain (Stinear et al. 2004).

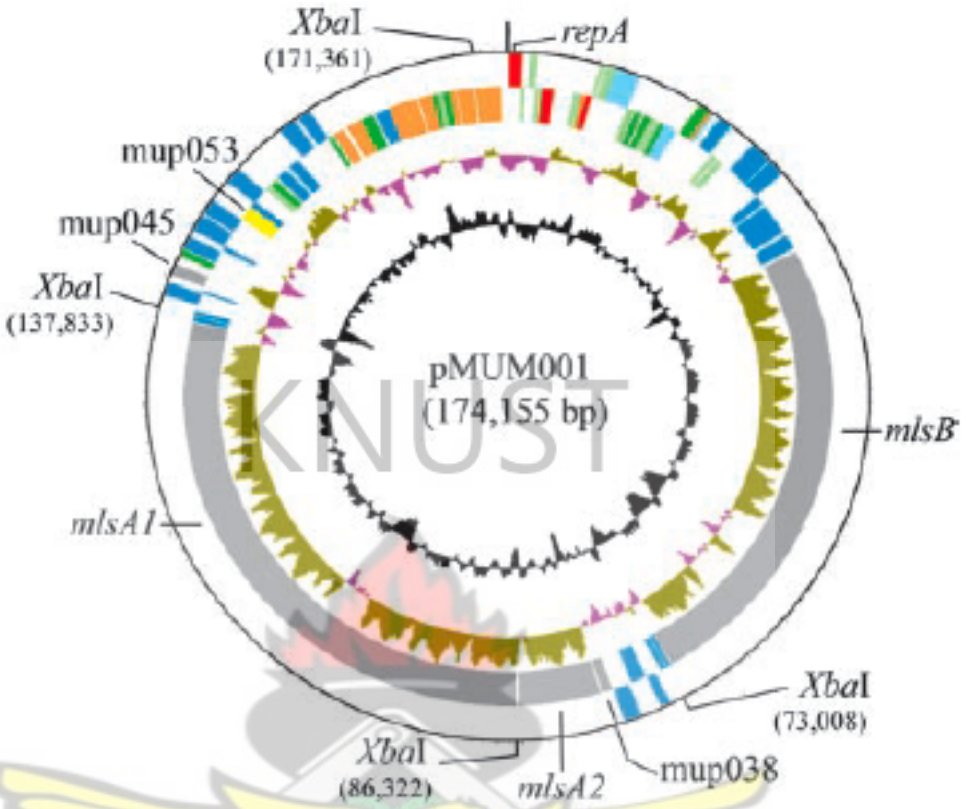


Figure 2.7. Circular representation of pMUM001.

Source: (Stinear et al. 2004).

Bacterial type I PKS are modular multi-enzymes and act as molecular assembly lines for the formation of polyketides (Weissman et al. 2005). These enzymes function in a sequential manner where each PKS module is responsible for one round of chain elongation via the addition of either acetate or propionate, supplied to the PKS as an activated malonyl or methylmalonyly-CoA thioester. Within each PKS module are a series of covalently linked enzymatic domains that process the growing polyketide chain before passing it downstream to the next module in the system (Moss et al. 2004). The minimum set of enzymatic domains required for PKS activity includes ketosynthase (KS), acyltransferase (AT) and an

acyl carrier protein (ACP) domain (Janke-Kodama et al. 2006). Ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains are also commonly found in modules and form a so-called reductive loop, providing reducing enzyme activities that modify the two or three-carbon unit being added to the polyketide (Janke-Kodama et al. 2006).

Mycolactone structural variations are quite restricted. Mycolactone-producing mycobacteria (MPM) recovered from the world for 70 years all produce mycolactones with an absolutely conserved core and all variations occur within the fatty-acyl side chain. For instance, mycolactone D produced by Chinese strains of *M. ulcerans* differs from mycolactone A/B by the substitution of methylene at C2′ of the acyl side chain . In this Chinese strain, the final extension module of MlsB possesses an AT domain with a propionate rather than acetate specificity suggesting natural recombination with *mlsB* (Hong et al. 2005). The role mycolactones play in the survival of MPM and why variation is only tolerated in the side chain remains to be resolved.

2.8.2 Biological effects of mycolactone in-vitro and in-vivo models

The pathogenesis of *M. ulcerans* disease involves responses to many different cell types to *M. ulcerans* and mycolactone. Fibroblasts, adipocytes, skeletal muscles, endothelial cells, and keratinocytes are all present in the affected structures. Additionally, different cellular infiltration at site of infection have also been observed, including macrophages, dendritic cells, neutrophils and T-cells. Various investigators have studied the biological activities and possible mechanism of action of mycolactone on immune and non-immune cell *in-vitro* to clarify its role in the

pathogenesis of *M. ulcerans* disease and are reviewed below. The biological activities of mycolactone in *in-vitro* and *in-vivo* models have been studied by using lipid extracts from *M. ulcerans*, purified or synthetic mycolactone, or comparing the effects of wild-type and mycolactone-negative mutant *M. ulcerans* strains.

2.8.2.1 *Non-immune cells*

Mycolactone has been shown to demonstrate significant cytotoxicity on murine fibroblasts (George et al. 1999 and George et al. 2000), adipose cells (Dobos et al. 2001), keratinocytes (Grönberg et al. 2010), and HeLa cells (Guenin-Macé et al. 2013) which are key cells in the skin.

(a) ***Fibroblasts***: Using fluorescently-labelled mycolactone, it was shown in fibroblasts that the uptake of mycolactone was both non-saturable and non-competitive with excess mycolactone indicating that mycolactone transfer occurs by passive diffusion across the cell membrane and was shown to localise in the cytosol (Synder et al. 2003). On L929 mouse fibroblast mycolactone was shown to induce profound cytoskeletal re-arrangement, followed by cell rounding and detachment at 12h, 24h and 48 hours respectively. After 3-5 days fibroblasts undergo apoptotic cell death (George et al. 2000) at concentrations as low as 2 pg/ml. It was subsequently shown that mycolactone exerted a concentration-dependent effect on cultured fibroblasts, in that at higher concentrations of 15μg/ml it caused rapid changes in cell permeability and caused cell death by necrosis as measured by increased lactate dehydrogenase (LDH) release and many swollen cells microscopically observed as

early as 4 hours post-treatment. However, at lower concentrations of 15ng/ml to 150ng/ml, cell death was predominantly by apoptosis (Adusumilli et al. 2005).

(b) Adipose cells: On human adipose cell line (SW872), mycolactone-mediated cell death was observed to occur predominantly by apoptosis but also by necrosis (Dobos et al. 2001). Importantly, histological examination of sections of *M. ulcerans*-infected guinea pig skin (George et al. 2000) and human skin tissue (Walsh et al. 2005) showed evidence of cell death by apoptosis.

(c) Keratinocytes: Keratinocytes are crucial in wound healing and repair by forming a protective epithelial barrier over the wound bed. On this cell line, Grönberg et al. (2010) demonstrated a concentration- and time-dependent reduction in cell numbers after exposure to mycolactone. It was found in their study that mycolactone-induced cytotoxicity on this keratinocyte cell line was associated with generation of reactive oxygen species (ROS) and was completely prevented by the ROS-inhibiting substance deferoxamine, which acts via chelation of Fe^{2+}(Grönberg et al. 2010). Although the mechanism of mycolactone-mediated toxicity is presently unknown, it appears like many other macrolide substances with cytotoxic and immunosuppressive properties to mediate their effects via production of reactive oxygen species (Navarro-Antolin et al. 1998; Karbowski et al. 1999; Ray et al. 2001; Hong et al. 2002; Gil et al. 2003; Zhou et al. 2004; Han et al. 2006; Choi et al. 2008a; Choi et al. 2008b; Han et al. 2008).

(d) **HeLa cells (mechanism for mycolactone-induced ulcer formation)**: The mechanism underpinning ulcer formation by mycolactone has been studied in HeLa cells, which is a prototype of anchorage-dependent epithelial cell line. Anchorage-

dependent cells such as fibroblasts and keratinocytes have demonstrated sensitivity to the cytotoxic effects of mycolactone (George et al. 1999; Grönberg et al. 2010) *in-vitro*. This cytotoxicity proceeds through cytoskeletal rearrangements and detachment of cells leading to a form of cell death referred to as anoikis. Anoikis is a form of programmed cell death which is induced by anchorage-dependent cells detaching from the surrounding extracellular matrix (Frisch et al., 2001). Guénin-Mace and Veyron-Churlet et al. (2013) have elucidated the mechanisms behind mycolactone-induced cytoskeletal rearrangement in HeLa cells by showing that mycolactone induce profound alterations in actin dynamics.

These investigators identified Wiskott-Aldrich syndrome protein (WASP) and neural WASP (N-WASP) as molecular targets of mycolactone. Along with Scar/WAVE-1 – WAVE-3, WASP and N-WASP constitute a family of scaffold proteins transducing a variety of signals into dynamic remodelling of actin cytoskeleton, via interaction of their C-terminal verprolin-cofilin-acidic (VCA) domain with the ARP2/3 actin-nucleating complex (Thrasher et al. 2010) (figure 2.8). In basal states, WASP and N-WASP are auto-inhibited by intramolecular interactions that sequester the VCA domain from ARP2/3. Binding of activated GTPases or phosphoinositide lipids to N-terminal target sequences triggers conformational changes resulting in release of the VCA, thereby enabling binding and activation of ARP2/3 (Padrick et al. 2010). The ARP2/3 complex has two subunits that closely resemble the monomeric actin and serve as nucleation sites (‘anchor sites’) for new actin filaments. This complex binds to the sides of existing (“mother”) filaments and initiates the growth of a new (“daughter”) filament at a distinctive 70° angle from the mother (see figure 2.9). These proteins play a crucial

role at the cellular level in endocytosis (Merrifield et al. 2004), immune function, synapse formation, signalling, adhesion, and migration (Thrasher et al. 2010; Hayward et al. 2006). At the tissue level, they are crucial for the maintenance of skin integrity (Kovacs et al. 2011; Otani et al. 2006; Lyubimova et al. 2010).

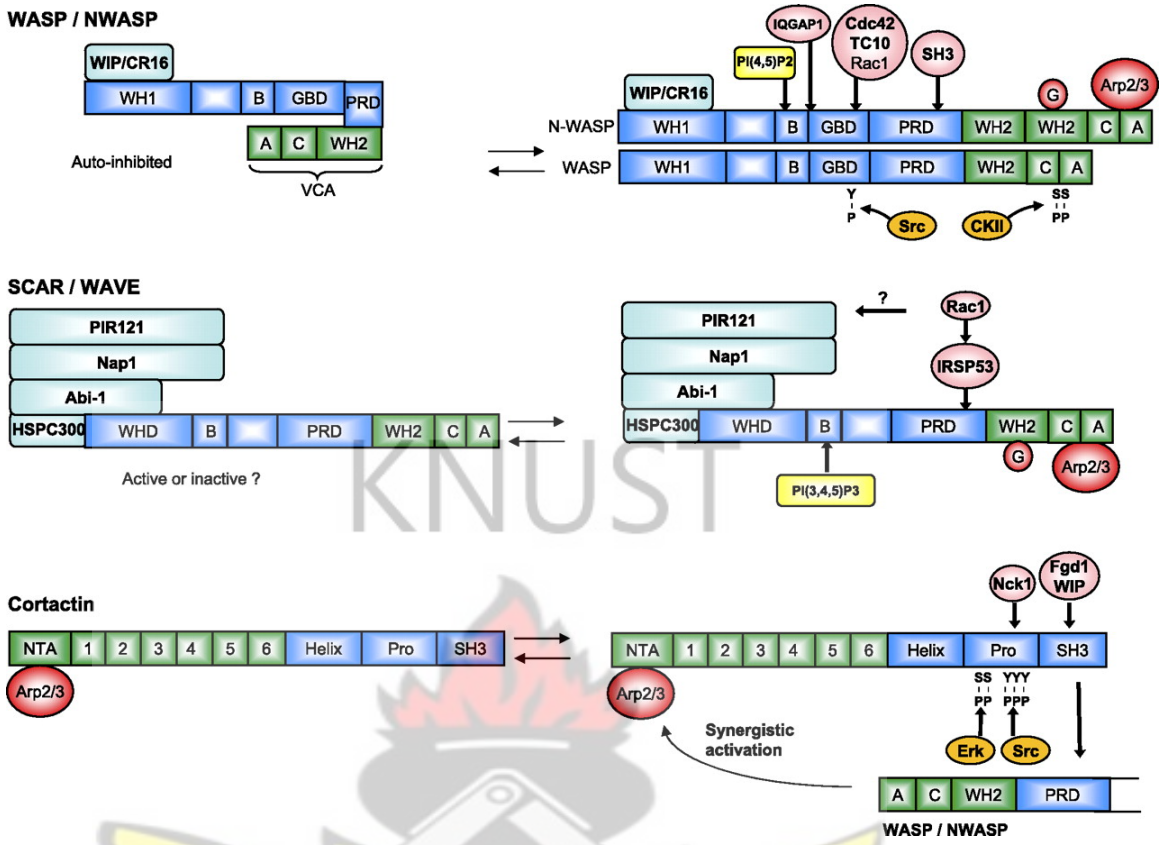


Figure 2.8. Regulation of Arp2/3 activators. Padrick et al. 2010.

Top: WASP and N-WASP are modular proteins that exist in an auto-inhibited conformation in which the VCA domain (green) is occluded by an intramolecular interaction. A variety of ligands synergistically activate WASP and N-WASP by disrupting the intramolecular interaction to expose the COOH-terminal domain that binds and activates the Arp2/3 complex. The phosphorylation of one tyrosine by Src and two serines by casein kinase II (CKII) also contribute to the activation of WASP.

Middle: WAVE1, -2, and -3 are part of a stable complex with four other proteins including Nap1 (Nck-associated protein), PIR121 (p53-inducible mRNA), HSPC300 (hematopoietic stem progenitor cell 300), and Abi1 (Abl-interactor 1).

Bottom: Cortactin displays a weak activity that is enhanced by the binding of WIP or Fgd1 to its COOH-terminal SH3 domain. This SH3 domain also binds and activates N-WASP, resulting in a synergistic activation of Arp2/3 by cortactin and N-WASP.

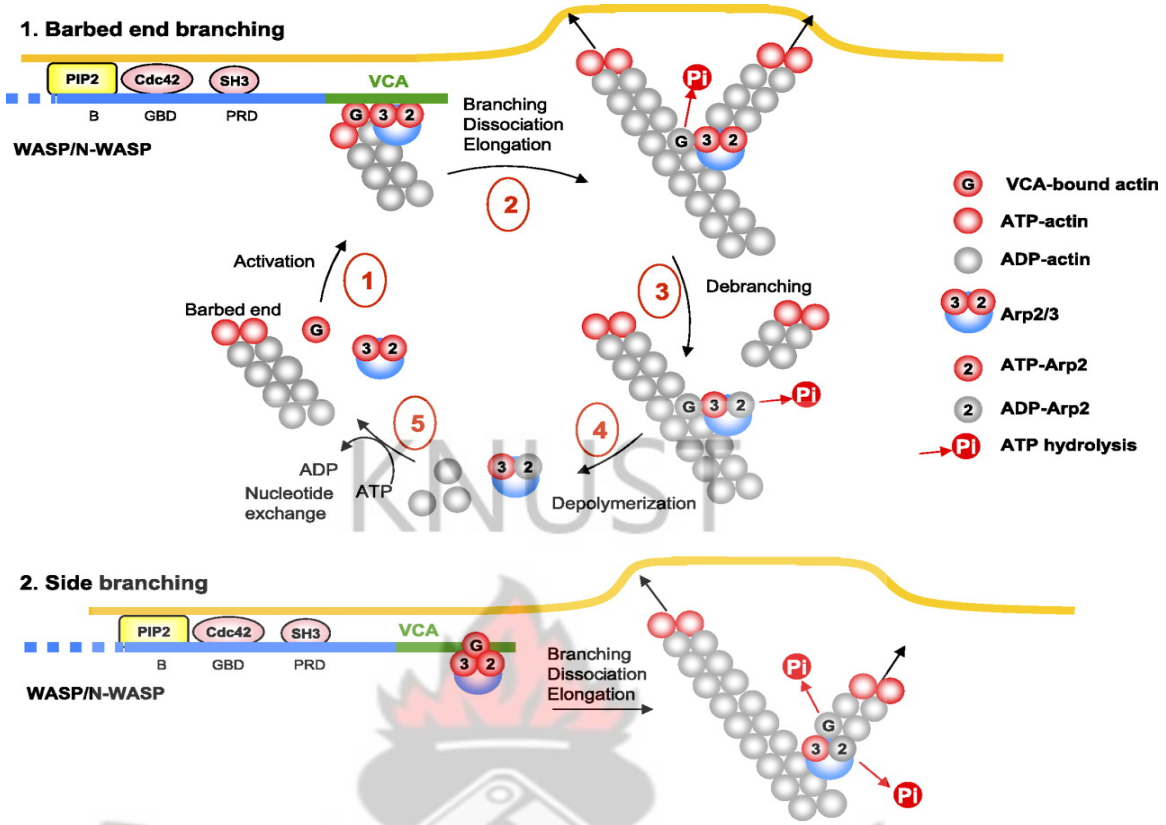


Figure 2.9. Model for the catalytic cycle of branching by Arp2/3. Padrick et al. 2010

Top: the barbed end branching model. *1*) The branching complex made of G-actin, Arp2/3, and the VCA domain of WASP/N-WASP (or WAVE isoforms) binds to the barbed end of the mother filament. This incorporation is mediated by the actin subunit of the branching complex. *2*) Arp2/3 nucleates a lateral branch. ATP hydrolysis on actin dissociates the branched junction from the membrane-bound activator. The growth of the mother and daughter filaments drives membrane protrusion. *3*) ATP hydrolysis on Arp2 induces debranching. *4*) After depolymerization of the branched filament, Arp2/3 and actin are released. *5*) Nucleotide exchange is required to recycle actin and Arp2/3. The duplication of barbed ends at the plasma membrane can easily account for the formation of a polarized dendritic actin array in which the barbed ends face the plasma membrane.

Bottom: the side branching model. This model proposes that Arp2/3 activated by the COOH-terminal domain of WASP/N-WASP (or WAVE isoforms) binds to the side of an actin filament. In this activated state, Arp2-Arp3-G-actin mimics an actin nucleus to initiate a lateral branch.

By using a combination of biochemical assays, cellular imaging and animal models, it was shown that mycolactone operates by hijacking WASP family of actin-nucleating factors. By disrupting WASP autoinhibition, mycolactone led to an uncontrolled activation of ARP2/3-mediated assembly of actin in the cytoplasm. In epithelial cells (HeLa cells), mycolactone-induced stimulation of ARP2/3 concentrated in the perinuclear region, resulting in defective cell adhesion and directional migration. Furthermore, *in vivo* injection of 5μg of mycolactone into mouse ears consistently altered the junctional organisation and stratification of keratinocytes, leading to epidermal thinning, followed by rupture to form an open ulcer. Interestingly, this degradation process was efficiently suppressed by co-administration of the N-WASP inhibitor wiskostatin. This piece of work has unravelled the molecular basis of mycolactone activity and provided a mechanism for Buruli ulcer pathogenesis (Guénin-Mace and Veyron-Churlet et al. 2013).

(e) **Peripheral nerves**: Despite the extensive necrosis and tissue destruction leading to the formation of deep ulcers, Buruli ulcers are characteristically painless. The absence of pain prevents patients from seeking early treatment leading to patients experiencing severe sequelae, including amputation. Goto et al. (2006) examined the pathology of BALB/c mice inoculated in the footpads with African strains of *M. ulcerans* and reported there was a loss of pain sensation and nerve degeneration. Nerve invasion occurred in the perineurium and extended to the endoneurium with vacuolar degeneration of myelin-forming Schwann cells and some swollen nerve bundles massively invaded by acid-fast bacilli. They concluded that the painlessness of Buruli ulcers could be partly due to the intraneural invasion of

bacilli in contrast to that of leprosy where nerve damage is due to invasion by inflammatory cells.

Furthermore injection of footpads of BALB/c mice with 100μg of mycolactone induced swelling, redness, erosion of footpads and importantly nerve damage. Nerve bundles showed intraneural haemorrhage, neutrophilic infiltration and loss of Schwann cell nuclei with ultrastructural evidence of vacuolar change of myelin on day 14 and gradually subsiding by day 42. Sensory testing by the von Frey test showed hyperesthesia on day 7, recovery on day 21 and hypoaesthesia on day 28. This study suggested that mycolactone was implicated in the direct destruction of nerves and was responsible for the absence of pain characteristic of Buruli ulcer (En et al., 2008).

(f) **Muscle cells**: One of the clinical features of severe and advanced forms of *M. ulcerans* disease is the development of skeletal muscle contracture and atrophy leading to significant impairment in function. Mycolactone has been found to be involved in this muscle destruction. Intramuscular injection of 300μg of mycolactone into the soleus muscles of mice had profound histological, biochemical and functional effects (Houngbédji et al., 2009). Histological observations demonstrated significant muscle necrosis and atrophy with limited signs of regeneration together with induction of acute and chronic inflammatory responses. In addition, muscle stiffness and total hydroxyproline content rose by 46% and 134% at day 42 relative to sham injections indicating an extensive fibrotic process in injured soleus muscles. Finally a 68% decrease in maximal isometric force production relative to sham injections was observed indicating that mycolactone not only

induces muscle damage but also prevents muscle regeneration and may explain why patients with Buruli ulcer experience muscle weakness and develop contractures. These findings were subsequently reproduced by subcutaneous injection of *M. ulcerans* in proximity to the right biceps muscles, avoiding direct physical contact between the infectious agent and the skeletal muscle (Houngbédji et al., 2011) and in another study involving mycolactone-producing *M. ulcerans* strains compared with an avirulent strain (Dufresne et al. 2013), thus implicating mycolactone in muscle damage in Buruli ulcer disease.

2.8.2.2 *Immune cells*

Mycolactone has been shown to exert significant immunosuppressive and immunomodulatory effects on a host of cells of that are central to both innate and adaptive immune responses against mycobacteria and is reviewed below:

(a) Keratinocytes*:* Although keratinocytes serve as a protective barrier in the epidermis of skin tissue, it has been hypothesized that keratinocyte Toll-like receptors (TLRs) may actively participate in the innate immune response to *M. ulcerans* (Lee et al., 2009). Toll-like receptors are pattern-recognition receptors expressed on surfaces of cells. It was shown in one study that human keratinocytes constitutively expressed TLRs 2 and 4 and induced Dectin-1 in response to *M. ulcerans* resulting in active sensing and internalisation of mycobacteria and culminating in rapid production of reactive oxygen species and production of the chemokines CXCL8, CCL2 and LL-37. It is not certain the extent to which

mycolactone would influence the innate immune response of keratinocytes since this was not directly assessed in their study (Lee et al., 2009).

(b) Neutrophils*:* There is a relative paucity of neutrophils in human and guinea pig *M. ulcerans* lesions compared with *M. marinum* lesions. To investigate the basis of these observations in-vitro, Adusumilli et al. (2005) demonstrated that there were significant defects in neutrophil chemotaxis towards *M. ulcerans* compared with *M. marinum* and again neutrophils were susceptible to cell death by necrosis upon exposure to incremental doses of mycolactone from 4ng to 1μg. The deficient neutrophil chemotaxis may not be a direct corollary of the effects of mycolactone since in their study, they showed that defects in neutrophil chemotaxis assay were observed in both wild type *M. ulcerans* and a mycolactone-negative mutant *M. ulcerans*. Thus even though neutrophils are short-lived with high rates of spontaneous apoptotic cell death, it appears their paucity at *M. ulcerans* infection foci may be a composite of lack of chemotactic stimuli as well as the demise of those attracted to the site of infection (Adusumilli et al., 2005).

(c) Macrophages*:* Macrophages are important antigen presenting cells involved in mycobacterial immunity and are responsible for the uptake and processing of foreign antigens. Macrophages which develop from monocytes gain new functions and receptors once they reside in the tissues. They express mannose receptors (MR), complement receptors (CR), and scavenger receptors and others including Toll-like receptors. Binding of mycobacteria to mannose receptors, complement receptors or scavenger receptors results in phagocytosis and uptake of bacteria into intracellular vesicles where they are destroyed. This destruction

involves formation of a phagosome as the mycobacterium is surrounded by the phagocyte membrane and then fusion of the phagosome with membrane bound granules called lysosomes to form a phagolysosome. Fusion with one of these granules results in killing of the pathogen but *M. tuberculosis,* for instance, arrests phagosome maturation at an early stage and strongly inhibits phagolysosome fusion (Kaufmann 2002) by blocking influx of Ca^{2+} into macrophages and interfering with proteins known as Rabs and their lipid kinases responsible for directing intracellular trafficking.

Additionally, macrophages produce several effector cytokines and chemokines such as TNF-α, interleukin-6, interleukin-1, interleukin-8, which have an impact on the recruitment of other inflammatory cells (Bendtzen 1988 and Dinarello 1992) as well as on the activation and phenotype of T-cells (Pasare et al., 2003 and Shibuya et al., 1998). TNF-α is a key effector produced by macrophages and has autocrine effect on the activation of the macrophage microbicidal activity against intracellular pathogens, including *M. tuberculosis* (Denis; 1991 and Flynn et al.; 1995), *M. bovis* (Kindler et al. 1989) and some strains of *M. avium* (Sarmento et al., 1995). Although a transient intra-macrophage growth phase has been shown for *M. ulcerans*, the organism is predominantly observed in tissue in extracellular colonies. Mycolactone has been shown to significantly influence various stages of the macrophage-pathogen interactions.

Purified mycolactone was shown to powerfully and dose-dependently inhibit production of cytokines (TNF, IL-1β, IL-6, IL-10 and interferon-γ-inducible protein-10), chemokines (IL-8) and intracellular effector molecules such as cyclooxygenase-

2 by macrophages in response to Toll-like receptor ligands such as lipopolysaccharide (LPS), Pam_3Cys, MALP-2, flagellin, poly(I:C) or R848 (Simmonds et al., 2009). Interestingly, mycolactone had no effect on the activation of signalling pathways such as the MAPK and NF-κB pathways which are important in the induction of genetic expression of these inflammatory cytokines. Furthermore, LPS-dependent transcription of TNF, IL-6 and cyclooxygenase-2 mRNA was found not to be inhibited, implying that mycolactone may mediate its effects by inhibiting the translation of a specific subset of protein in primary human monocytes. The inhibition of TNF-α secretion by macrophages by *M. ulcerans* has been shown to be determined by the virulence/ cytotoxicity of the strains of *M. ulcerans*. Production of TNF-α by macrophages was low, intermediate, or high when they were infected with high (mycolactone A/B producing strain), intermediate (mycolactone C producing strain), or noncytotoxic/nonvirulent strains respectively (Torrado et al., 2007). Notably, these effects on TNF-α production by bone marrow derived macrophages by highly virulent strains of *M. ulcerans* were not associated with premature death of macrophages in culture.

Mycolactone also significantly interferes with phagocytosis by macrophages. Firstly, whereas J774 macrophages were only able to internalise 0.7% of mycolactone-producing *M. ulcerans in vitro*, up to 90% of mycolactone-negative mutants of *M. ulcerans* were internalised (Adusumilli et al., 2005). Secondly, pre-treatment of J774 macrophages with incremental doses of mycolactone inhibited internalisation of *Candida albicans* in a dose-dependent fashion (Adusumilli et al., 2005). Thirdly, mycolactone modulates the macrophage microbicidal activity

triggered by interferon-γ through inhibition of phagosome maturation and nitric oxide production. The addition of mycolactone to cultures of IFN-γ-activated macrophages infected with mycolactone-negative avirulent *M. ulcerans* strain led to a dose-dependent inhibition of the IFN-γ-induced protective mechanisms, involving phagosome maturation/ acidification and decreased nitric oxide production with increased bacterial load (Torrado et al., 2010). Taken together mycolactone exerts profound suppressive effects on the functions of macrophages at non-cytotoxic concentrations as well as cell death at higher concentrations.

(d) Dendritic cells*:* Dendritic cells are key players in the initiation of cellular responses and the regulatory processes leading to cellular immunity. Coutanceau et al. (2007) showed that mycolactone at concentrations above 50ng/ml was found to cause cell death by apoptosis in both immature and mature human and murine dendritic cells. Below this concentration, mycolactone strongly affected the maturation of both mouse and human dendritic cells, evidenced by the failure to up-regulate the phenotypic markers of the mature dendritic cells CD83 and CD25. Additionally, mycolactone-treated dendritic cells showed a reduced ability to cross-present antigens to T-cells and limited migratory properties. However, their phagocytic activity measured by uptake of intracellular fluorescent beads after 4 hours was not reduced by co-incubation of the cells with 10-100 ng/ml of mycolactone. Taken together, mycolactone suppresses the capacity of dendritic cells to prime cellular immune responses. (Coutanceau et al., 2007).

Mycolactone at non-cytotoxic concentrations exerted a selective effect on the secretion of inducible chemokines by dendritic cells in response to Toll-like receptor stimulants. In particular, mycolactone blocked the production of monocyte and lymphocyte chemoattractants MIP-1α, MIP-1β, IP-10, RANTES and MCP-1 whereas the expression of the inflammatory cytokines TNF-α, IL-12, and IL-6 were marginally affected. This defective production of chemoattractants by dendritic cells is likely to interfere with both innate and adaptive immune responses to *M. ulcerans* infection. Firstly, decreased β-chemokine production by mycolactone-exposed dendritic cells may limit the recruitment and activation of immature dendritic cell precursors to the site of infection. Secondly, the defective production of these CCR5 ligands (Coutanceau et al., 2007), as well as the CXCR3 ligand IP-10 is likely to interfere with the homing capacity of IFN-γ-secreting T cells to infected tissues. These findings show that mycolactone interferes with the functional biology of dendritic cells in a unique fashion.

(e) T-cells*:* T-cells are crucial players in the development of an adaptive immune response to mycobacterial infections and are briefly reviewed before presenting a review on how mycolactone might interfere with the biology of T-cells.

Antigen recognition by T cells

Macrophages process mycobacterial peptides and present them on their surface to T lymphocytes resulting in their activation. These peptides are delivered to the surface of macrophages by major histocompatibility complex molecules that are peptide–binding glycoproteins encoded in a large gene complex called the major histocompatibilty complex (MHC). Cytosolic proteins undergo degradation by a

large protease complex called the proteosome and are carried by proteins called transporters associated with antigen processing-1 and 2 (TAP1 and TAP2) and then bound onto the MHC class I molecules in the endoplasmic reticulum. Once the peptides have been bound to class 1 molecules, the peptide/MHC complexes are released from the TAP transporters and pass out of the cells via the Golgi system and appear on the cell surface for presentation to the T-cell receptors (TCR). Peptides from the intracellular vesicles are generated by progressive decrease in pH thus activating proteases in the vesicles to degrade the engulfed bacteria. Such acid proteases include cathepsins B, D, S and L. The MHC class II molecules pass through these acidified vesicles and bind antigenic peptide fragments transporting them to the surface.

Activation of T lymphocytes

Classically T cells fall into two major classes depending on which cell surface proteins they express. They bear the surface molecules CD8 or CD4. Mycobacterial peptides complexed with MHC class 1 molecules are presented for activation of T cells expressing CD8 receptors whereas peptides complexed with MHC class II molecules are presented for activation of T lymphocytes expressing CD4 molecules (Fenton et al. 1996) (see figure 2.10 below).

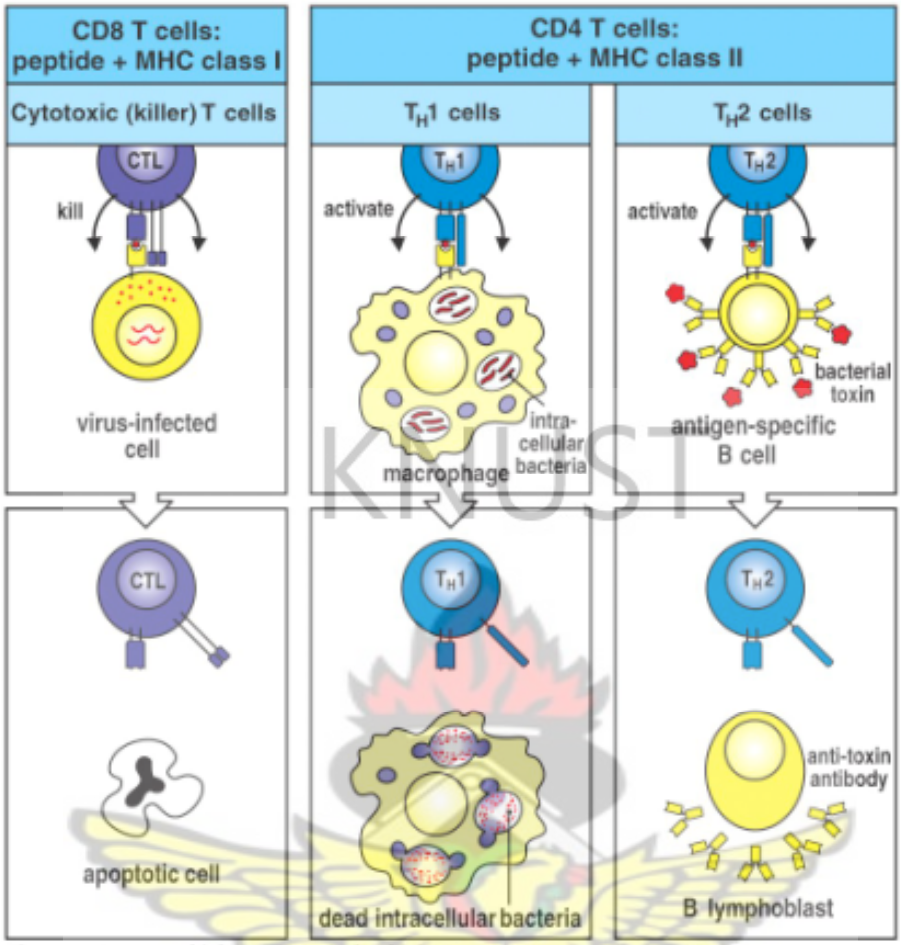


Figure 2.10. CD8 and CD4 positive T lymphocytes and their role in protection (Janeway et al., 2005)

T cells require two independent signals delivered by the same antigen presenting cells for activation. Binding of the foreign peptide/MHC II complex to the TCR:CD3 and CD4 co-receptor transmits the first signal but this can induce clonal expansion only when the co-stimulatory signal is given by binding of CD28 to B7 glycoprotein molecules like CD80 (B-7.1) or CD86 (B-7.2). On the contrary binding of B7 molecules to another protein CTLA-4 delivers inhibitory signals to the

activated T cells. CD8 T lymphocytes that encounter peptide:MHC I complexes are activated in similar fashion but require stronger co-stimulatory signals for activation. This happens if activation is by mature dendritic cells that have high intrinsic co-stimulatory activity and can directly stimulate CD8 T cells to synthesize IL-2 that drives T cell proliferation and differentiation. In some cases armed effector CD4 cells are required when there is inadequate co-stimulation of naïve T cells by the infected antigen presenting cell (APC). These armed effector CD4 cells then activate the APC by binding of CD40 ligand to CD40 on the APC to secrete high levels of co-stimulatory molecules. Activation of T cells results in secretion of IL-2 and expression of high affinity IL-2 receptors. IL-2 binds to the high affinity IL-2 receptors to promote T cell growth.

CD4 positive T lymphocyte

CD4 T lymphocytes have classically been divided into Th1 or Th2 and recognise peptides processed from the intracellular vesicles and displayed on the surface by MHC class II molecules. Activated Th1 lymphocytes express the CD4 ligand and produce cytokines such as IFN-γ, tumor necrosis factor-α (TNF-α) and lymphotoxin-α. Together these newly synthesized proteins activate the infected macrophage. Macrophages require two signals for activation one of which is provided by IFN-γ and the other by binding of CD40 ligand to CD40. TNF-α or TNF-β (lymphotoxin) also secreted by activated Th1 lymphocytes can substitute for CD40 ligand in macrophage activation. CD4 T lymphocytes also produce lymphotoxin-α, which participates in protection against tuberculosis (Kaufmann,

2001). In tuberculosis, for example, IFN-γ and TNF-α act in synergy to activate macrophages. Macrophages fuse their lysozomes more effectively with phagosomes after activation and production is induced of nitric oxide (NO) and superoxide ions (O_2) which have powerful antimicrobial activity. Th2 lymphocytes produce cytokines like IL-4, IL-5, IL-10 and IL-13 which act on B cells to induce antibody production (Mosmann et al. 1989)

CD8 positive T lymphocytes

CD8 T lymphocytes recognise mycobacterial peptides processed in the cytosolic compartment (York et al., 1996) and are referred to as cytotoxic T lymphocytes(CTL). They contribute to protective immunity in three ways. Like CD4 T lymphocytes they secrete cytokines and can be grouped into Tc1 or Tc2 depending on the cytokines they produce. Tc1 CD8 T lymphocytes are characterized by secretion of cytokines IL-2, IFN-γ and TNF-α (Croft et al., 1994; Li et al., 1997; Noble et al., 1995) whereas Tc2 CD8 T cells secrete IL-4, IL-5 and IL-10 (Croft et al., 1994; Li et al., 1997). It is the Tc1 CD8 T lymphocytes that are associated with protective immunity in tuberculosis as they secrete IFN-γ and TNF-α (Cooper et al. 1993; Flynn et al. 1993). This may account for the high frequency of IFN-γ-secreting CD8 T lymphocytes in the peripheral blood of healthy individuals who have been exposed to *Mycobacterium tuberculosis* (Pathan et al., 2000). They also cause cell-mediated cytotoxicity either in a Ca^{2+}-dependent release of cytolytic granules containing perforin and granzyme proteases leading to apoptosis of the target cell or in a receptor-mediated way which involves the engagement of Fas ligand (CD95L)

on the T cell membrane with the target cell surface receptor Fas (CD95) also resulting in programmed cell death (Bossi et al., 1999). Induction of apoptosis by these cells may account for the reduced viability of mycobacteria within such cells (Oddo et al., 1998). CD8 T lymphocytes also possess a granule associated molecule granulysin (protein 519) that has direct mycobactericidal activity against intracellular mycobacteria (Gamen et al., 1998; Pena et al., 1997; Stenger et al., 2001; Stenger et al., 1998).

Effects of mycolactone on T-cell biology: Using a crude preparation of mycolactone, it was suggested that the immunomodulatory properties of mycolactone may extend to lymphocytes due to the inhibition of production of IL-2 by activated T cell lines (Pahlevan et al., 1999). Mycolactone was subsequently shown to block the activation-induced production of IFN-γ, IL-4, IL-7, IL-10, TNF-α, IL-8 and MIP-1β by human peripheral blood $CD4^+$ T lymphocytes (Phillips et al., 2009) indicating that mycolactone effect was not restricted to IL-2 production. In a multiplex analysis of the immunological profile of patients with *M. ulcerans* disease the production of several Th-1, Th-2, and Th-17 cytokines were also altered (Phillips et al., 2009). Indeed, a defective systemic production of IFN-γ, in patients with active *M. ulcerans* disease has been severally reported by independent studies (Gooding et al., 2003; Phillips et al., 2006; Prevot et al., 2004; Westerbrink et al., 2005) and has been shown to resolve after either surgical excision of ulcers (Yeboah-Manu et al., 2006) or after curative antibiotic therapy (Schütte et al., 2007; Schütte et al., 2009; Sarfo et al. 2009) suggesting the association of these defects with the presence of *M. ulcerans*. Importantly mycolactone was detected by mass

spectrometry in peripheral blood mononuclear cells of mice after subcutaneous injection of mycolactone. (Hong et al., 2008).

Insights into the mechanisms underpinning the T-cell suppression by mycolactone have recently emerged from two studies by Boulkroun et al. (2010) and Guenin-Macé et al. (2011). A key observation from both studies is that primary T-cells are relatively resistant to the cytotoxic effects of mycolactone at concentrations of up to 1μg/ml.

Molecular basis of T-cell immunosupression by mycolactone:

Boulkroun et al., (2010) examined the effects of mycolactone on the functional biology of T-cells and identified two mechanisms by which mycolactone suppresses T-cell responsiveness to antigenic stimulation. Firstly, at non-cytotoxic concentrations, mycolactone blocked activation-induced production of the prototypical T-cell cytokine IL-2 by suppressing the TCR-induced transcription of the IL-2 gene and at a posttranscriptional level by blocking the translation of the IL-2 message.

The second mechanism involves mycolactone-mediated hyperactivation of an Src-family kinase, Lck, resulting in the depletion of intracellular calcium stores and down-regulation of TCR, culminating in impaired T cell responsiveness to stimulation. Phosphorylation of Lck is an early event of the TCR signalling cascade triggering the phosphorylation of ITAMs on the TCR/CD3 complex (Acuto et al., 2008), (see figure 2.11 below). Phosphorylated ITAMs create binding sites for the protein kinase ZAP-70, leading to its recruitment and activation. ZAP-70

phosphorylates the transmembrane adaptor protein, LAT, which recruits a broad range of signalling molecules leading to PLC-γ1 and MAPK activation. PLC-γ1 then initiates the release of calcium from intracellular stores. These investigators found that ZAP-70, LAT and PLC-γ1 were hyperphosphorylated in mycolactone-exposed T-cells and they showed that mycolactone-provoked accumulation of Lck in plasma membrane rafts may alter the balance of its regulators Csk and CD45, leading to kinase activation by autophosphorylation. They proposed that the mycolactone-mediated activation of Lck alters the capacity of T cells to respond to stimulation in two ways. First, activation of the Lck signalling cascade by mycolactone resulted in downregulation of TCR/CD3 complex, thereby impairing T cell responsiveness to anti-CD3/CD28 cross-linking. Secondly, mycolactone-induced activation of PLC-γ1 led to intracellular Ca^{2+} store depletion and failure to mobilize Ca^{2+} appropriately in response to T-cell stimulant ionomycin. These findings agree with other studies where increased basal Lck activity, or altered intracellular Ca^{2+} concentrations were described in association with anergic human Th2 or Th1 clones respectively (Faith et al., 1997, Gajewski et al., 1994). Taken together, mycolactone potently alters T-cell capacity to respond to antigenic stimulation.

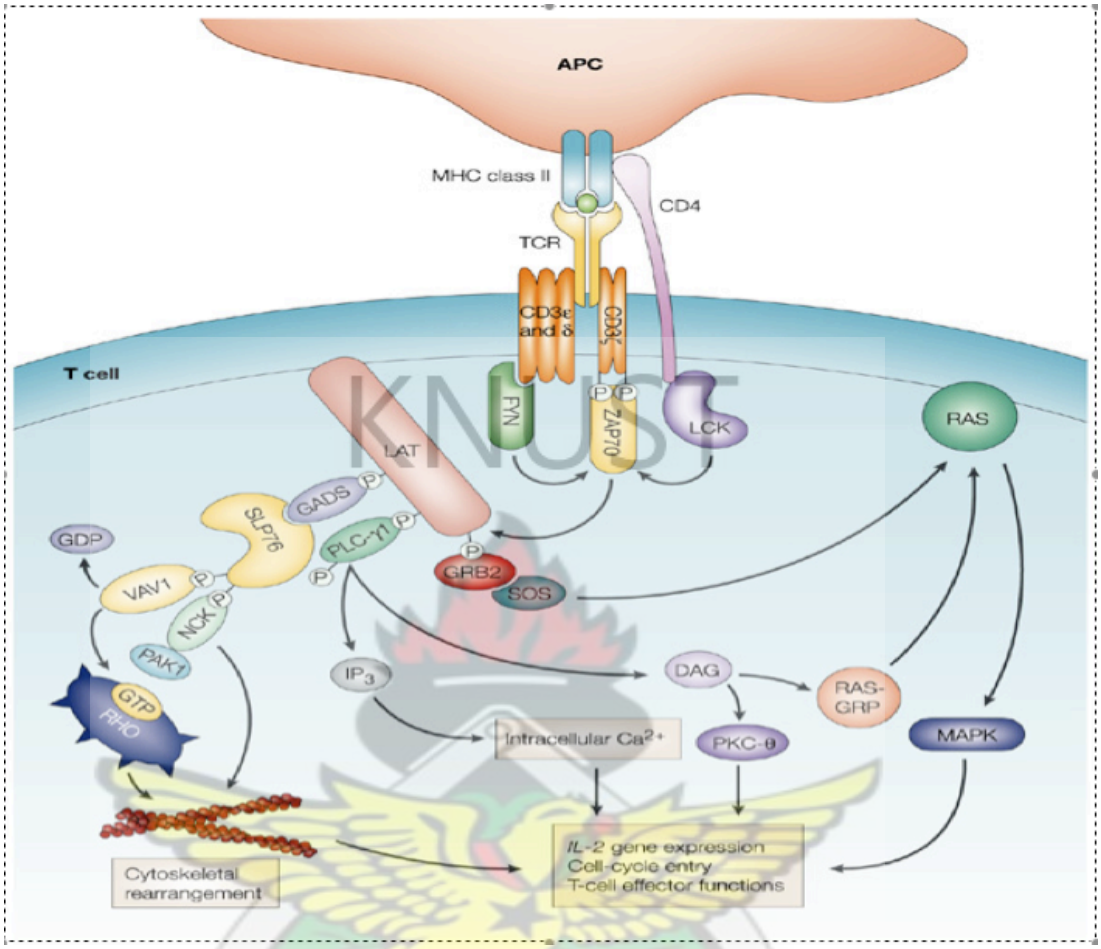


Figure 2.11. T-cell receptor signalling cascade. (Acuto et al., 2008)

Molecular basis of impaired T-cell homing by mycolactone:

Because T-cells are instrumental in the adaptive response to pathogens, it is also important to determine whether and if mycolactone interferes with the capacity of naïve T cells to gain access to peripheral lymph nodes (PLN) and make contact with antigen presenting cells. T-cell homing to PLNs is controlled by the expression of the receptors L-selectin (CD62-L), C-C chemokine receptor 7 (CCR7) and

lymphocyte function-associated antigen 1 (LFA-1). High expression of CD62-L on the surface of naïve T cells is essential for the initial steps of tethering and rolling of circulating lymphocytes in the high endothelial venules (HEV) of peripheral LNs, whereas CCR7 and LFA-1 play a crucial role in the subsequent adhesion of T cells to HEV (Warnock et al., 1998). Within the lymph nodes, T cells then migrate to specialised T-cell zones, where CCR7-driven motility is vital for scanning antigens presented by dendritic cells (Asperti-Boursin et al., 2007).

Guenin-Macé et al. (2011) investigated the effect of mycolactone on the process of T-cell homing using a murine model where mycolactone was subcutaneously delivered. They found that mycolactone injection led to a massive T-cell depletion in peripheral lymph nodes (PLNs) that was associated with defective expression of L-selectin. Pre-exposure of T-cells to mycolactone severely impaired the capacity of T-cells to reach peripheral lymph nodes after adoptive transfer, respond to chemotactic stimuli, and expand upon antigenic stimulation *in vivo*. Importantly, a mechanistic explanation of this mycolactone-induced suppression of CD62-L expression of human primary T cells was identified. Mycolactone was found to reduce both the mRNA and protein levels of CD62-L and these were correlated with reduced expression of one microRNA: let-7b. MicroRNAs generally function as gene silencers repressing translation and/or directing the sequence-specific degradation of complementary mRNA. They are found in some instances to enhance gene expression by interacting with promoter sequences (Place et al., 2008). It was found that let-7b regulated the expression of CD62-L positively. It is unlikely that let-7b activates CD62-L transcription directly but rather modulates the expression of transcriptional and/or translational regulators of CD62-L since no

complementary sequence of let-7b was identified on the CD62-L gene or its flanking regions. Indeed proteomic analyses have shown that variations in let-7b levels finely tune the production of thousands of proteins (Selbach M, et al. 2008). Hence, down-regulation of let-7b by mycolactone is likely to trigger a cascade of events leading to the modulation of CD62-L expression and potentially other factors which remain to be elucidated.

Summary of current knowledge on mycolactone: It is now thought that the pathogenesis of *M. ulcerans* disease is essentially mediated by the production of mycolactone at the site of infection. The extensive tissue necrosis and minimal inflammation in Buruli ulcers constitute the hallmarks of these lesions, and reflect the cytocidal and immunosuppressive properties of this toxic macrolide as has been reviewed above. In essence, mycolactone is rapidly diffusible across plasma membranes of target cells and accumulate in the cytosol. Mycolactone then triggers diverse cytopathic effects, including cytoskeletal rearrangements and cell cycle arrest, eventually culminating in apoptotic or necrotic cell death. The manifestations and levels of mycolactone cytotoxicity vary extensively among cell types, suggesting that the molecular target of mycolactone may be differentially expressed or have different functions in different cells. At noncytostatic and cytotoxic concentrations, mycolactone displays immunomodulatory properties on human primary monocytes and dendritic cells, indicating that it may limit the initiation of innate immune responses *in vivo*. By blocking the capacity of primary T cells to produce multiple cytokines upon activation and by impairing T-cell migratory and homing into lymph nodes, mycolactone may impact on the development of adaptive immune response as well.

2.9 Host immune response to *M. ulcerans* infection

The immune response to *M. ulcerans* infection has been studied in mice models and in patients. This section reviews the immune response to *M. ulcerans* into local, regional and systemic compartments as follows:

2.9.1. Local immune response to *M. ulcerans* infection

Quantitative studies of intralesional mRNA in patients by Prevot et al. (2004), Phillips et al. (2006) and Peduzzi et al. (2007) indicate that the innate immune system is activated at the site of *M. ulcerans* infection as evidenced by high mRNA levels for cytokines IFN-γ, IL-1β, IL-6, IL-10, IL-12, IL-15 and TNF-α, and the chemokine IL-8. Although these results would appear to contradict the notion that local production of mycolactone in the skin prevents the trafficking of inflammatory cells into the lesion and challenges the compelling evidence from *in vitro* studies, there are possible explanations for this discrepancy between *in vitro* and *in vivo* observations. First, insights into the mechanisms of mycolactone-mediated immunosuppression on monocytes and T-cells suggest that the toxin suppresses inflammatory cytokine production at the post-transcriptional level, by either inhibiting protein translation or secretion. Hence it is important that caution is exercised in interpretation of real-time PCR results obtained from patient samples since cytokine transcripts may not be necessarily correlated with protein concentrations. Secondly, results from mouse studies indicate that the lack of inflammatory infiltrates in Buruli ulcer is caused by the continuous destruction of inflammatory cells, rather than local immunosuppression. Developing assays to measure concentrations of mycolactone in human tissues would help further our

understanding in the interplay between mycolactone and the evolution of the local immune response.

2.9.2. Regional immune response to *M. ulcerans* infection

The initiation of a protective immune response against intracellular pathogens requires the activation and migration of antigen-presenting cells to the peripheral/draining lymph nodes in order to achieve T-cell priming (Reiley et al., 2008, Wolf et al., 2008). Fraga and co-workers (2010) showed that early in mouse infection with either mycolactone-producing or non-mycolactone producing strains of *M. ulcerans*, pathogen-specific IFN-γ-producing T cells developed in the draining lymph nodes. It was found that $CD4^+$ cells migrated to the infectious foci, but progressive infection with virulent *M. ulcerans* led to the local depletion of recruited cells. Furthermore the dissemination of mycolactone producing *M. ulcerans* to the draining lymph nodes was accompanied by extensive apoptotic cytopathology of the draining lymph nodes leading to depletion of $CD4^+$ T cells and abrogation of IFN-γ expression. This local/regional cytotoxic effect of mycolactone on T-cells also affected B-cells similarly.

The continuous lymphatic dissemination of *M. ulcerans*, either freely or shuttled in phagocytes via afferent lymphatic vessels into draining regional lymph nodes culminating in lymph node destruction and bacterial colonisation has clinical relevance. First, patients with Buruli ulcers seldom have palpable lymphadenopathy in spite of the presence of extensive skin lesion. Secondly, cases of disseminated Buruli ulcers may be due to this phenomenon. Thirdly, the high recurrence rates after

surgical excision of lesions alone with antibiotic therapy may be due to regional lymph node dissemination of *M. ulcerans* infections.

2.9.2. Systemic immune response to *M. ulcerans*

Studies in human subjects with *M. ulcerans* disease suggest that IFN-γ is associated with protection. This cytokine was first suggested to play a role in the control of *M. ulcerans* infection by Gooding et al. (2003) who reported that PBMCs from patients with previously healed Buruli ulcers displayed low capacity to produce IFN-γ compared with healthy contacts after stimulation with live *M. ulcerans* or *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). Westerbrink and co-workers (2005) reported that purified protein derivative (PPD)-stimulated PBMCs from BU patients produce significantly higher levels of IFN-γ in the late stages of the disease but not in early stages, as compared with matched community controls. When PCR analysis was used to quantify the expression of this cytokine in nodular or ulcerative lesions, there was a higher expression of IFN-γ in the nodular lesions while a lower expression was detected in the more severe ulcerative lesions (Prevot et al., 2004). However, Phillips and co-workers (2006) using a larger number of patients, reported a strong expression of IFN-γ in both nodular and ulcerative lesions with significant inter-patient variation in IFN-γ concentrations for each of these forms of lesions.

Evidence of the existence of protective cellular-mediated immune and delayed-type hypersensitivity responses, associated with IFN-γ production in the context of Th-1 responses, has come from the observation that as *M. ulcerans* disease progresses to healing granuloma formation occurs (Connor et al., 1965, Hayman et

al., 1993, van der Werf et al., 1999, Thangaraj et al., 1999, Gooding et al., 2002, Gooding et al., 2003, Prevot et al., 2004, Westerbrink et al., 2005 and Schipper et al., 2007) and the positivity of the Burulin skin test increases (Marston et al., 1995, Dobos et al., 2000). In addition, BCG vaccination was found to protect humans against BU osteomyelitis (Portaels et al., 2002 and Portaels et al., 2004), and BCG or DNA vaccine encoding Ag85A from BCG confer some degree of protection against *M. ulcerans* experimental infections (Tanghe et al., 2001). These studies have suggested a protective role for IFN-γ in *M. ulcerans* disease.

On the basis of these lines of evidence, it is probable that the evolution of a protective systemic adaptive immune response to *M. ulcerans* is a gradual process. The initial suppression of systemic T-cell responses in *ex-vivo* stimulation assays observed in the early stages of the disease could be due to a local build up of mycolactone which would impair macrophage activation and or induce the cell death of incoming and resident leukocytes as well as destruction of draining regional lymph nodes and T-cell homing into these nodes. Alternatively, as has been shown in mice (Hong et al. 2008), mycolactone may reach the systemic circulation to interfere with adaptive protective immune responses.

There is restoration of the systemic IFN-γ secretion *ex-vivo* in response to both mycobacterial as well as non-mycobacterial antigens after surgical excision of lesions (Yeboah-Manu et al., 2006). Secondly, the administration of combination of rifampicin and streptomycin for the treatment of *M. ulcerans* disease, has been shown to increase phagocytosis of bacilli at the infection foci and to promote inflammatory cellular responses (Schutte et al., 2007) as well as increased

production of IFN-γ in *ex-vivo* stimulation assays (Sarfo et al., 2009). These would support the argument that the reduction of mycolactone concentrations would allow macrophage activation and further containment of the infection. Without any form of curative intervention, this process would occur slowly.

In spite of the apparent systemic anergic state induced by infection with *M. ulcerans*, most patients are constitutionally well and are not at increased risk of opportunistic infections. Indeed, mice infected with mycolactone-producing strains of *M. ulcerans* were not more susceptible to a systemic co-infection with sub-lethal doses of *Listeria monocytogenes,* an infection for which the host requires T-cell-mediated immunity for protection (Fraga et al., 2011). Thus at least in mice, the evidence shows that even though progressive infection with highly virulent strains led to a local and regional suppression of mycobacterium-specific T-cell response, a state of systemic immunosuppression was not induced. The study by Fraga and co-workers has important implications for vaccine development for *M. ulcerans* disease in that prophylactic and/or therapeutic interventions that could prevent dissemination of *M. ulcerans* to regional lymph nodes during the early phase of infection would contribute to the maintenance of a protective immunity and hence disease control.

2.10. Vaccine development for *M. ulcerans* disease

The prevention of Buruli ulcer disease in endemic countries has been hindered by incomplete knowledge on the exact mechanisms of transmission as well as the lack of an effective protective vaccine. The review in this section presents the

current state of the art knowledge on attempts at vaccine development for *M. ulcerans* and is focused on potential *M. ulcerans* vaccine targets and vaccination methods.

2.10.1. The immune response to *M. ulcerans*. Is it Th-1 or Th-2 driven?

M. tuberculosis and *M. leprae*, the causative agents of tuberculosis and leprosy respectively, are both intracellular pathogens. Hence Th1-type cellular immune responses are essential for control of both infections, while humoral (antibody) responses have little benefit or potentially adverse effects to the host (Touw et al., 1982, Demkow et al., 2005). While the immune response to *M. ulcerans* is not fully understood, Th1-type cellular immune responses appear to be important for the control of *M. ulcerans* (reviewed under section 2.9 above).

However, there is debate regarding the induction of Th2-type and anti-inflammatory cytokines by *M. ulcerans*, which seems to predominantly persist in infected foci as an extracellular pathogen. Gooding et al. (2002) reported expression of IL-4 and IL-10 among other cytokines from Buruli ulcer patients from Australia (Gooding et al., 2002), while Prevot et al. (2004) detected IL-10 and not IL-4 among patients from French Guyana. Westerbrink et al. (2005) did not detect IL-4 nor IL-10 in *ex-vivo* stimulation assays using PPD which is not specific for *M. ulcerans*. However, Phillips et al. (2006) demonstrated clearly using a crude sonicate of *M. ulcerans* antigens that among Ghanaian patients with active *M. ulcerans* disease, a mixed Th1-type (measured using IFN-γ secretion) and Th2-type (measured using IL-10 secretion) cytokine phenotype was observed with a Th1 response emerging as the dominant phenotype among healed patients. Admittedly, these differences could be

due to genetic and ethnic differences in patients or *M. ulcerans* strains, severity of lesions and differences in experimental designs such as the types of antigens used for stimulation as well as techniques employed in the measurement of respective cytokines.

Thus said the greatest challenge to vaccine development for *M. ulcerans* disease appears to that posed by mycolactone. *M. ulcerans* is distinct from other mycobacteria in that it expresses a toxin that is the main virulence factor (Hong et al., 2008). As reviewed earlier mycolactone has been shown to interfere with most of the immune defense mechanisms the host has evolved to contain mycobacterial infections. In particular, the fact that mycolactone provokes a prolonged extracellular phase of infection means that potentially humoral responses may be more important for protection than in other mycobacterial infections, with obvious implications for vaccine development. Furthermore because mycolactone is a lipid toxin, it is poorly immunogenic in both mice and humans (Huygen 2003) and mycolactone-specific antibodies have not been found. However, a range of potential targets, coupled with recent advances in the field of vaccine development, offers a wide variety of vaccine candidates against *M. ulcerans* and are reviewed below.

2.10.2. *M. ulcerans* vaccine targets

(a) Mycolactone: The obvious vaccine candidate for *M. ulcerans* is mycolactone since it is the primary virulence factor for the disease. While a vaccine aimed at neutralising the toxin or preventing it from binding to its target(s) would not prevent colonisation of the bacterium, it could most likely prevent pathological

symptoms. In this regard the recent identification of Wiskostatin, which is able to competitively inhibit mycolactone binding to N-WASP and thus prevent degradation of skin by mycolactone, represent an advancement for rationale design of mycolactone inhibitors with anti-Buruli ulcer therapeutic potential (Guénin-Mace and Veyron-Churlet et al. 2013). However attempts at generating neutralising antibodies have so far not been possible (Huygen, 2003) due to the poor immunogenicity of this lipid toxin.

An alternative strategy is to target the polyketide synthase responsible for mycolactone synthesis by generating synthase-specific antibodies or synthase-specific T-cell responses to prevent toxin production by bacterium or help eliminate *M. ulcerans*-infected cells, respectively (Einarsdottir et al., 2011). Pidot et al. (2010) have shown that patients with Buruli ulcer and endemic controls exposed to *M. ulcerans* but who remain disease-free, can produce antibodies against several of the PKS enzymatic domains.

(b) Live, attenuated bacteria: The BCG vaccine or mycolactone-deficient *M. ulcerans* represent two attractive strategies for a vaccine developed using a live, attenuated bacteria approach.

(i) BCG: Bacille Calmette-Guerin (BCG) is a live, attenuated strain of *M. bovis*, used as vaccine against tuberculosis, that induces significant cross-reactive immune responses against other mycobacteria. The BCG vaccine has been administered in over four billion doses, and is routinely given to newborns in developing countries because of the protection it confers against the childhood forms of tuberculosis (meningitis, military TB). However, the vaccine does not prevent *M.*

tuberculosis infection and its protection against the classical, pulmonary form of TB is variable (Martin, 2005).

In two large clinical trials in Uganda involving 2,500 and 9,000 people respectively, BCG was shown to confer transient protection against Buruli ulcer in 47% of vaccinated persons that can last for up to a year (Uganda Buruli group, 1969 and Smith et al., 1976) and lessen the severity of ulcers in patients (Smith et al., 1976). In another study, children and adults with BCG vaccination were less likely to develop osteomyelitis, the most severe form of *M. ulcerans* disease. (Amofah et al., 1993). The fact that Buruli ulcers tend to be commoner in the younger age population in whom BCG vaccination was administered in infancy suggests that the protection provided by BCG against Buruli ulcer is limited.

Antigenic differences between BCG and disease-causing mycobacterial strains have been posited as a theoretical explanation for the limited protection of BCG. This is supported by the finding that recombinant BCG expressing *M. tuberculosis* antigens confer greater protection than standard BCG upon challenge with *M. tuberculosis* (Pym et al., 2003). Perhaps a similar strategy of developing a recombinant BCG strain expressing immunodominant, protective *M. ulcerans* antigens could be considered to increase BCG-mediated protection against Buruli ulcer.

(ii) Mycolactone-deficient *M. ulcerans*: The advantage with this alternative Buruli ulcer vaccine strategy is that the bacteria express all antigens expressed by virulent *M. ulcerans* bacteria, except for mycolactone. Therefore, they can induce a broad spectrum of humoral and cellular *M. ulcerans* specific immune responses.

Several Mycolactone-deficient *M. ulcerans* have been generated either spontaneously or through random transposon mutagenesis. (Sinear et al., 2004) Random transposon mutagenesis has revealed that several genes are involved in mycolactone synthesis (Sinear et al., 2004 and Adusumilli et al., 2005) and that mutations of some of these genes ablate toxin production completely, while strains with mutations in other genes produce incomplete toxin (Adusumilli et al., 2005).

To assure the safety of this vaccine candidate particularly for immunocompromised individuals, it has been recommended that at least two targeted gene deletions should be introduced into live attenuated mycobacterial vaccines (Kamath et al., 2005). While this may negatively affect the persistence and immunogenicity of bacteria by over-attenuating the vaccine strain, it is a necessary safety precaution as the bacteria are alive and could potentially revert to a virulent state.

Fraga et al. (2012) used a mouse model to characterise the histological and cytokine profiles triggered by vaccination with either BCG or mycolactone-negative *M. ulcerans*, followed by footpad infection with virulent *M. ulcerans*. They observed that compared with unvaccinated mice, BCG vaccination significantly delayed the onset of *M. ulcerans* growth and footpad swelling through the induction of an earlier and sustained IFN-γ T cell response in the draining lymph nodes. BCG vaccination resulted in cell-mediated immunity in *M. ulcerans*- infected footpads with a preponderant chronic mononuclear infiltrates with increased and sustained levels of IFN-γ and TNF-α mRNA. No significant IL-4, IL-17 or IL-10 responses were detected in the footpad or the draining lymph node, in either infected or vaccinated

mice. Despite this protective Th1 response, BCG vaccination did not avoid the later progression of *M. ulcerans* infection, an observation which had been reported by Converse et al. (2011). Similarly, immunisation with mycolactone-deficient *M. ulcerans* also significantly delayed the progression of footpad infection, swelling and ulceration, but ultimately *M. ulcerans* pathogenic mechanisms prevailed (Fraga et al., 2012). The delayed emergence of pathology observed in vaccinated mice appears to be determined by a protective Th1 recall responses against *M. ulcerans*. Exactly how this cell-mediated immunity induced by vaccination is compromised remains to be elucidated.

(c) Sub-unit *M. ulcerans* vaccines: The complete genome sequence of the plasmid and the genome of *M. ulcerans* is now available (genolist.pasteur.fr/BuruList/). Unfortunately, the complete genome sequence of *M. ulcerans* revealed the loss of immunodominant proteins such as mycobacterial proteins ESAT-6, CFP-10 and HspX which represent potential antigens for sub-unit vaccine and immunodiagnostic tests. This has supported the notion that the loss of these immunodominant proteins by *M. ulcerans* is an evolutionary strategy to help the bacterium to evade the host's immunological responses and that this may represent part of an ongoing adaptation of *M. ulcerans* to survival in host environments that are screened by immunological defense mechanisms (Huber et al., 2008).

These notwithstanding, several *M. ulcerans* specific proteins have recently been identified and shown to be immunogenic (Pidot et al., 2010). In addition, there are many proteins with strong homology to other mycobacterial proteins, such as

antigen 85A (Ag85A) that show an 84% amino acid sequence identity with Ag85A from *M. tuberculosis*. (Tanghe et al., 2001). Tanghe et al. (2008) showed that a DNA vaccine coding for Ag85A from either *M. tuberculosis* or *M. ulcerans* was able to delay BU progression in mice, with DNA encoding Ag85A from *M. ulcerans* giving significantly more protection than DNA encoding Ag85A from *M. tuberculosis*. In addition, Coutanceau et al. (2006) showed that DNA vaccination with *M. ulcerans* heat shock protein 65 (HSP-65) conferred some protection against *M. ulcerans* in mice. The serious drawback with developing vaccines targeting HSP-65 is that whilst it is immunogenic, it shares strong homology with human HSP-60.

(d) Carrier proteins: Data published by Marsollier et al. (2007) indicate that immune responses to insect saliva proteins can confer protection against *M. ulcerans* when mice were subsequently exposed to *M. ulcerans* coated with insect saliva proteins. Even though the mechanism of protection is unclear, it would seem that the coupling of *M. ulcerans* antigens to saliva antigens might be strongly immunogenic. However, because transmission through aquatic insect bites may not be sole route of infection, Buruli ulcer vaccines in this category would have to be customised to different regions, based on the predominant transmission route of the pathogen.

2.11. Laboratory confirmation of *M. ulcerans* disease

Laboratory confirmation of a clinical diagnosis of *M. ulcerans* disease is currently performed using either culture for *M. ulcerans*, staining for acid-fast bacilli, PCR for IS2404 or histology.

(a) Smear microscopy for detection of acid-fast bacilli: This is the simplest diagnostic technique available in endemic communities in sub-Saharan Africa. Swab samples obtained from the undermined edges of an ulcer or biopsy specimens obtained surgically or by a punch biopsy are used to prepare a smear on a slide which is then stained using the Ziehl-Neelsen stain for the presence of acid-fast bacilli (Portaels et al. 1997). The sensitivity of microscopy of varies between 29%-78% (Phillips et al., 2005 and Herbinger et al., 2009) with an average sensitivity of approximately 50%. It should be noted that higher sensitivity of microscopic diagnosis is noted for samples obtained from surgically excised tissues. With the advent of antibiotic therapy for *M. ulcerans* disease, minimally invasive techniques are required to obtain samples for laboratory confirmation. Microscopic examination has a high specificity of up to 96.6% (Herbinger et al., 2009) compared with the gold standard PCR for IS2404.

Given that smear microscopy for diagnosis of *M. ulcerans* disease is cheap, rapid and has high specificity with potential for wide applicability in endemic communities, measures to enhance the sensitivity of this diagnostic approach are urgently required. Yeboah-Manu et al. (2011) reported that by optimising the release of mycobacteria from swab specimen and concentrating the bacterial suspension before smearing, they were able to improve the detection rate of acid-fast bacilli by

microscopy to up to 58.4% compared with PCR. They also showed that a stepwise laboratory confirmation algorithm with detection of AFB as first-line method and IS2404 PCR performed only with those samples that were negative in microscopic analysis would reduce the unit cost of diagnosis by more than 50%. Perhaps for ulcerative Buruli lesions, obtaining at least three swabs for microscopy would enhance the sensitivity further.

(b) Culture for *M. ulcerans*: *M. ulcerans* grows slowly in the laboratory on Lowenstein-Jensen slopes, taking on average 9 to 12 weeks or up to 6 months for a positive result. The sensitivity of culture for diagnosis has been reported to range from 34% to 79% (Herbinger et al., 2009). The main drawback with culture is the prolonged time it takes to obtain a positive result which in the antibiotic therapy era constitutes a challenge but at the moment culture remains the only test to assess viable organisms in tissues. Newer techniques to assess the presence of viable organisms in tissue are urgently required.

(c) Histology: Histology was perhaps the most sensitive of the conventional diagnostic methods before the advent of PCR for *IS2404*. In a Ghanaian study, it was 82% sensitive (Phillips et al., 2005). However histopathology services require expertise and are often not available in endemic areas.

(d) PCR for *IS2404*: A major advance for the purpose of research studies is the application of PCR for the insertion sequence *IS2404*, (Ross et al., 1997), one of the two multi-copy insertion sequences found in the genome of *M. ulcerans* (Stinear et al., 1999). With an analytical specificity of 100% and a sensitivity of 79-98%, *IS2404* PCR is considered to be the most reliable technique for the detection of *M.*

ulcerans in human diagnostic samples such as to swabs or punch biopsies (Stinear et al., 1999, Guimares-Peres et al., 1999, Stienstra et al., 2003, Phillips et al., 2005, Herbinger et al., 2009). Recently, fine needle aspirates obtained from lesions have been used for PCR diagnosis of *M. ulcerans* disease. Phillips et al. (2009) reported a sensitivity of up to 90%. Hence even though sensitivity of PCR is somewhat slightly compromised with FNA samples, its acceptance and tolerability by patients has pushed it as first line sampling strategy for non-ulcerative lesions (Eddyani et al., 2009, Herbinger et al., 2010).

PCR can give results within 24 hours enabling clinicians to reach a treatment decision quickly provided it is done in a laboratory with high standards to avoid false positives (Phillips et al., 2005). The PCR technique has been refined by the addition of uracil DNA glycosylase and deoxyuridine triphosphate instead of deoxythymidine triphosphate to the reaction mixture, which reduces the risk of false positives due to amplicon contamination (Phillips et al., 2005). A dry-reagent PCR technique with lyophilised PCR reagents and transport buffers has also been developed to overcome technical difficulties with PCR in the tropics (Siegmund et al., 2005). For purposes of quality control, quantitative real-time PCR for *IS2404* with improved sensitivity has been introduced. (Rondini et al., 2003, Rondini et al., 2006). Clearly cost and local expertise are the main determinants of the availability of these laboratory methods.

(e) Loop mediated isothermal amplification: A more recent approach to diagnosis of *M. ulcerans* disease is the use of Loop Mediated Isothermal Amplification (LAMP). LAMP is a novel nucleic acid amplification method for

molecular detection and identification (Notomi et al., 2000). The principle of LAMP is autocycling strand displacement DNA synthesis in the presence of Bst DNA polymerase with high strand displacement activity under isothermal conditions between 60-65°C within 60 minutes (Wang et al., 2008). LAMP is a highly specific assay due to the recognition of target DNA by 4 to 6 independent sequences with an amplification efficiency equivalent to that of PCR-based methods (Notomi et al., 2000, Nagamine et al., 2002, Poon et al., 2004). Amplification products can be visually identified in reaction tubes through the addition of intercalating dyes such as SYBR green or hydroxynapthtol (Iwamoto et al., 2003). Two other features places LAMP as a potential point of care test for *M. ulcerans* disease. First, the closed tube format of this assay reduces the problem of carry over contamination (Jayawardena et al., 2007) and second the assay is less affected by a number of inhibitors of conventional PCR (Kaneko et al., 2007).

The feasibility of using LAMP for laboratory confirmation of Buruli ulcer has been explored recently. Njiru et al. (2012) found that the test was robust and specific with a detection limit equivalent to 20 copies of the target sequence. In that study, the investigators used LAMP primers targeting IS2404 and the ketoreductase B domain (KR-B) and reported that the LAMP assay could detect *M. ulcerans* in clinical specimens and spiked environmental samples. In 79 clinical samples obtained from Ghanaian patients, 50 were positive by conventional PCR, 42 were positive by the LAMP assay giving a percent ratio of LAMP-to PCR-positive of 84% and a kappa value of 0.74 indicating a moderate agreement between the two tests (Njiru et al., 2012). Two other studies both conducted in Ghana support these findings (Ablordey et al., 2012, de Souza et al., 2012). BU LAMP assay has

potential to be used as a point-of-care test, which would contribute immensely towards early detection and confirmation of clinically diagnosed cases of Buruli ulcer.

2.12 Management of *M. ulcerans* disease

2.12.1. *Surgical management*

Until the recent introduction of antibiotics, the mainstay of treatment for *M. ulcerans* disease had been surgery. Techniques used in surgical treatment consist generally of two stages. In the first stage the aim is to excise all dead tissue, including a healthy tissue margin around the lesion- typically 3-4cm (van der Werf et al., 1999). This procedure results in a large surgical lesion that needs to be skin-grafted in a second stage. Surgery involving the head and neck region, especially the face and eyes, poses difficult reconstructive plastic surgical problems for surgeons (Agbenorku et al., 2000, Agbenorku et al., 2005).

As an approach to management, surgical treatment is beset with two important limitations. First, at the practical level, surgery is difficult to perform in settings with poor resources, as there are very limited possibilities for general anaesthesia and blood transfusion (Barogui et al., 2009). Moreover, it requires technical and surgical skills and patients often have to undergo several months of wound dressing. Added to these is the occurrence of severe sequelae typified by contractures resulting from the extensive surgical excisions required for large lesions. Thus, surgery would be available to a tiny fraction of patients afflicted with Buruli ulcer in poor rural endemic communities. Second, at a more scientific level, it has been shown that infection with *M. ulcerans* reaches beyond the margins of Buruli lesions into normal looking tissue where an experienced surgeon would make the incision for removal of

the lesion, so recurrence is likely despite good surgical technique (Rondini et al., 2003). In practice, recurrence rates after surgery vary between 6% and 17% (Amofah et al., 1999, Debacker et al., 2005), depending on the type and extent of lesion and on the experience and skill of the surgeon.

Evidence that antibiotic have a role in treatment and the limitations imposed by surgery as discussed above have resulted in shift in favour of antibiotic therapy for *M. ulcerans* disease, with limited surgical intervention where necessary.

2.12.2. ***Antibiotic treatment***

1.12.2.1. *In-vitro* antibiotic sensitivity testing: *In vitro, M. ulcerans* has been shown to be susceptible to rifamycins (rifampicin, rifabutin and rifapentine) (Havel et al., 1975), aminoglycosides (amikacin and streptomycin) (Thangaraj et al., 2000), macrolides (clarithromycin, azithromycin but not telithromycin) (Portaels et al., 1998) and quinolones (moxifloxacin but not levofloxacin nor sparfloxacin) (Thangaraj et al., 2000). In these *in vitro* studies rifampicin, rifabutin, rifapentine, amikacin and streptomycin demonstrated bactericidal activity whilst clarithromycin, azithromycin and moxifloxacin were found to be bacteriostatic. A brief review of the mechanism of action of these four classes of antimicrobials is presented below:

(a) **Rifamycins**: The antibacterial activity of rifamycins relies on the inhibition of bacterial DNA-dependent RNA synthesis (Calvori et al., 1965). This is due to the high affinity of rifamycins to prokaryotic RNA polymerase. Crystal structure data of the antibiotic bound to RNA polymerase indicates that rifamycins block synthesis by causing strong stearic clashes with the growing oligonucleotide in what is referred to as "stearic-occlusion" mechanism (Campbell et al., 2001, Feklistov et al., 2008).

Rifamycins are particularly effective against mycobacteria, and are therefore used to treat tuberculosis, leprosy, Buruli ulcer and *Mycobacterium avium* complex (MAC) infections.

(b) **Aminoglycosides**: Although the exact mechanism of action of aminoglycosides is not fully known, it is thought to have several potential mechanisms including:

i. binding to the 30S ribosomal subunit and thereby inhibiting protein synthesis (Levison, 2009).

ii. interference with the proof-reading process, causing increased rate of error in synthesis with premature termination of m-RNA.

iii. disruption of the integrity of bacterial cell membrane (Lorian, 1996). Aminoglycosides are thought to competitively displace cell biofilm-associated Mg^{2+} and Ca^{2+} that link the polysaccharides to adjacent lipopolysaccharide molecules. This results in the shedding of cell membrane blebs, with formation of transient holes in the cell wall and disruption of the normal permeability of the cell wall.

(c) **Macrolides**: The macrolides inhibit protein synthesis. Their mechanism of action involves preventing peptidyltransferase from adding a peptidyl attached to transfer RNA to the next amino acid (Tenson et al., 2003). Macrolide antibiotics bind reversibly to the P site on the subunit 50S of the bacterial ribosome, an action considered to be bacteriostatic.

(d) **Fluoroquinolones**: The fluoroquinolones work by inhibiting the topoisomerase II ligase domain, leaving the two nuclease domains intact. This

modification, coupled with the constant action of the topoisomerase II in the bacterial cell, leads to DNA fragmentation via the nuclease activity of the intact enzyme domains (Elsea et al., 1992).

2.12.2.2. In-vivo antibiotic susceptibility testing in mouse footpads**:** The mouse footpad model was developed by an Australian virologist Frank Fenner in the 1950s (Fenner, 1956, Leach et al., 1954). In this model, *M. ulcerans* bacilli are injected just under the surface of the skin into a relatively cool area of the mouse body, approximating the peripheral location of most human lesions. Gross pathology shows progressive swelling of the footpad that often leads to ulceration and loss of the foot and leg if left untreated, with no or limited pain. Footpads can easily be monitored for both worsening of infection and response to antibiotic treatment. A reduction in swelling is observed with effective curative therapy and also assessment of microbiological response is feasible because the whole lesion can be cultured to evaluate for bacteriostatic and bactericidal drug activity.

Using the mouse footpad model, treatment with rifampicin, aminoglycosides, macrolides and quinolones, showed favourable responses with lesions becoming smaller and the total number of *M. ulcerans* bacilli in the tissue reducing (Stanford et al., 1972, Dega et al., 2000, Bentoucha et al., 2001). The combination of rifampicin with amikacin or streptomycin was the most effective at preventing relapse after treatment for 12 weeks, suggesting a bactericidal effect, but cultures for *M. ulcerans* were not successful in that study (Bentoucha et al., 2001). Subsequent studies showed no viable bacteria in mouse tissue after 7 weeks treatment with the combination of rifampicin plus amikacin (Marsollier et al., 2003). Another reason

for using more than one drug was that resistant mutants were found after rifampicin monotherapy in mice (Marsollier et al., 2003).

Though the mouse footpads model is a useful tool for evaluating the activity of drugs against *M. ulcerans* in vivo, some important caveats have been raised (Converse et al., 2011). For instance, the metabolism of drugs in mice may not be similar to that in humans. A dose that is potent in humans may not be in mice, although rifampicin and streptomycin are comparable. The clarithromycin dose is quite different and therefore requires different dosing regimens in mice to avoid interference with the activity of rifampicin. Finally, although the mouse footpad model has some semblance to human lesions, there are forms of human disease such as oedema that are not reproduced in mice.

2.12.2.3. Efficacy of antibiotics for treatment of *M. ulcerans* disease in humans:

The combination of rifampicin with streptomycin was evaluated for its ability to kill *M. ulcerans* in early human lesions of *M. ulcerans* disease (Etuaful et al., 2005). This was the first study to define the role of antibiotics for the treatment of *M. ulcerans* disease. Treatment with rifampicin 10mg/kg orally and streptomycin 15mg/kg intramuscularly daily was administered for 0, 2, 4, 8 or 12 weeks and lesions were excised for quantitative culture. The findings were definite, showing that cultures for *M. ulcerans* were positive at baseline with no treatment and after 2 weeks but negative thereafter, indicating that this combination of antibiotics might be useful for the management of nodules and plaques which were the only lesion types studied. Furthermore, it was observed that no lesions enlarged during therapy with most lesions regressing during treatment. (Etuaful et al., 2005). Although this

study did not prove that the disease could be cured by antibiotic treatment alone because lesions were excised, it provided data for the WHO to issue interim guidelines for treatment of *M. ulcerans* disease in 2004. According to the guidelines issued for clinicians by the WHO Technical Advisory Group on Buruli ulcers, the combination of rifampicin and streptomycin can be given to ambulant patients of any age with Buruli ulcers and early lesions for 8 weeks under careful observation and that limited surgery be reserved for essential debridement, grafting to accelerate healing of large ulcers, or to excise lesions that continue to enlarge despite antibiotic therapy (WHO, 2004).

The guidelines for Buruli ulcer treatment by the WHO were first applied in Benin to assess the effectiveness of the antibiotic combination. Among the 224 patients for whom antibiotic treatment was initiated, 215 (96%) were categorised as treatment successes and 9, including 1 death and 8 losses to follow up were classified as treatment failures. The 215 successfully-treated patients had 102 (47%) treated solely with antibiotics and 113 (53%) treated with antibiotics plus surgical excision and skin grafting. A key determinant of the need for surgical intervention was the size of lesion at treatment initiation as evidenced by the observation that 73% of patients with lesions of >15cm in diameter underwent surgery, whereas only 17% of patients with lesions of <5cm had surgery. Importantly, only 3 out of 208 patients retrieved after 1 year of antibiotic completion had recurrent lesions. These findings led to the conclusion that the WHO-recommended streptomycin-rifampicin combination was highly efficacious for treating *M. ulcerans* disease although only 47% of cases were cured by chemotherapy alone (Chauty et al., 2007).

A study conducted in Ghana involving 160 PCR-positive small and large lesions provided data on the efficacy and practicality of antibiotics for treatment of *M. ulcerans* disease (Sarfo et al., 2010). Notably, 95% of lesions healed solely on antibiotic chemotherapy alone with only eight (5%) patients requiring skin grafting after completion of the 2-month course of rifampicin plus streptomycin treatment. Wide variations in time to complete healing and the healing rate as measured by reduction in ulcer diameter were reported. Another observation was the complete healing of large oedematous forms of the disease solely on antibiotic therapy without recourse to surgery. Antibiotics were well tolerated with three reported adverse events: two were vestibular toxicity attributable to streptomycin and one case of skin rash from rifampicin. The streptomycin toxicity was managed by substitution with moxifloxacin whilst the rifampicin toxicity was managed by stopping the treatment and continuing wound dressings till lesions eventually healed. (Sarfo et al., 2010).

A randomised controlled trial comparing 8-weeks of rifampicin and streptomycin or 4-weeks of rifampicin plus streptomycin followed by a 4-week course of rifampicin plus clarithromycin (7.5 mg/kg) has confirmed the efficacy of antibiotic therapy for the management of early, limited lesions. An intent-to-treat analysis found that 96% of participants on the standard regimen compared with 91% in the other arm had healed lesions at 1 year after the start of treatment. Again no recurrences were observed. This study showed that the regimens had similar efficacy, possibly allowing for a reduction of injections. (Nienhuis et al., 2010).

2.12.2.4. Towards an oral antibiotic regimen for *M. ulcerans* disease:

Despite the high efficacy of rifampicin plus streptomycin, there is a drive to have an all-oral treatment regimen, which means replacing streptomycin, the most bactericidal drug in the combination. An oral replacement could be less costly and safer without the requirement for sterile needles and providers to administer the injections. The potential advantage of an injected drug is that patients and providers must both comply with the treatment which enhances adherence to treatment which is important for a mycobacterial disease. The potential replacements for streptomycin would be clarithromycin or moxifloxacin and the long-acting rifamycin, rifapentine for the replacement of rifampicin.

A pilot study from Benin showed that 30 patients with lesions ≤ 10cm in diameter were successfully treated with rifampicin 10 mg/kg and oral clarithromycin 12mg/kg once daily for 8 weeks without adverse events (Chauty et al., 2011). However limited surgery was carried out on 11 patients whilst four patients had extensive surgery comprising of major excision followed by skin grafting. There were no relapses or recurrences after 18 months after treatment completion. Additional evidence for the promising efficacy of an all-oral combination therapy came from case reports from Benin (Dossou et al., 2008) and Australia (Jenkin et al., 2002, Gordon et al., 2010).

2.12.2.5. Paradoxical reactions associated with antibiotic therapy:

Treatment of *M. ulcerans* disease with combination antibiotic may be accompanied by an apparent worsening of the lesion after an initial improvement or the appearance of new lesions away from the primary lesion. This phenomenon has been term as “paradoxical reaction” and was first described for *M. ulcerans* disease

in Australian patients who underwent surgery accompanied by rifampicin and ciprofloxacin therapy (O'Brien et al., 2009). A study involving 151 participants reported that >30% (n=134 patients eligible for analysis) of participants showed an increase in lesion size as compared with their previous assessment and 9 participants developed new lesions either during or after completion of antibiotic therapy (Nienhuis et al., 2012). Of note, the events were observed towards the end of the 8-week antimicrobial treatment and since all lesions subsequently healed should not be misinterpreted as treatment failure.

The basis of this phenomenon is beginning to be unravelled. It has been observed that following antibiotic therapy, bacteria are located primarily intracellularly rather than extracellularly and that there is a development of organised cellular infiltration in the lesions. This suggests a reversal of the local immune suppression possibly due to a declining tissue concentration of mycolactone and is indicative of a healing process (Schütte et al., 2007, Schütte et al., 2009). Furthermore, analysis of whole blood cells stimulated overnight with *M. ulcerans* sonicate indicated that during antibiotic treatment there was also a general enhancement of the immune response with a significant increase in interferon gamma secretion (Sarfo et al., 2009). Ruf et al. (2011) described by histopathological and immunohistochemical analysis, two young patients who after 12 and 409 days after completing antibiotic treatment for a single category III lesion, developed multiple new PCR-positive, culture negative skin lesions 5 to 30 cm from the primary lesions. However, the AFB had a degenerated appearance and there were massive leukocyte (monocytes/macrophages, T and B cells) infiltrates, also typical of cured lesions. Although a re-infection could not be excluded, the new infection

foci were removed by limited debridement or excision and spontaneously resolved without additional antibiotic therapy.

2.12.3. ***Other treatment modalities***

M. ulcerans is predominantly a skin infection hence topical treatment would be an obvious choice in resource-limited settings. A cream generating topical nitric oxides was shown to promote healing in one small controlled trial (Phillips et al., 2004a). Nitric oxide was also shown to kill *M. ulcerans in vitro* (Phillips et al., 2004b). Phenytoin powder also was found to promote healing of lesions; although it did not probably kill the organism, healing perhaps occurred through the acceleration of fibrogenesis (Adjei et al., 1998).

Exploiting the temperature sensitivity of *M. ulcerans*, localised application of heat to lesions has been reported to help healing with or without surgery in a few cases (Glynn et al., 1972, Meyers et al.,1974, Junghanss et al., 2009, Braxmeier S., et al., 2009). However, the sophisticated nature of the devices and dressings, as well as discomfort, may prevent it from widespread routine use in a tropical setting. Although hyperbaric oxygen at partial pressures of 2.5 kPa administered to mice infected with *M. ulcerans* appeared to be beneficial compared with controls, its effectiveness in human beings needs to be demonstrated in controlled trials and the cost of such treatment may prevent its use in clinical practice. (Krieg et al., 1975, Krieg et al., 1979).

Local traditional methods of treatment usually involve application of herbal preparations but none are known to be effective and secondary bacterial infection is

a risk. A treatment protocol for Buruli ulcer using French green clays was developed and demonstrated healing of lesions (Brunet de Courssou, 2002; Williams et al., 2004) in the Ivory Coast. The stages of healing (see figure 2.12 below) show that after one day of treatment with one green clay (called CsAr02), the therapeutic properties of the clay were demonstrated with the initiation of rapid, non-surgical debridement of the necrotic tissue following topical application. After this, the second green clay (called CsAg02) was administered. Extended treatment with the CsAg02 clay resulted in continued tissue regeneration and wound healing with supple scarring and return of normal motor function (Brunet de Courssou, 2002). The absorptive properties of clay minerals are well documented for healing skin ulcers (Droy-Lefaix et al., 2006). Scientific validation of the possible mechanism of action showed that none of the components were directly bactericidal against *M. ulcerans* (Williams et al., 2008). CsAr02 has two properties of interest- first, its smectite component has absorbent properties which could bind to and inactivate mycolactone present in lesions and second, CsAr02 was shown to promote growth of a natural consortium of bacteria, typified by *E. coli*, potentially present in the open wounds to compete with *M. ulcerans* within lesion and also to provoke the natural immunity against *M. ulcerans* in infected tissues. The CsAg02 clay sterilized wounds by its demonstrated antibacterial action.

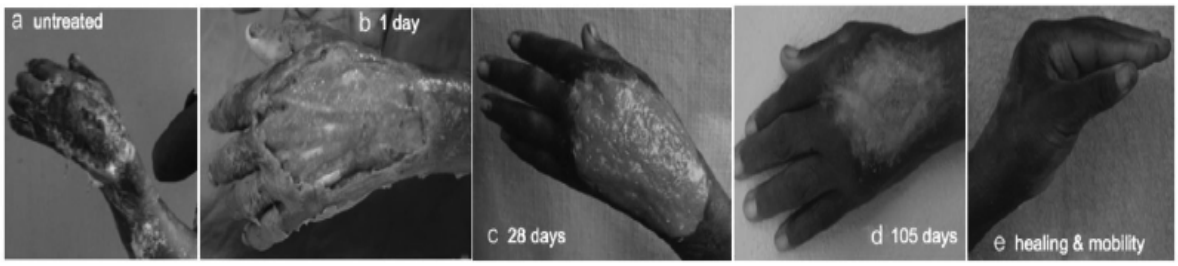


Figure 2.12. A photographic example of the stages of healing by clay: destroyed tissue (a) is easily removed by one treatment with CsAr02 to expose raw muscle and bone (b). The progression of healing is shown (c,d) with daily treatments of CsAgO2. After 3-4 months, the infection is healed with soft, supple and a return of normal motor function (e).

(Williams et al. 2008).

2.12.4. ***Physiotherapy and functional limitation prevention***

In rural Africa, patients tend to report to the hospital late in the course of the disease, some with extensive and long-standing ulcers with joint and bone involvement leading to fibrosis, scarring, calcification, and contractures with permanent disabilities may result. A system has been devised to assess functional limitation in individual patients, which may be helpful in clinical trials of treatment (Stienstra et al., 2005). Using this system, 362 (57%) of 638 patients in Benin and Ghana had persistent functional limitation after 4 years (Stienstra et al., 2005). Indeed patients with Buruli ulcer have lower quality of life scores compared with healthy controls (Hamzat et al., 2011). Thus simple physiotherapy administered by

health care workers with minimal training may have an important role in prevention of fixed joint deformities.

CHAPTER 3

METHODOLOGY

3.1. The bacteriological and clinical responses of buruli ulcer to either daily treatment with rifampicin-streptomycin for 2 weeks followed by rifampicin-clarithromycin for 6 weeks compared with rifampicin-streptomycin for 8 weeks: Pilot study.

Between July 2009 and July 2010, patients were recruited from three BU endemic districts of Ghana at Tepa Government Hospital, Agogo Presbyterian Hospital and Nkawie Government Hospital after active case search by district outreach teams. Ethical approval of the study protocol and consent forms was obtained from the Committee on Human Research Publications and Ethics of the Kwame Nkrumah University of Science and Technology and Komfo Anokye Teaching Hospital (CHRPE/05/18/09).

Clinical Trial Design

In a non-blinded manner, patients with a strong clinical diagnosis of Buruli ulcer were randomized based on a pre-generated coin tossed sequence concealed in envelopes. After giving informed consent, patients received either rifampicin-streptomycin for 7 days per week for 8 weeks or rifampicin (10mg/kg)-streptomycin (15mg/kg) for 2 weeks followed by rifampicin and clarithromycin (7.5mg/kg) for 6 weeks (RS2RC6). The randomly assigned allocation for enrolled patients was generated and provided by the principal investigator (RP). Verbal and written

informed consent was obtained from all participants, and from parents, carers, or legal representatives of participants aged 18 years or younger.

Patients were included if they were more than 5 years old, if they were willing to participate and if they met the WHO clinical case definition for *M. ulcerans* disease with a nodule, plaque, oedema or ulcer with maximum diameter less than 15cm. Patients were excluded if they had tuberculosis, leprosy, clinical and/or laboratory evidence of significant renal or hepatic impairment or auditory problems and if they were already under treatment with antibiotics or herbal preparations. Patients were also excluded if they were pregnant or known to be intolerant of the antibiotics. Patients were not tested for HIV infection. A detailed history and clinical examination was performed and recorded. An informal assessment of functional limitation was performed before therapy was initiated.

Diagnostic Confirmation

The diagnosis was confirmed by obtaining two fine needle aspirates (FNA) for PCR from non-ulcerative lesions or a swab from the undermined edge of ulcers which was examined for the presence of acid fast bacilli (AFB), cultured for *M. ulcerans* and tested by PCR for the IS2404 insertion sequence of *M. ulcerans* (Phillips et al., 2005, Phillips et al., 2009). Before treatment one 4mm diameter punch biopsy was taken from non-ulcerative lesions and from the margin of viable tissue in ulcerative lesions for AFB and semi-quantitative culture. Punch biopsies were also obtained at week 6 and week 12 if the lesion had not healed. Local anaesthetic in the form of 1% lidocaine was applied before punch biopsies were obtained. Additional swabs, punch biopsies or FNA were taken from any lesion

deemed to be exhibiting a paradoxical reaction for culture and PCR in order to distinguish recurrent or new infection from sterile inflammatory lesions. All females above 10 years were tested for early pregnancy before recruitment (Guangzhou Wondfo Biotech Co., Ltd, China).

Treatment Administration and Monitoring

Upon initiation of therapy, patients returned daily to their nearest health centre where treatment was administered under the direct observation of an existing team of village health workers. Participants were educated not to use herbal medication. Clinical response was assessed fortnightly during antibiotic treatment to monitor the time to complete healing. Serial photographs were taken until healing was complete and lesions were traced onto acetate paper by study nurses who were blinded to the treatment regimen patients were receiving. The surface area of ulcers was measured with a specially adapted digital camera (Aranz technology; Aranz Medical Limited, New Zealand). The mean diameter was calculated by measuring the maximum diameter and that at right angles to it. The rate of reduction in mean diameter per week was calculated as the pre-treatment mean diameter divided by the number of weeks to complete healing. Patients were questioned about side effects from the antibiotic treatment at each fortnightly clinical assessment and asked to report any problems to the health centre between periodic reviews. Patients were questioned about symptoms of vertigo and abnormal hearing, and examined for scleral icterus. Side effects were judged as mild (grade 1), moderate (grade 2), severe (grade 3) or life threatening (grade 4) according to the NIH/NCI Common Toxicity Criteria. Oral treatment with rifampicin and clarithromycin was given in two weekly batches at study centres and adherence was monitored by pill counts and by inspecting patient

treatment cards. Patients defaulting scheduled follow-up visits during the treatment period were traced by village health workers who assessed their compliance to therapy.

After completion of antibiotic treatment, daily saline dressing was continued until the ulcer healed and patients were reviewed monthly for a year after complete healing to detect recurrence. Surgical excision and grafting was offered to patients whose lesion enlarged during or after treatment by more than 150% of the initial size or which had not healed by week 52. New or paradoxical lesions occurring during the course of therapy or follow up period were cultured to distinguish recurrent or new infection from sterile inflammatory lesions.

Outcome Measurements

The primary clinical outcome was bacterial load in tissue samples after treatment for 6 weeks and 4 weeks post treatment. The secondary endpoint was recurrence free healing 12 months after completing treatment. The investigators who took measurements of the lesions, were not blinded to treatment assignment.

Definitions

Treatment failure was defined either as failure of complete wound closure by week 52, or enlargement of a lesion to more than 150% of its initial size during or after therapy requiring surgery, or loss to follow up in a patient whose lesion had not yet healed at the last assessment.

Paradoxical reaction was defined as an increase in inflammatory changes with increase in lesion size of greater than 100%, after initial improvement and decrease

in size; and/or the appearance of new lesions following or during antimycobacterial treatment.

Microbiological Procedures

All specimens were processed at Komfo Anokye Teaching Hospital in Kumasi, Ghana. As described previously, for semi-quantitative culture 4mm punch biopsies were cut into pieces with a scapel blade and homogenized with a sterile mortar and pestle in 2 ml of normal saline. Ziehl-Neelsen stained smears of 10 µl of supernates were examined under a microscope in duplicate. Tissue homogenates were decontaminated by the sodium hydroxide (modified Petroff) method (Palomino et al., 1998). An equal volume of 1 M NaOH was added to 1 ml of homogenized specimen at a final concentration of 0.5 M, and the mixture left to stand for 10 minutes with occasional shaking and then neutralised with 1 ml of 1 M HCl. The mixture was centrifuged at 3,000 x g for 20 min, and the supernatants decanted. Sediments were diluted 10-fold up to 10^6 fold. 0.2ml of undiluted and 10-fold dilutions were inoculated onto Löwenstein Jensen (LJ) slopes in triplicates. All inoculated culture media were incubated at 31^{o}C and checked weekly for 6 months. In positive cultures, colonies were enumerated. PCR was performed targeting the *IS2404* insertion sequence (Phillips et al., 2005, Phillips et al., 2009).

Sample Size and Power

With *M. ulcerans* culture positivity for RS8 at 6 weeks in this population being 18 % (Sarfo et al., 2010) it was expected that a sample size set at 32 patients per treatment arm will give an 80% power to achieve a significant difference with culture positivity in tissue samples after treatment. Based on an assumption that there would be losses to follow up and that some of the lesions would have healed after 6

weeks of therapy and could not be sampled for viability, the sample size was increased to 83.

Stopping rule

The trial was to be stopped if at 12 months the non-healing or relapse rate was 50% or more. An interim report was discussed at the annual WHO Buruli ulcer meeting in Geneva in 2011. A formal data safety monitoring board was not in place but experienced doctors at the study sites and MWJ who is experienced with the disease reviewed the data on patients that were recruited.

Statistical analyses

Median time to complete healing and rate of healing of lesions were compared using the Mann-Whitney U test. A Kaplan-Meier curve with log rank test was used to compare time to healing of BU in the two treatment arms. Chi-squared tests were used to compare the proportion of lesions which were culture-positive at 0, 6 and 12 weeks. ANOVA with Kruskal-Willis analysis was used to compare more than two group medians with a p value less than 0.05 considered statistically significant.

3.2. Optimising techniques for extraction, detection and quantification of mycolactone in infected human skin tissue.

Eighty (80) patients with a PCR-confirmed diagnosis of *M. ulcerans* disease were recruited between July 2009 and July 2010 from villages around the Ahafo Ano

North, Ashanti Akim North and Nkawie districts at the Tepa Government Hospital, the Agogo Presbyterian Hospital and the Nkawie Government Hospital of the Ministry of Health, Ghana. After the patients had given informed consent, two or three 4 mm punch biopsies were obtained from the centre of non-ulcerated lesions or from the edge of the viable skin around ulcers after application of a topical anaesthetic for 15 minutes. One biopsy was used to establish the diagnosis of *M. ulcerans* disease; another biopsy was placed in amber-coloured tubes, snap frozen in liquid nitrogen and stored at -70^{o}C for lipid extraction to detect and quantify mycolactone. Patients were randomized to start either standard antibiotic treatment with oral rifampicin 10mg/kg and intramuscular streptomycin 15mg/kg daily for 8 weeks (RS8) or oral rifampicin and intramuscular streptomycin for 2 weeks followed by oral rifampicin 10mg/kg and clarithromycin 7.5mg/kg for 6 weeks (RS2RC6). In consenting adults two further biopsies were obtained, one for quantitative culture and one for lipid extraction and mycolactone quantification at 6 weeks during antibiotic therapy and, from lesions which had not healed, at 12 weeks.

Extraction of lipids from human skin for mycolactone detection and quantification.

Lipids were extracted from Buruli lesions using chloroform : methanol 2:1 (vol/vol) followed by a Folch extraction with 0.2 volumes water as described for extracting lipids from bacterial pellets (George et al., 1999). Briefly, the punch biopsy was weighed and placed in a 1.5ml green top matrix tube containing 500µl of diatoms (Q-bio) and homogenised in extraction solution for 45 seconds at a power of 6.5 in a Fast Prep Ribolyser. The homogenate was transferred into a microfuge tube

and allowed to stand for 30 minutes. After centrifugation at 10,000g for 15 minutes the organic phase was harvested. The organic phase was dried in a roto-evaporator and re-suspended in ice-cold acetone for 1 hour to precipitate phospholipids. Acetone soluble lipids (ASL) suspended in 100µl ethanol for mycolactone detection by thin layer chromatography, mass spectrometry and cytotoxicity assays were kept in amber-coloured Eppendorf tubes at -80^{0}C until analyses were performed. To determine the efficiency of the extraction technique, 4mm punch biopsies from an excised human breast tissue (obtained with permission from Department of Surgery) were spiked with 1µg of synthetic mycolactone A/B and subjected to extraction as described above.

Confirming the presence of mycolactone in lipid extracts from infected human skin tissue:

Thin Layer Chromatography**:** Synthetic mycolactone (positive control), ASL extracts from infected skin lesions and lipid extracts from uninfected human skin (negative controls) were spotted 1cm from one edge of silica thin layer chromatography (TLC) plate (Merck, Germany) and eluted using chloroform-methanol-water (90:10:1 vol/vol/vol) as the mobile phase of the solvent system. After the solvent front had run 10cm on the plate, the plate was taken from the developing tank and air dried in a hood cabinet. Lipids bands were visualised by oxidative charring using 5% ethanolic phosphomolybdic acid (Sigma, UK).

Mass spectroscopy: The aims of mass spectroscopic analyses were to specifically detect the presence of intact mycolactone A/B in lipid extracts and to quantify the concentration of mycolactone. Serial dilutions of synthetic mycolactone

A/B standards from 80ng/ml to 1.25ng/ml and ethanolic lipid extracts diluted 1:10 in 96-well plates (Nunc) and liquid chromatographic separation and tandem mass spectrometric detection were performed using an ABI Sciex 3200 Q Trap mass spectrometer (ABI Sciex, UK) interfaced with a Shimadzu UFLC system (UFLC XR system with CBM-20A controller, SIL-20AC XR Prominence autosampler, CTO-20AC Prominence column oven, LC-20AD XR and LC-20 AD pumps (Shimadzu, UK).

Mycolactone A/B was characterized by Enhanced Product Ion (EPI) detection. EPI conditions were optimized by infusing a 1µg/mL ethanolic solution of mycolactone A/B standard via a Harvard syringe pump at flow rate of 10 µL/min. Characteristic ions in mycolactone A/B EPI spectra were m/z 765.7 [M+Na^+], 429.3 and 359.2. EPI parameters for mycolactone A/B were: declustering potential (DP) 20V, entrance potential (EP) 10V, collision cell entrance potential (CEP) 32.35V and collision energy (CE) 65 eV.

Mycolactone was quantified by Multiple Reaction Monitoring (MRM). MRM transitions of mycolactone were identified by the compound optimization tool in Analyst 1.5 during the infusion of 1µg/mL mycolactone A/B standard. From these, MS-MS fragmentation of the mycolactone A/B sodium adduct [M+Na^+] (m/z 765.7, Q1 mass) to m/z 429.6 (Q3 mass) was selected for quantification. MRM parameters were dwell time 150 ms, DP 126V, EP 10V, CEP 34V, CE 57eV, cell exit potential (CXP) 6V. Source parameters for LC-MS-MS were: curtain gas 30 psi, collision gas medium, ion spray voltage 5500 V, temperature 400°C, nebulizer gas 30 psi, turbo gas 40 psi.

For the analysis of lipid extracts from infected human skin tissue, UPLC was combined with EPI methods (LC-EPI) or MRM methods (LC-MRM). Solvent A was 0.1% formic acid and 10-µM ammonium acetate in water. Solvent B was 0.1% formic acid and 10-µM ammonium acetate in acetonitrile. Samples (10µL) were injected via autosampler and eluted at 0.5mL/min on a Shima-Pack XR-ODS II column (2.1µm, 2.0 mm x 75 mm) by a 20-98% linear gradient of B over 2 min. Solvent composition was then held for 4 min before returning to the starting conditions. Standard curves (1.25ng/mL to 100 ng/mL) were prepared using ethanolic stock solutions of synthetic mycolactone A/B.

Cytotoxicity Assays**:** The objectives for performing cytotoxicity assays were to confirm that mycolactone in lipid extracts were biologically active and to employ the dose-response relationship between mycolactone concentration and cytotoxicity of cultured cells to quantify mycolactone in lipid extracts. Cytotoxicity of ASL from infected human skin tissue was tested using the MTT assay, a colorimetric test assessing cellular viability as shown schematically in Figure 3.1. Briefly, HELF cells (kindly donated by Dr. Kay Capaldi of St. Georges University of London, United Kingdom) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2mM L-glutamine in the presence of penicillin and streptomycin 100mU/ml and 100mg/ml respectively and incubated in 5% carbon dioxide at 37^0C. For cytotoxicity assays proliferating HELF cells were seeded at a density of 10^5/well in microtiter plates overnight.

ASL were dissolved in 100µl of absolute ethanol and 2-fold dilutions performed up to 1:32. 5µl of each dilution was used to treat HELF cells in triplicate.

After incubation for 48 hours, 20µl of 5mg/ml of MTT (Sigma) was added to each well and incubated for a further 4h for purple colored formazan crystals to develop following which 100µl of detergent solution of isopropanol:HCl (2N) in a ratio of 49:1 was used to dissolve formazan crystals for spectrophotometric quantification in a multiplate well reader at 570-690nm. Time-course and dose-response curves were derived by treating HELF cells with serial dilutions of purified mycolactone A/B from 250 ng/ml to 0.3 ng/ml (kind gift of Professor Y. Kishi, Harvard University, USA]. All calibration and quantification experiments were performed at least twice. Concentration of mycolactone was determined within the linear portion of the dose response curve since cytotoxicity followed a sigmoidal dose-response relationship.

In order to evaluate non-specific cytotoxicity caused by other skin lipids, lipid extracts from non-infected human skin were tested for cytotoxicity and this was set as the lower limit of detection for the cytotoxicity assay. All calibration and quantification experiments were performed three times.

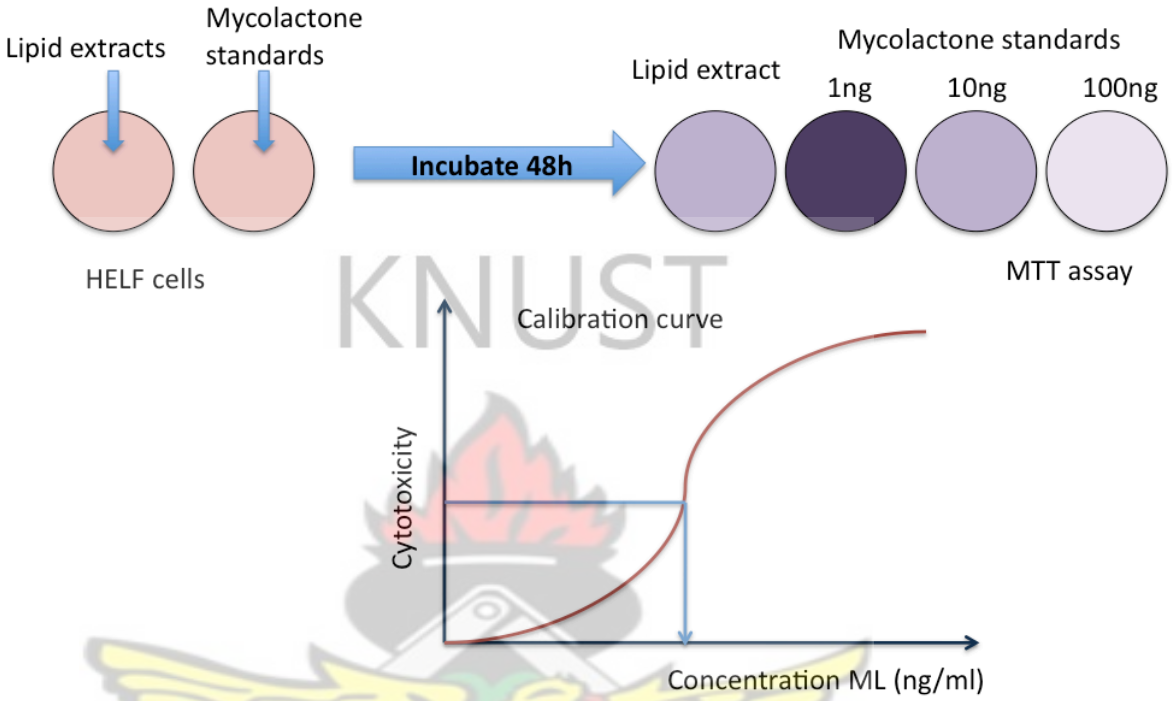


Figure 3.1. Schematic presentation of the cytotoxicity-based assay for quantifying mycolactone in lipid extracts. Human embryonic lung fibroblasts (HELF) were treated with either serial dilutions of mycolactone standards or lipid extracts. After incubation for 48 hours viability of HELF cells was assessed using an MTT assay. Cell death was characterised by inhibition of mitochondrial succinate dehydrogenases of HELF cells from reducing dimethythiazolyl diphenyl tetrazolium bromide (MTT) dye to form purple formazan crystals.

Characterisation of pattern of cell death: To characterize the pattern of cell death caused by mycolactone in patient samples, HELF cells were stained with 8µl of a combination of 100µg/ml of ethidium bromide (Sigma) and 100µg/ml of acridine orange (Sigma) (EB/AO) dye mix in microtitration plates as described for adherent cells. (Ribble et al., 2005). Cells were observed for fluorescence using a DM1 6000B Leica fluorescent microscope and images taken at x10 magnification.

Assessing the immunosuppressive properties of lipid extracts from *M. ulcerans*-infected human skin tissues: To confirm the immunosuppressive properties of mycolactone extracted from infected human tissues, J774 macrophages were treated with ASL from patient lesions and TNF-α secretion after stimulation with lipopolysaccharide (LPS) were measured. Briefly, J774 macrophages (kindly donated by Dr. Rajko Reljic, St. Georges University of London) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum and 2mM L-glutamine in tissue flasks and incubated in 5% carbon dioxide at 37°C. Proliferating macrophages were seeded at a density of 10^5 in 96-well microtitration plates and allowed to adhere to culture plates over 4 hours. Cells were exposed to 5µl of ASL from lesions or to purified mycolactone as positive control for 6 hours and stimulated with 0.5µg/ml of LPS (Sigma) with a 16 hour incubation in 5% CO_2 at 37°C. Samples were analysed in triplicates and experiments were repeated twice. Supernates were harvested and TNF-α quantities assayed in duplicate with a Quantikine ELISA kit (R & D Systems) according to the manufacturer's instructions. The limit of detection of TNF-α was 23.4 pg/ml. Viability of macrophages was determined using the MTT assay.

Statistical Analysis: Medians were compared using either the Mann-Whitney's U-test or the Wilcoxon signed rank test for two independent continuous variables or Kruskal Wallis test for more than 2 continuous variables. Correlations between 2 independent variables were analysed using Pearson's or Spearman's correlation test. Agreement between mycolactone concentrations measured by mass spectrometry and cytotoxicity assays was assessed using Bland-Altman plots. GraphPad Prism version 4 software was used for all analysis with $p<0.05$ considered statistically significant.

3.3. IFN-γ and IL-5 kinetics with antibiotic therapy in humans

Whole blood assays and ELISAs for IFN-γ and IL-5: Whole blood assays were performed as previously described (Sarfo et al 2008). Briefly, six milliliters of venous blood was collected in a sodium heparin vacutainer tubes (Becton Dickinson, United Kingdom) at 0, 6, 12 and 32 weeks. Samples were processed within 4 hours after collection from the field at a laboratory at the Komfo Anokye Teaching Hospital. One-milliliter aliquots of undiluted blood were distributed in duplicate in 24-well tissue culture plates (BD, UK) and incubated with *M. ulcerans* sonicate at a concentration of 10ug/ml, 10 μg/ml of phytohaemagglutinin (Sigma, UK) or unstimulated as negative controls after which 10 μg/ml of gentamicin was added to each well. Plates were swirled gently and incubated at 37^{o}C in 5% CO_2 for 24 hours. Supernatants (200 – 300 μl per well) were collected and stored at -70^{o}C for enzyme-linked immunosorbent assay to measure IFN-γ and IL-5 concentrations using

OptEIA set for human IFN-γ and IL-5 (BD Biosciences, Pharmingen, San Diego, CA) according to manufacturer's instructions. The mean absorbance of duplicate standards, samples, and controls were calculated for each plate, and the mean zero standard absorbance was subtracted. Results were analysed with GraphPad Prism 4 software (GraphPad Software, Inc.) and a standard (best-fit) curve was plotted. Values for unstimulated cultures were subtracted from those for stimulated cultures. The lower detection limit was 4.7 pg/ml for gamma interferon and 1.25 pg/ml for IL-5.

3.4. Studying the kinetics of mycolactone, microbiological, clinical and immunological responses in a murine model of *M. ulcerans* infection.

Bacteria

M. ulcerans 1615 (Mu1615), an isolate originally obtained from a patient in Malaysia in the 1960s, (Pettit et al., 1966) was kindly provided by Dr. Pamela Small, University of Tennessee. Previous studies have confirmed that this strain produces mycolactone and kills macrophages and fibroblasts (Converse et al., 2011, Zhang et al., 2011). The strain was passaged in mouse footpads before use in these studies. The bacilli were harvested from swollen footpads at the grade 2 level, i.e., swelling with inflammation of the footpad (Dega et al., 2002).

Infection and CFU/Histopathology analyses

BALB/c mice, age 4-6 weeks (Charles River, Wilmington, MA), were inoculated in the right hind footpad with approximately 5.5 $\log_{10}$ ($3x10^5$) CFUs of Mu1615 in 0.03 ml PBS. Footpads were harvested from 9 mice (3 for CFU count, 3 for mycolactone detection, 3 for histopathology) at different time points after infection (Table 3.1) up to ≥ grade 3 swelling. After the onset of swelling (~ day 21-23), treatment with rifampicin (R, Sigma, St. Louis, MO, 10 mg/kg by gavage) and streptomycin (S, Sigma, 150 mg/kg by subcutaneous injection) began on day 24, and was administered 5 days/week for 8 weeks. Groups of the treated mice were also sacrificed for these analyses. For CFU count footpad tissue was harvested, minced with fine scissors (Shepard, 1967), suspended in 2.0 ml PBS, serially diluted, and plated on Middlebrook selective 7H11 plates (Becton- Dickinson, Sparks, MD). Alternatively, footpads were harvested and placed in 10% buffered formalin for histopathological analysis by hematoxylin and eosin (H&E) or acid-fast (AF) staining. Footpads from a third group of mice at each time point were placed in freezer vials and frozen at -80°C. These latter footpads were shipped on dry ice to St. George's University of London for mycolactone detection experiments. Mice were evaluated for footpad swelling weekly using an established scoring system (Dega et al., 2002) with grade 1 showing footpad swelling, grade 2 swelling with inflammation, and grade 3 swelling and inflammation of the entire foot (Dega et al., 2002).

Table 3.1. Scheme of study.

Day	3	7	13	20	27	34	40	48	55	62	70	78	98	**Total**
Control mice														
CFU	3	3	3	3	3	3	3	3	3					27
Mycolactone	3	3	3	3	3	3	3	3	3	3				30
Histopathology	3	3	3	3	3	3	3	3	3					27
Treated mice														
CFU					3	3	3	3	3	3		3	3	24
Mycolactone					3	3	3	3	3	3				18
Histopathology					3	3	3	3	3				3	18
Total	9	9	9	9	18	18	18	18	18	9		3	6	144

Control and treated (i.e., Rifampicin, 10 mg/kg, and streptomycin, 150 mg.kg) mice were sacrificed at the indicated days after infection for CFU analysis, mycolactone detection, or histopathology. Treatment began on day 24 after infection.

Immunological analysis

Spleens were harvested to assess responses to mycobacterial culture filtrate proteins (CFP) and to the concanavalin A (ConA) mitogen (Sigma) by multiplex ELISA (Bio-Plex Pro™ Mouse Cytokine 23-plex Assay, Biorad, Hercules, CA) as described (Converse et al., 2011). The 23 analytes included Th1 cytokines: IL-2, IFN-γ, IL-12p40, IL-12p70; Th2 cytokines: IL-4, IL-5, IL-10, and IL-13; proinflammatory cytokines: TNF-α, IL-1α, IL-1β, and IL-6; IL-9; IL-17; colony stimulating factors: GMCSF, GCSF, IL-3; and chemokines: CXCL-1, CCL-2, CCL-3, CCL-4, CCL-5, and CCL-11. All animal procedures were conducted according to relevant national and international guidelines. The study was conducted adhering to

the Johns Hopkins University guidelines for animal husbandry and was approved by the Johns Hopkins Animal Care and Use Committee, protocol MO08M240. The Johns Hopkins program is in compliance with the Animal Welfare Act regulations and Public Health Service (PHS) Policy and also maintains accreditation of its program by the private Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Mycolactone detection assays

Extraction of lipids from mouse footpads for mycolactone detection and quantification: Lipids from footpads of mice were extracted as described earlier. Briefly, mouse footpads were weighed, diced with a scalpel blade and homogenised in 500µl extraction solution of chloroform: methanol 2 :1 vol/vol with ceramic beads in a Fastprep ribolyser at power 6.5 for 45 seconds and again at power 5.0 for 45 seconds. After homogenisation, an additional 500µl of extraction solution was added and samples were placed on ice for 4 hours. 0.2 ml of 0.3% sodium chloride solution was added for Folch's extraction and samples were centrifuged at 13,000g for 5 minutes. The organic phase was harvested, dried down under vacuum and lipids re-suspended in ice-cold acetone for an hour and centrifuged at 13,000g for 5 minutes to remove phospholipids. Acetone soluble lipids (ASL) were dried down under vacuum and re-suspended in 100% ethanol.

Cytotoxicity Assay for mycolactone quantification: Cytotoxicity of ASL from infected mouse tissue was tested using the MTT assay described earlier.

Mass spectroscopy: Liquid chromatographic separation and tandem mass spectrometric detection were performed as described earlier.

Statistical analysis: GraphPad Prism 4 was used to assess significance of differences by student T-test and analysis of variance.

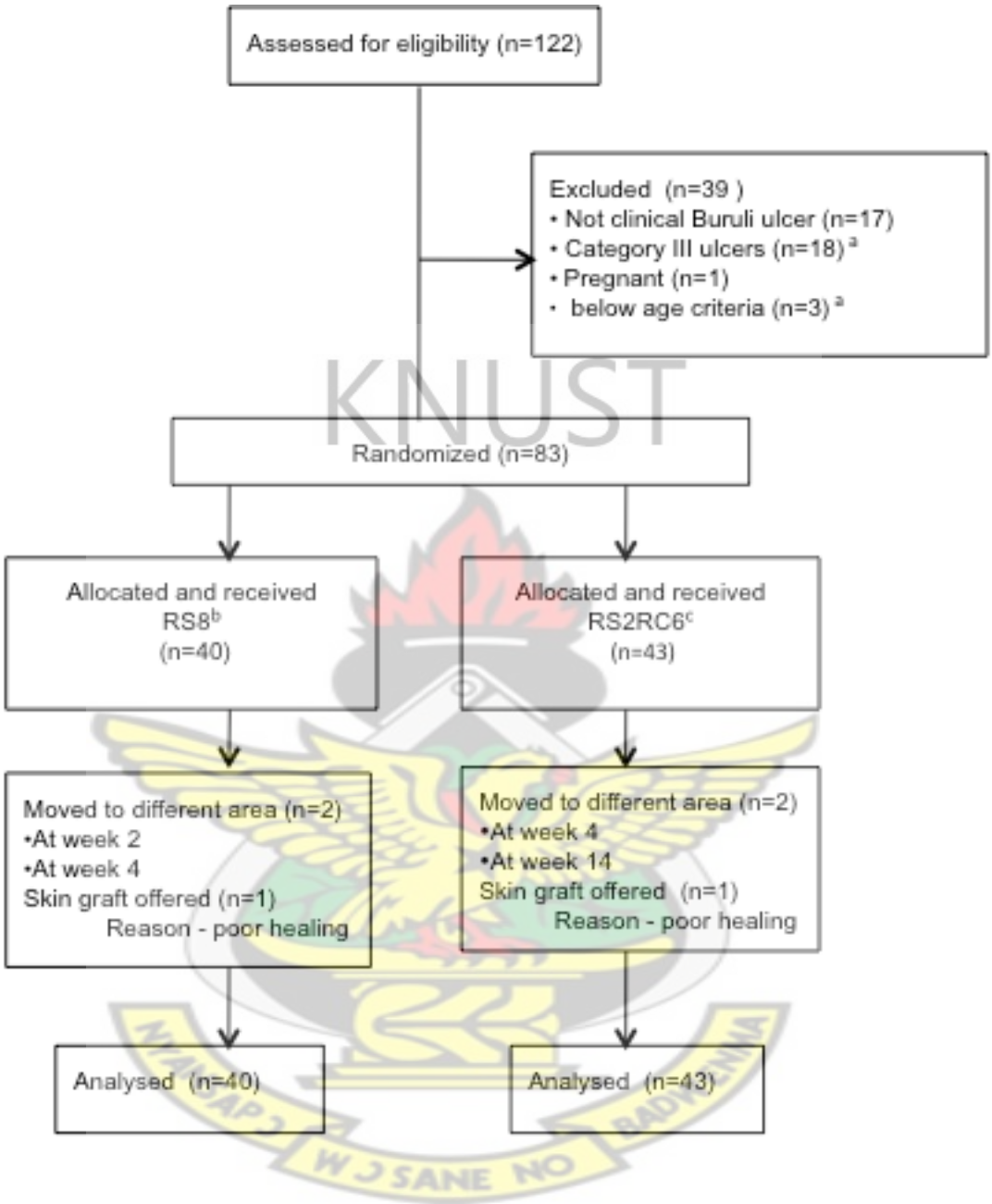
CHAPTER 4

RESULTS

4.1. Efficacy of RS8 compared with RS2RC6.

Figure 4.1 shows the study profile. Of 122 patients screened for *M. ulcerans* disease, 39 were excluded of which 17 did not meet clinical and / or epidemiological criteria of *M. ulcerans* disease; 18 were large category III ulcers; 1 was pregnant and 3 were below the age criteria for inclusion. The 3 below the age of inclusion were found to have had their ages misrepresented by their parents. Twenty-one participants who were too young or had large lesions received standard streptomycin and rifampicin for 8 weeks (RS8). The pregnant participant was observed on wound dressing until after delivery when standard RS8 was administered. Diagnostic samples were obtained only from those who had clinically-suspected *M. ulcerans* disease. Out of 83 patients recruited all of whom had a positive PCR for *M. ulcerans* IS2404, 40 were randomised to receive RS8 and 43 to receive RS2RC6. Table 4.1 shows the baseline characteristics of the study participants. The two groups were well-matched demographically. All lesions were <15cm in diameter but 4 in the RS2RC6 group and 1 in the RS8 group had multiple lesions that were not up to 15 cm but could be classified as WHO category III. Six participants in the RS8 group and 3 in the RS2RC6 group had mild to moderate functional limitation before treatment was commenced. Adherence to the treatment regimen was assessed by use of Directly Observed Therapy (DOT) forms, signed by health personnel at the health facilities. Adherence was 94% in the RS2RC6 group and 96% in the RS8 group.

Figure 4.1. Trial flow



[a]Patients not enrolled but given 8 weeks of treatment with streptomycin and rifampicin

[b]RS8 Rifampicin-streptomycin combination administered daily for 8 weeks

[c]RS2RC6 Rifampicin-streptomycin combination administered daily for 2 weeks followed by rifampicin-clarithromycin daily for 6 weeks

TABLE 4.1. Demographics, clinical features and diagnostic data of study participants in pilot study

	RS8[c] (n = 40)	RS2RC6[d] (n = 43)
Sex (male/female)	24/16	18/25
Median (range) age	12 (6 – 59)	14 (5 – 70)
Site of lesion		
Head and neck	0 (0%)	1 (2%)
Upper limbs	20 (50%)	21 (49%)
Trunk	1 (3%)	1 (2%)
Lower limbs	19 (47%)	20 (47%)
Clinical form		
Nodule	12 (30%)	14 (32%)
Plaque	7 (18%)	9 (21%)
Ulcer	21 (53%)	20 (47%)
Category of lesion		
I	28 (70%)	27 (63%)
II	11 (28%)	12 (28%)
III	1 (2%)	4 (9%)
Laboratory confirmation		
[a] AFB detection (+/-)	15/15	11/21
[b] Mu culture (+/-)	18/15	14/22
PCR for IS2404 (+/-)	40/0	43/0

[a] Ziehl Neelsen stain for acid fast bacilli (AFB) not performed at baseline in 21 patients

[b] *M. ulcerans (Mu)* culture not performed at baseline in 14 patients .

[c]Patients in the 8 week-streptomycin group were assigned to receive oral rifampicin and intramuscular (I/M) streptomycin daily for 8 weeks (RS8).

[d]Patients in the RS2RC6 group were assigned to receive rifampicin orally and streptomycin I/M daily for 2 weeks followed by rifampicin and clarithromycin orally each day for 6 weeks.

Healing after treatment with RS8 or RS2RC6

There was no significant difference in the proportion healed in each group after 4, 8, 12, 16, 20, 24 and up to 52 weeks (Figure 4.2). In an intention-to-treat analysis, 37 participants in the RS8 group and 40 in the RS2RC6 group reached the end-point of recurrence-free healing by 52-weeks without recourse to surgery with no significant difference between the two groups. The success rate was 93% in each group.

There were 3 treatment failures in both groups. Two were lost to follow up in each treatment arm because they moved from the study area, one at week 4 and another at week 14 in the RS2RC6 group; one at week 2 and another at week 4 in the RS8 group. In addition 1 in each group was offered skin grafting at week 32 due to poor healing (Table 4.2). Lesions that had not healed upon completion of therapy subsequently went on to heal with observation and wound care.

The median time to healing of lesions in the RS2RC6 group was 16 weeks (interquartile range (IQR) 4–36) which was not significantly different from 14 weeks (IQR 4–48) for the RS8 group. The median (IQR) rate of reduction in diameter of lesions was similar in the two groups: 2.5 (1.1–5.1) mm/week on RS2RC6; 2.6 (1.7–5.8) mm/week on RS8. There were no recurrences after 12 months of follow-up.

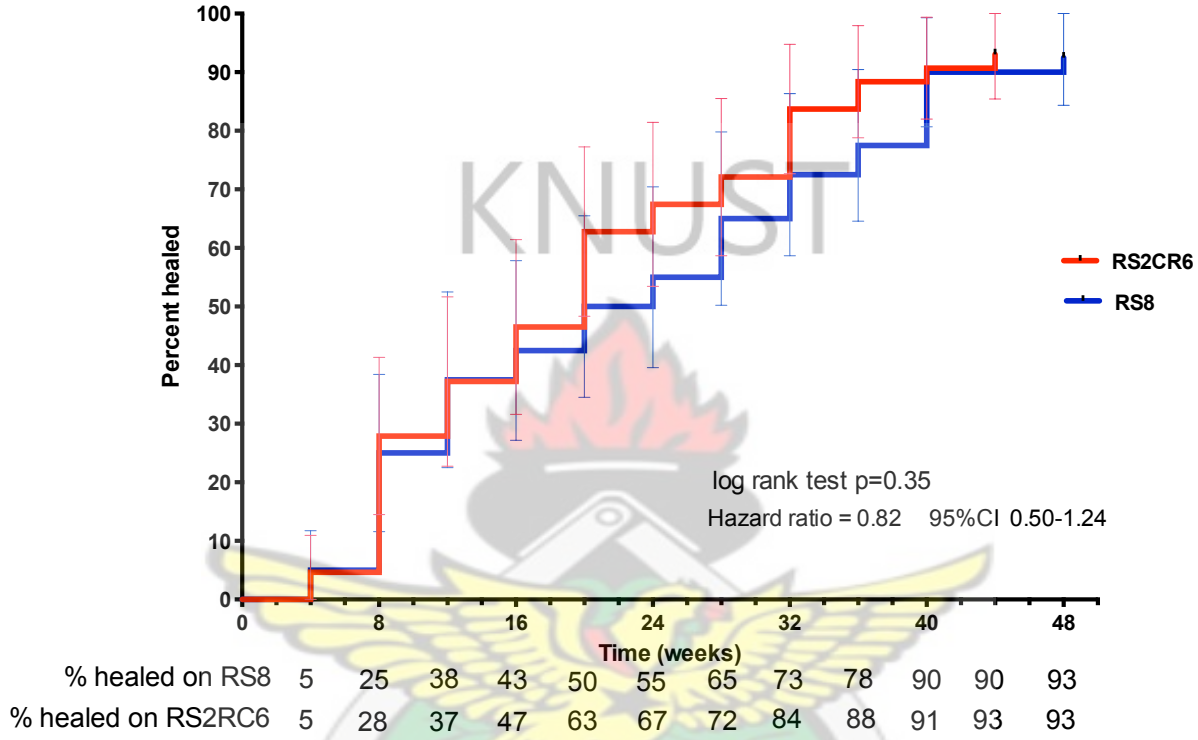


Figure 4.2. **Survival analysis curve of cumulative complete healing**. Time to complete healing defined as complete re-epithelialisation ± stable scab formation was determined for each patient and cumulative healing in the two-groups compared by Kaplan-Meier analysis. There were 6 treatment failures, 3 in each group: 2 patients in each group were lost to follow-up when their lesions had not yet healed while 1 patient in each group had surgery with skin grafting. The log rank test comparing the two groups was not significant.

TABLE 4.2. Summary of trial outcomes and events

Event/outcome	RS8[a] (n=40)	RS2RC6[b] (n=43)	Total n=83
Healed without surgery	37 (93%)	40 (93%)	77 (93%)
Healed with surgery	1 (2%)	1 (2%)	2 (2%)
Lost to follow up	2 (5%)	2 (5%)	4 (5%)
Healed with functional limitation	0	0	0
Paradoxical reactions	3 (8%)	4 (9%)	7 (8%)
Adverse events:			
Vestibulo-cochlear toxicity	0	1	1 (1%)

[a]Patients in the 8 week-streptomycin group were assigned to receive intramuscular streptomycin and oral rifampicin daily for 8 weeks (RS8).

[b]Patients in the 2-week streptomycin plus 6-week clarithromycin (RS2RC6) were assigned to receive streptomycin and rifampicin daily for 2 weeks followed by clarithromycin and rifampicin, both orally for 6 weeks.

Paradoxical reactions:

Paradoxical reactions were observed in 3 patients in the RS8 group and 4 in the RS2RC6 group (Table 4.3). They occurred at a median time of 12 weeks (range 4-32) and were associated with ulcer enlargement in all 7 cases. Lesions were painful in 4/7 cases and there was pus requiring aspiration in 2 cases. In 6 of 7 cases the reaction occurred at the original site but one was at a new site on the same limb. Culture was negative in 6 out of 7 cases but positive from one ulcer that enlarged at week 4. In four cases, lesions which had completely healed by week 6, 8, 8 and 12 broke down at week 20, 14, 12 and 20 respectively. In one of these cases the lesion healed at week 12, broke down at week 20, improved but further enlarged at week 32. Paradoxical reactions were managed by observation, wound care and aspiration of pus but no additional antibiotics were administered to any patient. The median time to healing in patients who developed paradoxical reactions was 24 weeks (range 18 - 39). In the four patients whose lesions healed before they further broke down the time to healing was prolonged by 8 to 19 weeks from the time of the initial paradoxical reaction.

TABLE 4.3: Clinical features of patients with paradoxical reactions

Treatment group		Primary lesion form	Type of reaction	Reaction site in relation to primary lesion	Pus collection	Pain	Time of initial Healing (weeks)	onset of reaction (weeks)	*Mu* Culture result[c]	Treatment administered	Time to healing
RS8[a]	1	plaque	enlargement	same site	no pus	no	6	20	negative	observation and wound care	30
	2	plaque	enlargement	same site	no pus	yes	8	14	negative	observation and wound care	24
	3	nodule	enlargement	same site	pus filled	yes	N/A	4	negative	observation, aspiration of pus, continuation of antibiotic course	22
RS2RC6[b]	1	ulcer	enlargement	new site	no pus	yes	8	12	negative	observation and wound care	20
	2	ulcer	enlargement	same site	no pus	no	12	20,32[d]	negative	observation and wound care	39
	3	plaque	enlargement	same site	no pus	no	N/A	4	positive	observation and wound care continuation of antibiotic course,	30
	4	nodule	enlargement	same site	pus filled	yes	N/A	6	negative	aspiration of pus, continuation of antibiotic course	18

[a]RS8 Rifampicin-streptomycin combination administered daily for 8 weeks

[b]RS2RC6 Rifampicin-streptomycin combination administered daily for 2 weeks followed by rifampicin-clarithromycin daily for 6 weeks

[c] *Mu* represents *Mycobacterium ulcerans*

[d] lesion broke down at week 20, improved but further enlarged at week 32.

N/A not applicable

Functional Limitations:

Six participants in the RS8 group and 3 in the RS2RC6 group had functional limitation before therapy was initiated. All had a complete recovery of function with simple exercises during and after antibiotic treatment carried out according to WHO guidelines (WHO, 2004).

Side effects/toxicity:

Treatment was well tolerated in both study groups but a 70-year-old man who had been randomized to receive RS2RC6 developed vestibulotoxicity in the second week of treatment. His symptoms subsided when he was switched to the rifampicin and clarithromycin combination. The main clinical outcomes of the trial are summarized in table 4.2.

Bacterial Viability after Treatment with SR8 or RS2RC8

M. ulcerans culture was performed at baseline for 69 out of 83 participants as initial samples were difficult to obtain in 14. Thirty-three (33) of these were assigned to the RS8 group and 36 to the RS2RC6 group. Table 4.4 shows that at initiation of therapy 18 of 33 (55%) cultures from the RS8 group and 14 of 36 (39%) from the RS2RC6 group were positive for *Mu* with mean colony forming units per gram of tissue 2.2 log for RS8 and 3.2 log for RS2RC6. 11 of 16 (69%) cultures from the RS8 group were positive after 6 weeks and 3 of 5 (60%) after 12 weeks compared with 10 of 16 (63%) and 3 of 7 (43%) at 6 and 12 weeks, respectively in the RS2RC6 group. There was no significant difference in the number of bacteria cultured at the different time points. All positive *Mu* cultures were confirmed by *Mu* specific *IS2404* PCR. Samples were not obtained for all patients during and after antibiotic therapy because some lesions were assessed as healed or nearly healed.

TABLE 4.4. Culture results of lesions before, during and after treatment

Time of culture/culture result	RS8	RS2RC6	Total	p-value
Week 0	n=33	n=36	n=69	
Positive n (%)	18 (55)	14 (39)	32 (46)	0.42
Negative n (%)	15 (45)	22 (61)	37 (54)	
not done n (%)	7 (21)	7 (19)	14 (20)	
Mean log10 cfu/g of tissue	2.2 (1.6-3.3)	3.2 (1.6-4.3)	2.7 (1.6 – 4.3)	ns
Week 6	n=16	n=16	n=32	
Positive n(%)	11 (69)	10 (63)	21 (66)	0.71
Negative n(%)	5 (31)	6 (38)	11 (34)	
Mean log10 cfu/g of tissue	2.3 (1.6-3.8)	3.0 (2.0-4.0)	2.7 (1.6-4.0)	ns
Week 12	n= 5	n=7	n=12	
Positive n(%)	3 (60)	3 (43)	6 (50)	0.56
Negative n(%)	2 (40)	4 (57)	6 (50)	
Mean log10 cfu/g of tissue	3.3 (1.8 – 3.8)	1.6 (1.0 - 2.3)	2.5 (1.0-3.8)	ns

4.2 Kinetics of mycolactone in human skin during antibiotic therapy for *Mycobacterium ulcerans* disease.

Demographics, Diagnostic And Clinical Data Of Study Participants

Eighty patients with clinically-confirmed *M. ulcerans* disease were included with median age 14 years (range 5 – 70 years) and male to female ratio 1:1. There were 18 (22.5%) patients with nodules, 14 (17.5%) patients with plaques, 44 (55.0%) patients with ulcers and 4 (5.0%) patients with oedematous lesions (Table 4.5). There were 50 (62.5%) category I lesions, 23 (28.8%) category II lesions and 7 (8.7%) category III lesions. The median duration of onset of lesions before presentation was 3 weeks (range 1 – 32 weeks) with no significant differences between the clinical forms of *M. ulcerans* disease. PCR for *IS2404* was positive in all patients; 40 out of 60 cultures for *M. ulcerans* (66%) were positive and acid-fast bacilli were positive in 25 out of 60 (42%) samples tested before antibiotic therapy.

The patients included in this study were a subset from the pilot study presented in section 4.1. Forty-two patients were randomised to receive RS2RC6 and 38 to receive RS8. There was no difference in clinical outcome between the two groups and overall the median time to healing was 12 weeks with a range of 2-48 weeks.

Table 4.5: Demographic, diagnostic and clinical data of study participants in mycolactone study

Characteristic	Form of *M. ulcerans* lesion				
	Nodule	Plaque	Oedema	Ulcer	Total
	n=18	n=14	n=4	n=44	n=80
Male:Female	12:6	5:9	2:2	22:22	41:39
Median (range) age	13.5 (5-48)	12 (5-70)	16(7-55)	16 (5-59)	14(5-70)
Lesion category					
Category 1(<5cm)	18	10	0	22	50
Category 2 (5-15cm)	0	4	2	17	23
Category 3 (>15cm)	0	0	2	5	7
Median (range) duration of lesion (weeks)	4 (1 – 12)	3 (1-12)	3.5 (3-4)	3 (1 – 32)	3 (1 – 32)
PCR result: no. of positive tests	18	14	4	44	80
Culture result (+: - : nd)	8:6:4	8:5:1	1:1:2	23:8:13	40:20:20
Microscopy result (+: - : nd)	7:7:4	6:7:1	0:2:2	12:19:13	25:35:20
Median (range) time to healing (weeks)	8 (4–32)[1]	31 (2–48)[2]	8 (6-12)[3]	13(5-44)[4]	12(2-48)
Recurrence 1 year after treatment	0	0	0	0	0

nd = not done

[1]Excluding one patient lost to follow up

[2]Excluding one patient whose lesion had not healed after 52 weeks and required surgery

[3] Excluding two patients with extensive oedematous disease who required skin grafting

[4] Excluding four patients lost to follow up and 2 ulcers that required skin grafting.

Detection of mycolactone in infected human tissues

***Mass spectrometry**:*

Mass spectral analysis of acetone soluble lipid extracts from infected human lesions revealed the presence of an ion with mass-to-charge ratio (m/z) of 765.7 [M+Na^+] which corresponded to the sodium adduct of mycolactone A/B (Figure 4.3). Fragmentation of this ion yielded ions with m/z 429.6 corresponding to the conserved core lactone ring present in all mycolactone congeners and another prominent ion with m/z of 359.5, corresponding to the polyketide side chain of mycolactone A/B. In addition ions were also observed at m/z 341.2, 565.3, 579.4, 659.4, 677.4, 707.4, 721.3, 747.7 and 766.4 (Figure 4.3A).

Mycolactone detected in skin extracts showed the same mass spectra as those of synthetic mycolactone A/B and also contained a number of other mycolactone associated ions (e.g. m/z 659.6 and 747.7) (Figure 4.3B). For instance, m/z 747.7 is derived from m/z 765.7 with loss of water and generates m/z 659.6 with loss of $C_4H_9O_2$.

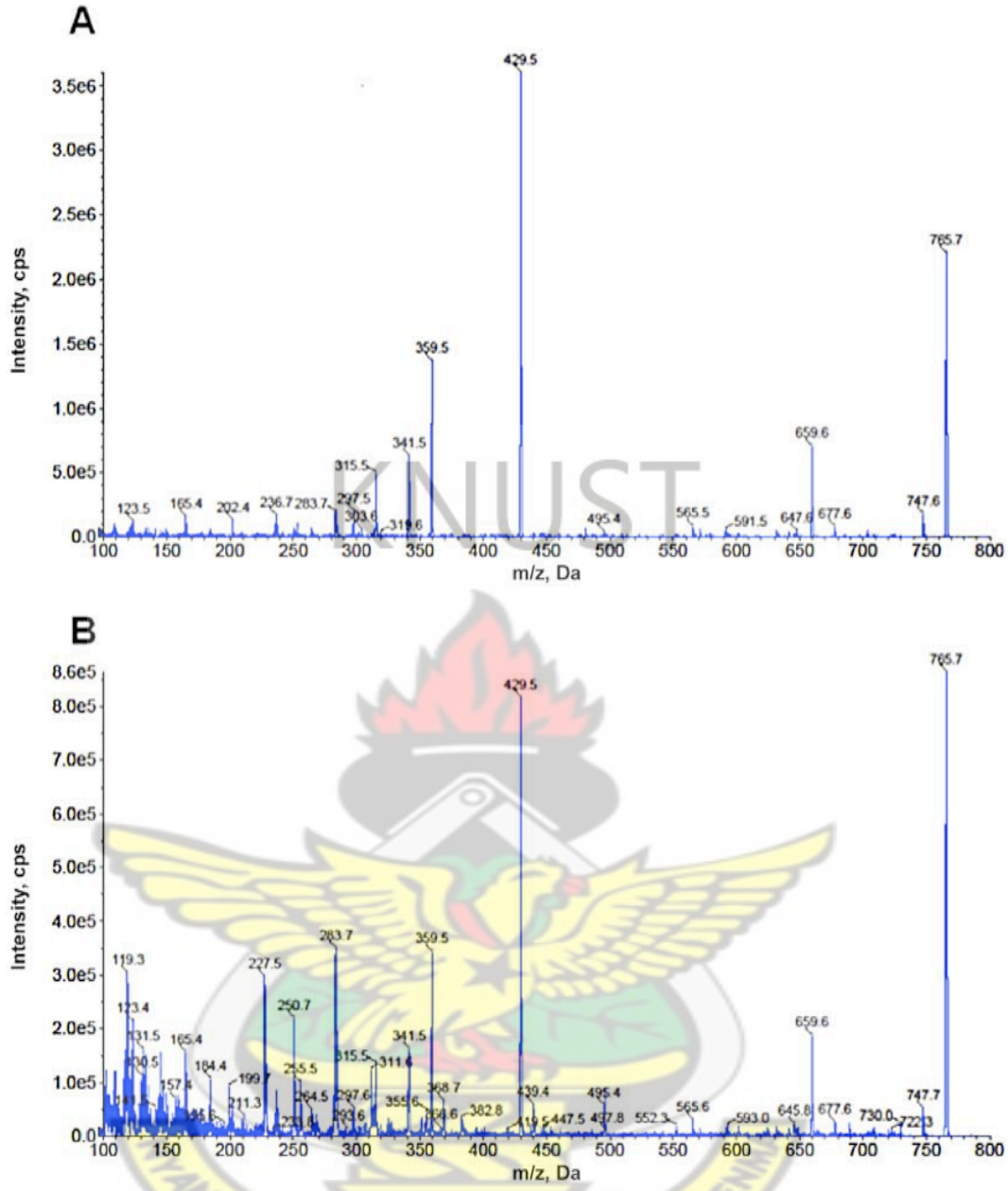


Figure 4.3. Mass spectroscopic analysis using LCQ coupled tandem mass spectrometry for characterisation of mycolactone A/B; Enhanced Product Ion analysis of the sodiated mycolactone A/B adduct with m/z 765.7, showing core lactone with m/z 429.6 and polyketide side chain with m/z 359.5 from lipid extracts from a representative skin biopsy from an infected human lesion (4.3A) and from synthetic mycolactone standard (4.3B).

Biological activity of mycolactone in lipid extracts as assessed by cytotoxicity assay.

Mycolactone exhibits a characteristic cytotoxic phenotype which includes cell rounding at 10 hours, cell cycle arrest at 36 hours and apoptotic cell death at 72 hours. This classic cytotoxic phenotype of mycolactone was observed within 48 hours upon exposing human embryonic lung fibroblasts (HELF) to lipid extracts from infected human skin tissue. ASL from patient extracts caused significant apoptotic cell death comparable to 5μg/ml of synthetic mycolactone after treatment of HELF for 48 hours (Figure 4.4). No qualitative differences were observed in the cytopathicity or proapoptotic activities of lipid extracts from different forms of *M. ulcerans* infected lesions (Figure 4.4).

Inhibition of induction of TNF-α release by J774 macrophages treated with lipid extracts from infected human skin.

Mycolactone is known to potently inhibit the release of cytokines at nanomolar, non-cytotoxic concentrations. Figure 4.5 shows that ASL from infected human lesions significantly inhibited TNF-α release by murine macrophages compared with negative controls. There was no significant macrophage cytotoxicity despite the inhibition of cytokine release.

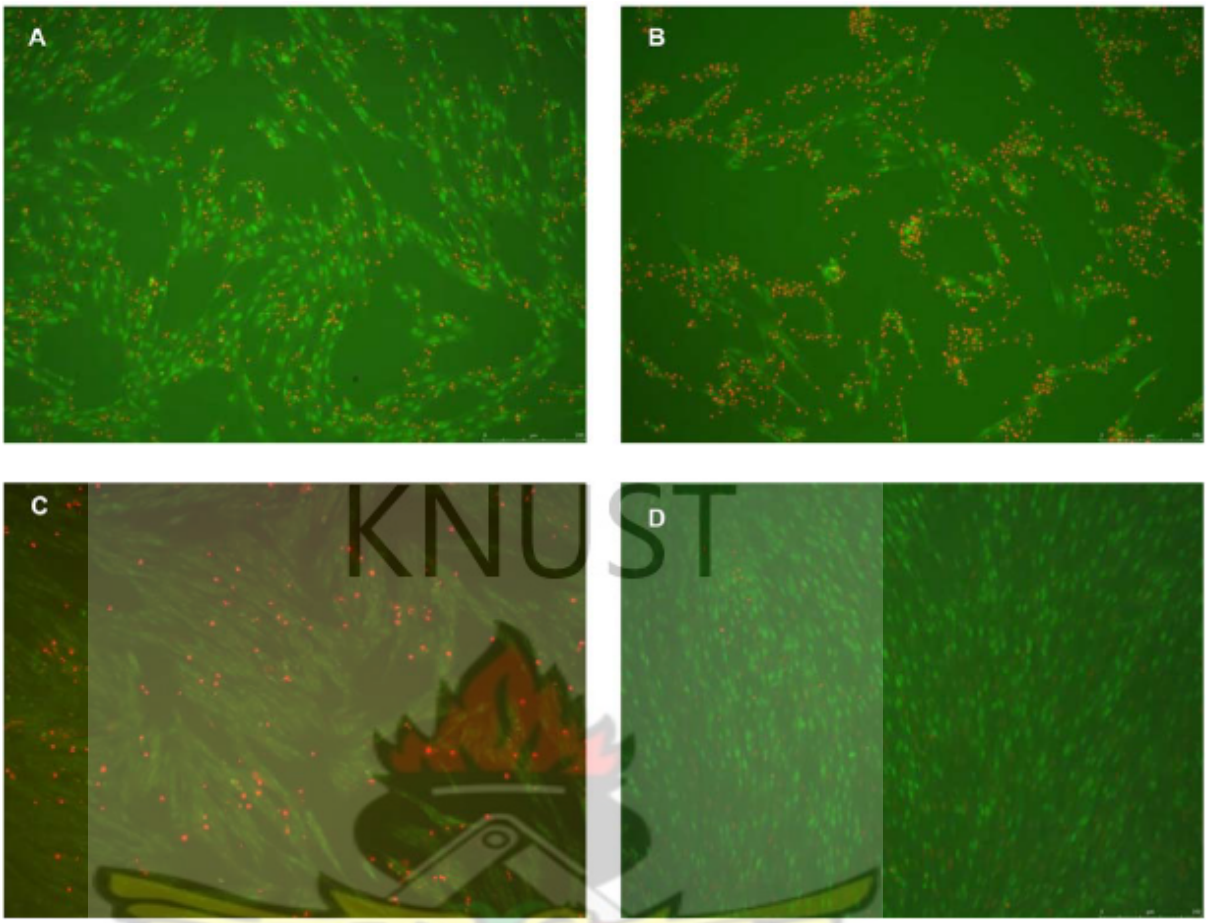


Figure 4.4. Mycolactone-mediated cell death on HELF cell cultures. A and B show the effect of ASL from *M. ulcerans* infected skin on HELF cells after 48 hours with detachment of cells from culture plate, numerous apoptotic cells (orange nuclei stained with acridine orange) and few spindle shaped cells (green nuclei with ethidium bromide). A and B represent ASL from a nodular lesion and an ulcerative lesion respectively. C shows the effect of synthetic mycolactone at a concentration of 5μg/ml and D is a negative control demonstrating the effect of ASL from uninfected human skin. Pictures were taken at x10 magnification with a DM1 6000B Leica fluorescent microscope.

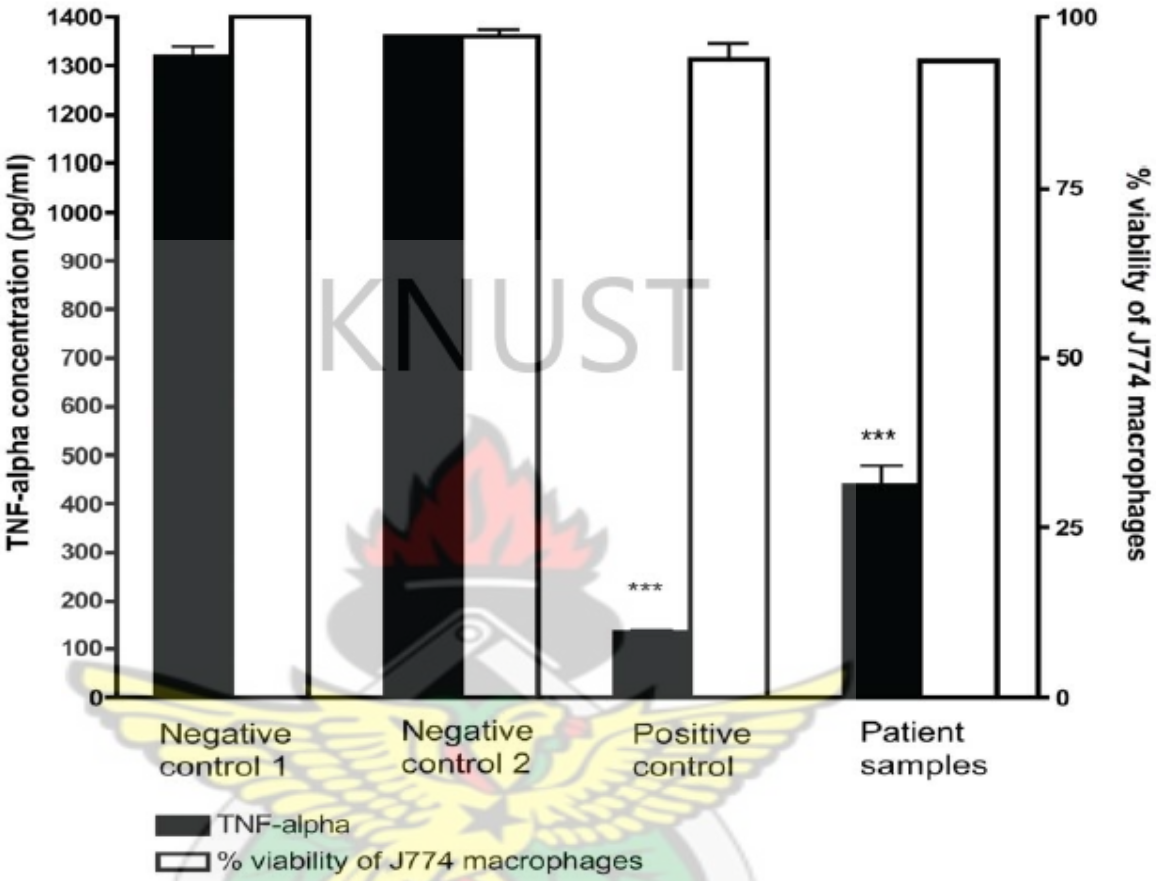


Figure 4.5. The effect of ASL from human *M. ulcerans* infected lesions on TNF-α release by J774 macrophages. J774 macrophages were stimulated with 0.5 μg/ml of LPS. Negative control 1 is untreated J774 macrophages, negative control 2 is J774 treated with ASL from uninfected skin. Positive control refers to synthetic mycolactone at a concentration of 500 ng/ml and patient samples refers to 10 representative patient lesions. ASL from infected lesions significantly inhibited TNF-α release compared to both negative controls with ***$p<0.001$. Error bars are ±SEM of duplicate assays. Although TNF-α release by J774 macrophages was significantly inhibited by synthetic mycolactone and lipid extracts from patient lesions, this occurred without significant cytotoxicity.

***Thin layer chromatography**:*

Using TLC, synthetic mycolactone A/B yielded a major UV active band at a retention factor (Rf) value of 0.23 and two minor bands (Figure 4.6A). The detection limit for synthetic mycolactone was 2µg/ml corresponding to 160ng of mycolactone. Figure 4.6B shows that ASL from human lesions had detectable bands at Rf of 0.23 which were ultraviolet active and corresponded to the synthetic mycolactone A/B ladder M. However, a faint band was also seen at R_f 0.23 in lipids extracted from normal skin (lane C). Out of 31 samples from *M. ulcerans* infected skin, 21 (68%) showed strong bands aligning with the synthetic mycolactone band as in lanes 1, 2 and 4.

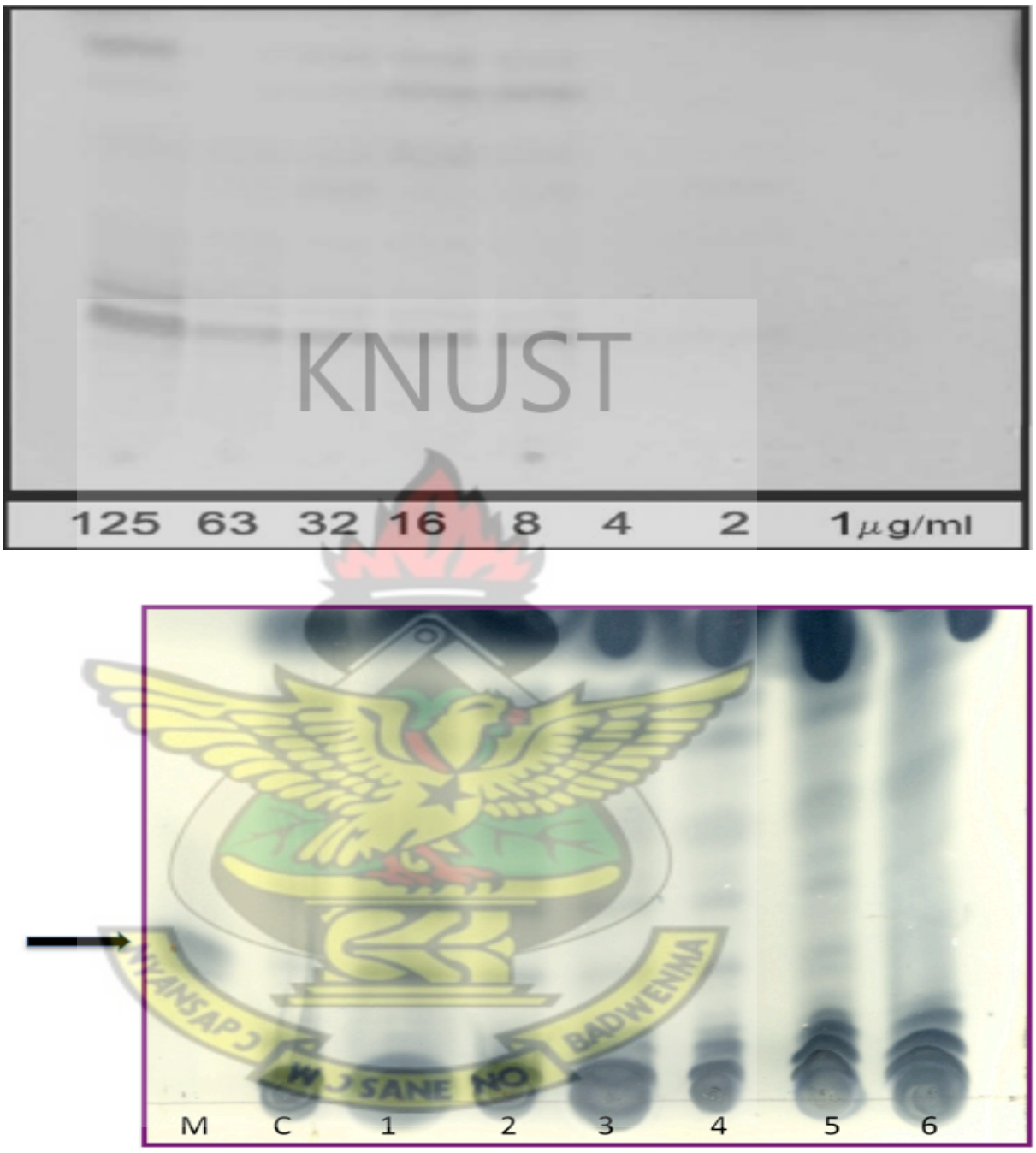


Figure 4.6. Detection of mycolactone A/B by TLC. Upper panel: 20µl of two-fold serial dilutions of mycolactone A/B at concentration from 125 to 1 µg/ml were spotted and examined under UV-light and by oxidative charring. The detection limit on TLC was 8µg/ml (corresponding to 160ng of mycolactone A/B). Lower panel: Each track represents one sample. M is synthetic mycolactone A/B, C is lipid extracts of normal skin tissue and tracks 1-6 are extracts from infected human skin samples.

Assays for quantification of mycolactone among lipid extracts from infected human tissue

Mass spectrometry

Figure 4.7 shows extracted ion chromatograms (XIC) of the m/z 429.6 ion selected for quantification of mycolactone by LC-MRM analysis. The lower limit of detection by this method was 10ng/ml.

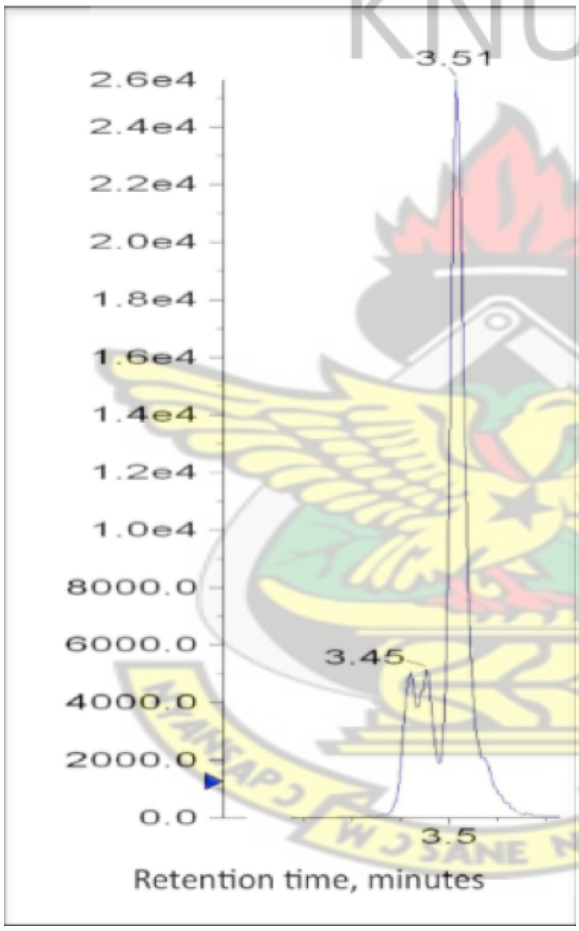


Figure 4.7. The extracted ion chromatograms (XIC) for m/z 429.6 which was selected for quantification in LC-MRM analyses of tissue extracts.

Cytotoxicity assay:

Mycolactone-mediated cytotoxicity on human embryonic lung fibroblasts followed a sigmoidal dose response (Figure 4.8) and incubation for 48 hours resulted in optimal best-fit plots (r^2=0.98). Synthetic mycolactone induced 50% cytotoxicity at a concentration of 4.7ng/ml. The lower limit of detection was set at 20% cytotoxicity to exclude non-specific cytotoxicity (Figure 4.9). This lower limit was set by measuring the cytotoxicity induced by lipid extracts from uninfected skin tissue used as negative control.

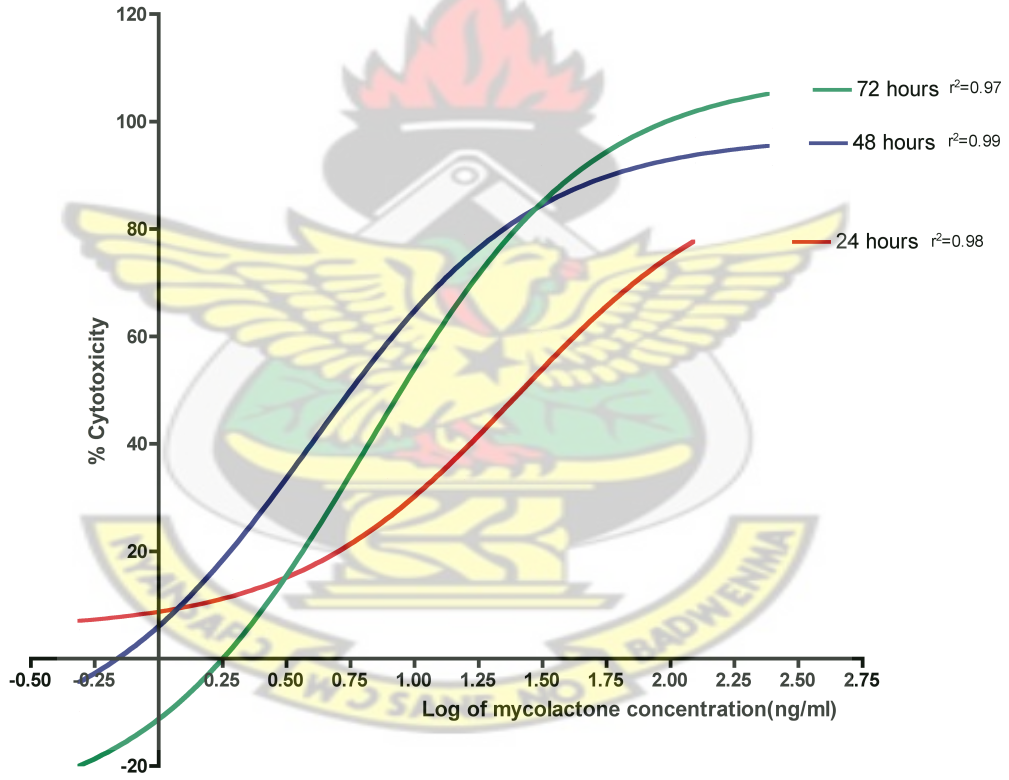


Figure 4.8. Mycolactone-mediated cytotoxicity on human foetal lung fibroblasts (HELF) over 24, 48 and 72 hours. Cytotoxicity was measured using the MTT assay, a colorimetric test which measures cellular metabolic activity of HELF as a reflection of viability.

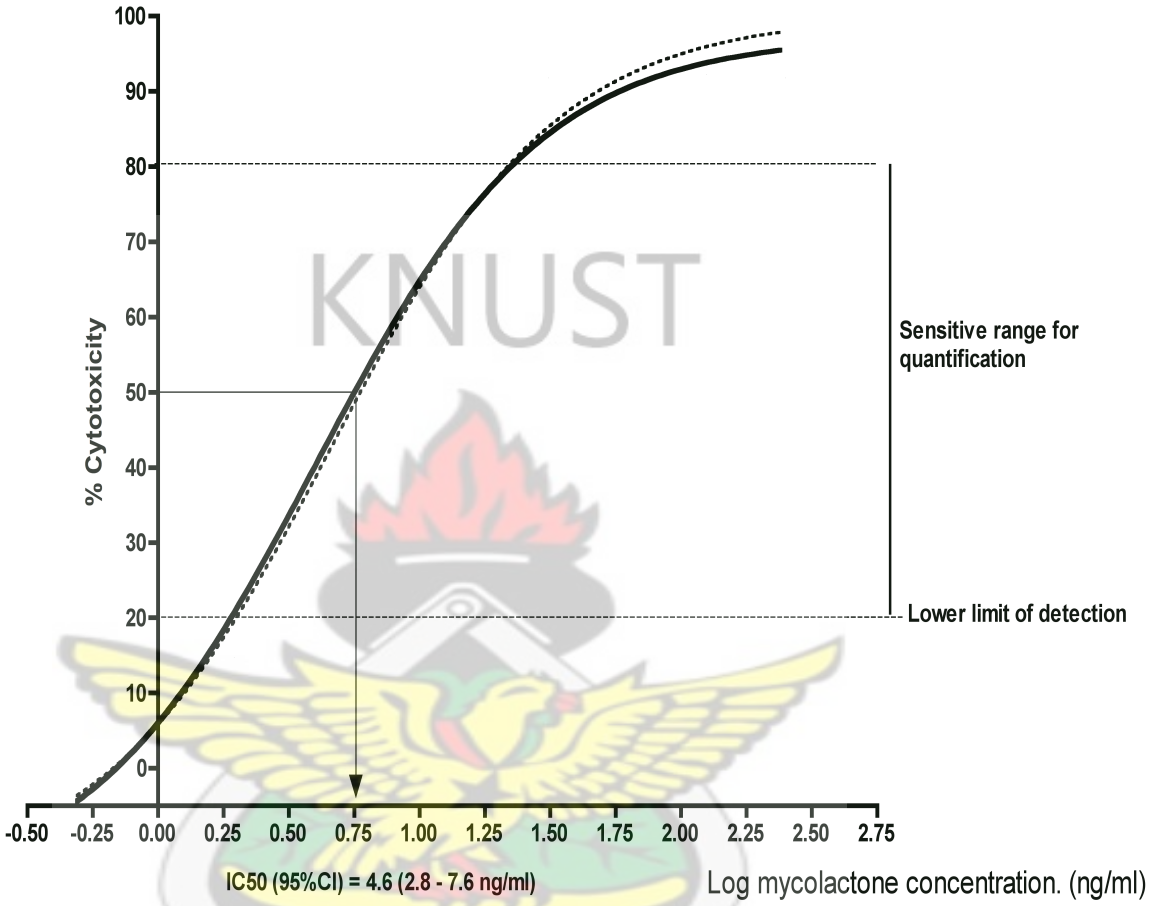


Figure 4.9. Mycolactone-mediated cytotoxicity on human foetal lung fibroblasts after 48 hours of incubation. Best-fit plots of two independent experiments. IC-50 is the concentration of mycolactone that induced 50% cytotoxicity.

Recovery of mycolactone from spiked skin tissue:

The recovery of synthetic mycolactone A/B from spiked skin tissue was 11-18% for a 1µg spiked skin sample (n=3 independent experiments) using mass spectroscopy compared with a recovery of 64-100% (n=3 independent experiments) using the cytotoxicity-based method of quantification. Mycolactone concentration from lipid extracts from each 4mm skin biopsy was expressed as ng/ml.

Comparison of mycolactone detection and quantification by mass spectrometry and cytotoxicity assays:

Compared with the conventional PCR for *IS2404*, the sensitivity of mycolactone detection was 92% by cytotoxicity and 77% by mass spectrometry. Bland-Altman analysis for agreement between the two quantitative assays gave a bias (± SD) of 543 (± 604) ng/ml indicating poor agreement on concentration values as shown in figure 4.10.

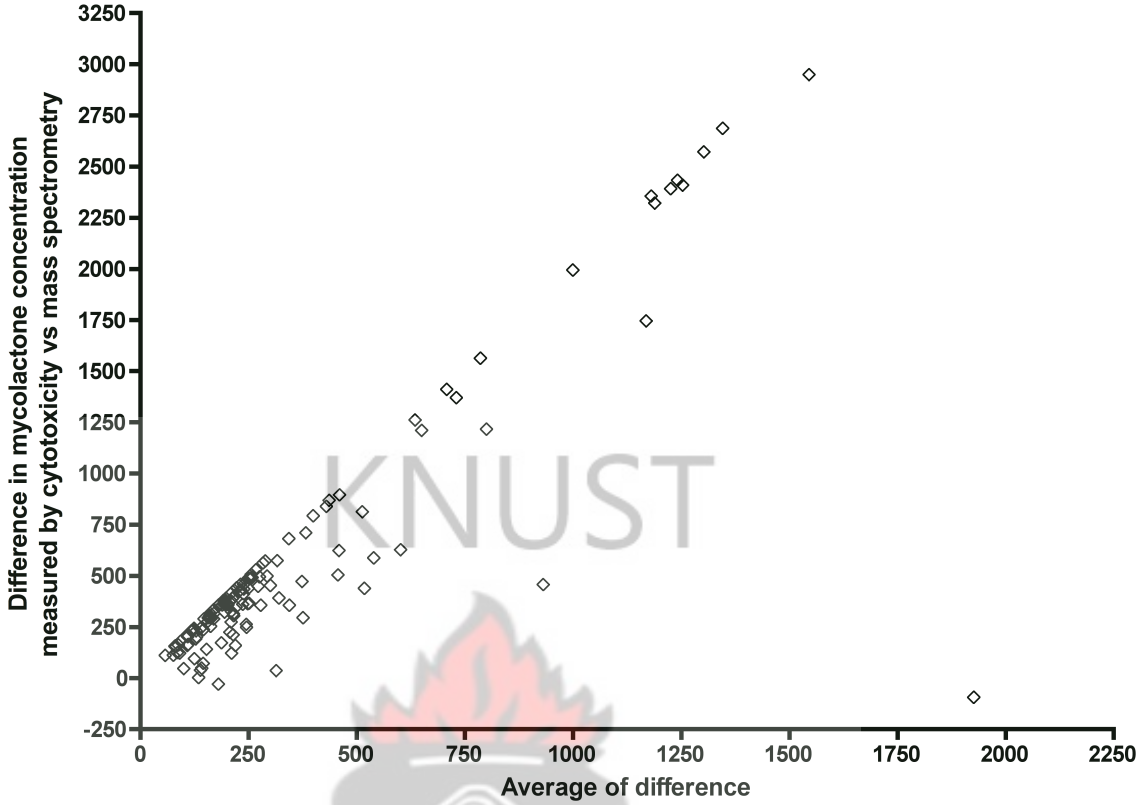


Figure 4.10. Analysis of agreement between cytotoxicity assay and mass spectrometry for measurement of mycolactone concentration in lipid extracts from skin (Bland-Altman analysis). This analysis compared the difference in concentration of mycolactone measured by the two assays (y-axis) with the average of the difference (x-axis) to determine the level of bias in the measurement between the two methods for mycolactone quantification.

Mycolactone concentration in *M. ulcerans* infected human skin lesions

***In untreated nodules, plaques, ulcers and oedematous lesions**:*

The median (range) concentration of mycolactone in 80 untreated lesions measured by mass spectrometry was 26ng/ml (0-1970), significantly lower than that measured by cytotoxicity at 439 ng/ml (136 – 3020) ($p<0.0001$). Figure 4.11 shows the concentration of mycolactone in four forms of Buruli lesions before antibiotic treatment. There was a wide range of concentration in all forms of lesions with median (range) of 437ng/ml (136–2589; n=18) in nodules, 311ng/ml (148-834; n=14) in plaques, 443ng/ml (114–3020; n=45) in ulcers and 895ng/ml (457-2689; n=4) in oedematous lesions using the cytotoxicity assay (Figure 4.11). Using mass spectrometry, the median concentration in nodules and plaques was significantly higher than that in ulcers (Figure 4.12) whereas there was no significant difference by cytotoxicity (Figure 4.11). Cytotoxicity was high among the four oedematous lesions but the amounts of intact mycolactone detected by mass spectrometry were low.

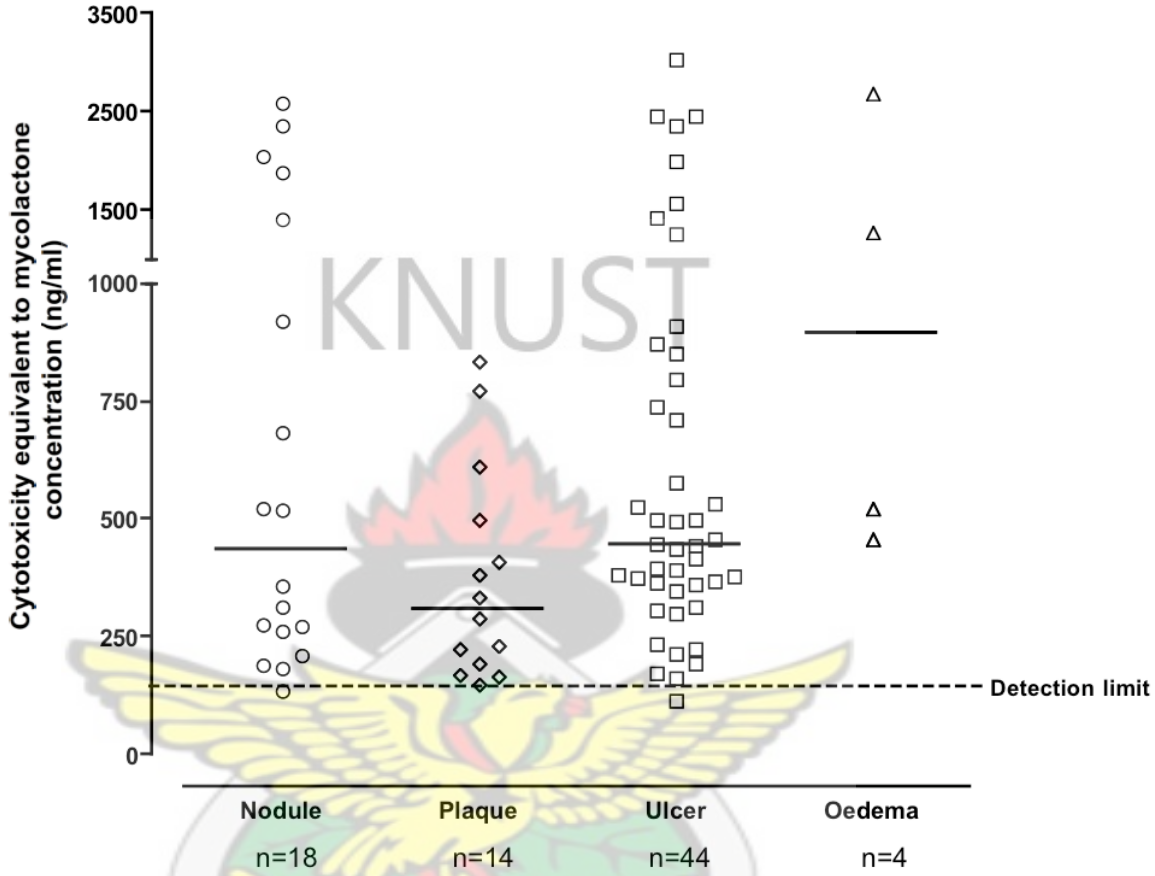


Figure 4.11. **Mycolactone concentration measured by cytotoxicity assay in punch biopsies of untreated *M. ulcerans* disease lesions before antibiotic treatment.** Mycolactone concentration in ASL measured by cytotoxicity assay using synthetic mycolactone A/B generated calibration curves. Horizontal lines represent medians and each dot represents one lesion.

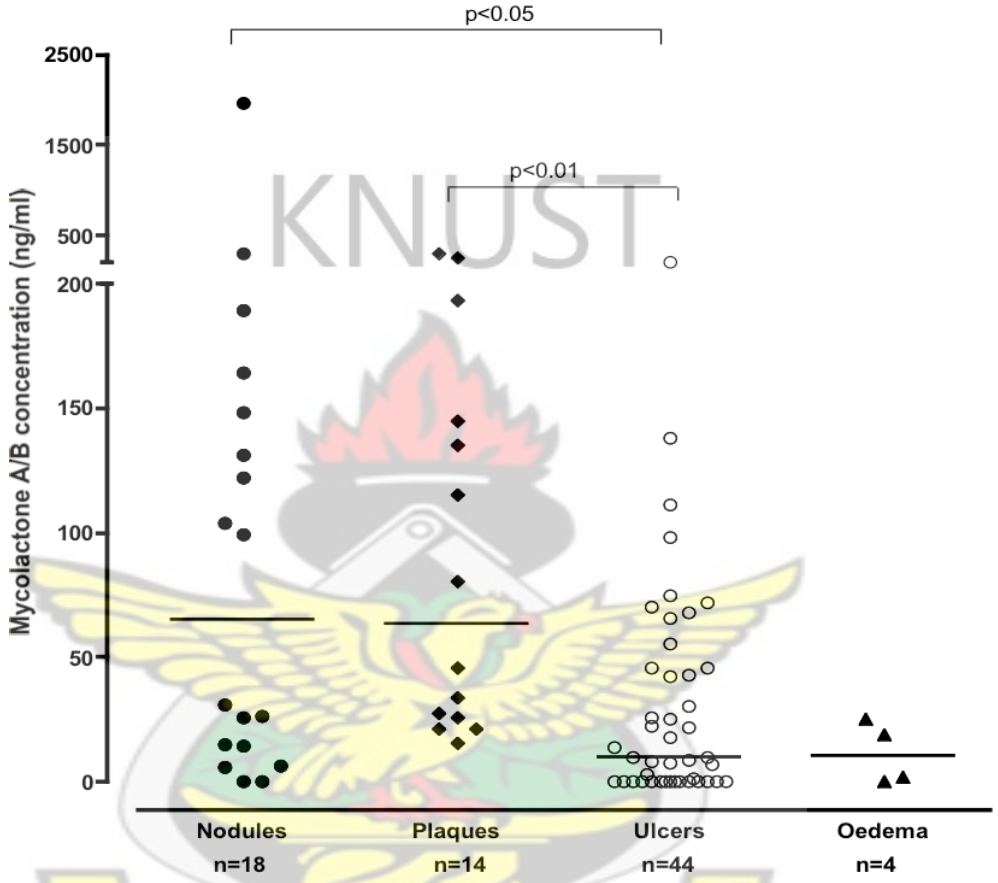


Figure 4.12. Mycolactone concentration measured by mass spectrometry in punch biopsies of untreated *M. ulcerans* disease lesions before antibiotic treatment. Mycolactone A/B concentration in ASL measured by multiple reaction monitoring (MRM) methodology with liquid chromatographic separation coupled to a tandem mass spectrometer. Horizontal lines represent medians and each dot represents one lesion.

Distribution of mycolactone within infected tissues:

Mycolactone concentration was measured by cytotoxicity in paired biopsies taken from the centre and the periphery of six pre-ulcerative lesions (3 nodules and 3 plaques) (Table 4.6). There was a trend towards a centripetal gradient in 5 out of 6 lesions. By cytotoxicity the median concentration at the centre was 397.6ng/ml (132.6-610.3) compared with 263.7ng/ml (123.9- 430.1), $p>0.05$ at the periphery. The median (range) concentration of mycolactone by mass spectrometry at the centre was 110ng/ml (18-285), significantly higher than 26ng/ml (0-75) at the periphery ($p<0.05$).

Table 4.6: Mycolactone distribution within *M. ulcerans* infected lesions

Patient code	Lesion form	Time of sampling (weeks)	Concentration at centre of lesion (ng/ml)	Concentration at periphery of lesion (ng/ml)
A51	nodule	0	915.5	355.8
A52	nodule	0	378.4	313.2
T11	nodule	0	132.6	211.3
T10	plaque	0	167.1	123.9
T14	plaque	0	181.8	147.9
T15	plaque	0	610.3	430.1
			Concentration at edge 1 of ulcer[1]	**Concentration at edge 2 of ulcer[1]**
NK01	ulcer	0	2458.1	2421.8
NK05	ulcer	0	434.7	320.5
NK06	ulcer	0	376.9	565.2
NK07	ulcer	0	442.6	393.6
T13	ulcer	0	157.8	246.2
A33	ulcer	6	158.4	1160
A55	ulcer	6	242.2	374.4
A40	ulcer	12	244.8	443.6

Data shown is mycolactone concentration as measured by cytotoxicity assay.

[1] Two biopsies were obtained from different parts of the ulcer border to investigate variations in mycolactone concentration.

Association with size and severity of lesion:

There was no significant difference between the median (range) concentration of mycolactone in category I lesions (417ng/ml; 136– 3020; n=50), category II

lesions (394ng/ml; 161 – 2689, n=23) or category III lesions (457ng/ml; 314 – 2458, n=7) measured by cytotoxicity or by mass spectrometry (data not shown).

***Mycolactone concentration during and after antibiotic therapy**:*

Further biopsies were taken at 6 and/or 12 weeks from patients whose lesions were not healing rapidly (Figures 4.13 and 4.14). Median concentration of mycolactone by cytotoxicity decreased from 479ng/ml (range 159-3020; n=26) at week 0 to 385ng/ml (153-1160, n=23)($p<0.05$) at 6 weeks and to 320ng/ml (169-604; n=7)(NS) at 12 weeks (Figure 4.13). By mass spectrometry, the median concentration decreased from 34ng/ml (range 0–296) at week 0 to 16ng/ml (0-226)($p<0.05$) at week 6 and to 3ng/ml (0-27)(NS) at week 12 (Figure 4.14). There was no significant difference in mycolactone concentration by either method at any time point between patients receiving the RS8 or RS2RC6 treatment regimens.

The mycolactone concentrations measured by mass spectrometry increased from baseline value to a higher value at 6 weeks for 3 patients whilst an increase from a 6-week value to 12 weeks was noted for another patient as shown in Figure 4.14. All four patients had ulcerative lesions which regressed during RS8 treatment and healed without recurrence.

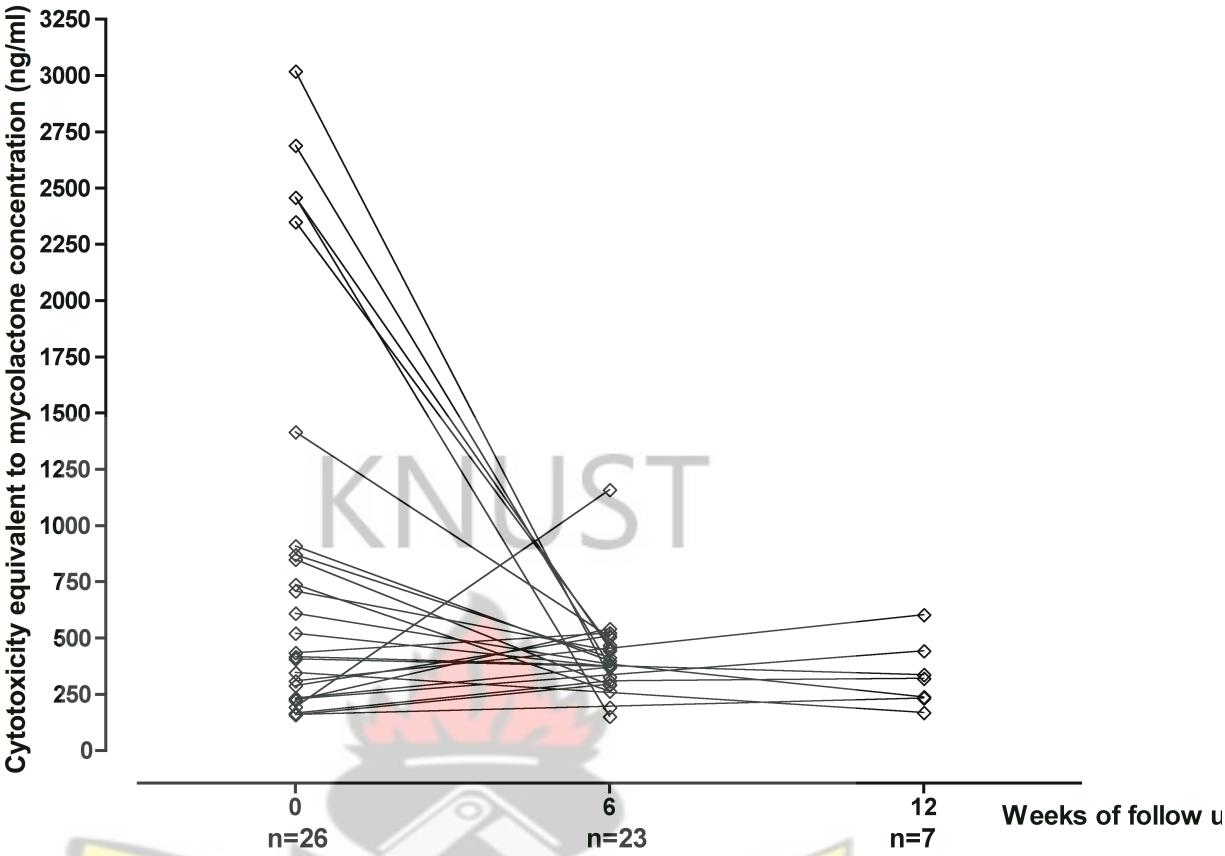


Figure 4.13. Tissue mycolactone concentration measured by cytotoxicity assay in serial biopsies during and after antibiotic treatment. Antibiotic treatment was started at week 0 and completed at week 8. Biopsies were taken at weeks 0 in all patients and at week 6 and/or 12 when it was clinically indicated. Mycolactone concentration in ASL measured by cytotoxicity assay using synthetic mycolactone A/B generated calibration curves.

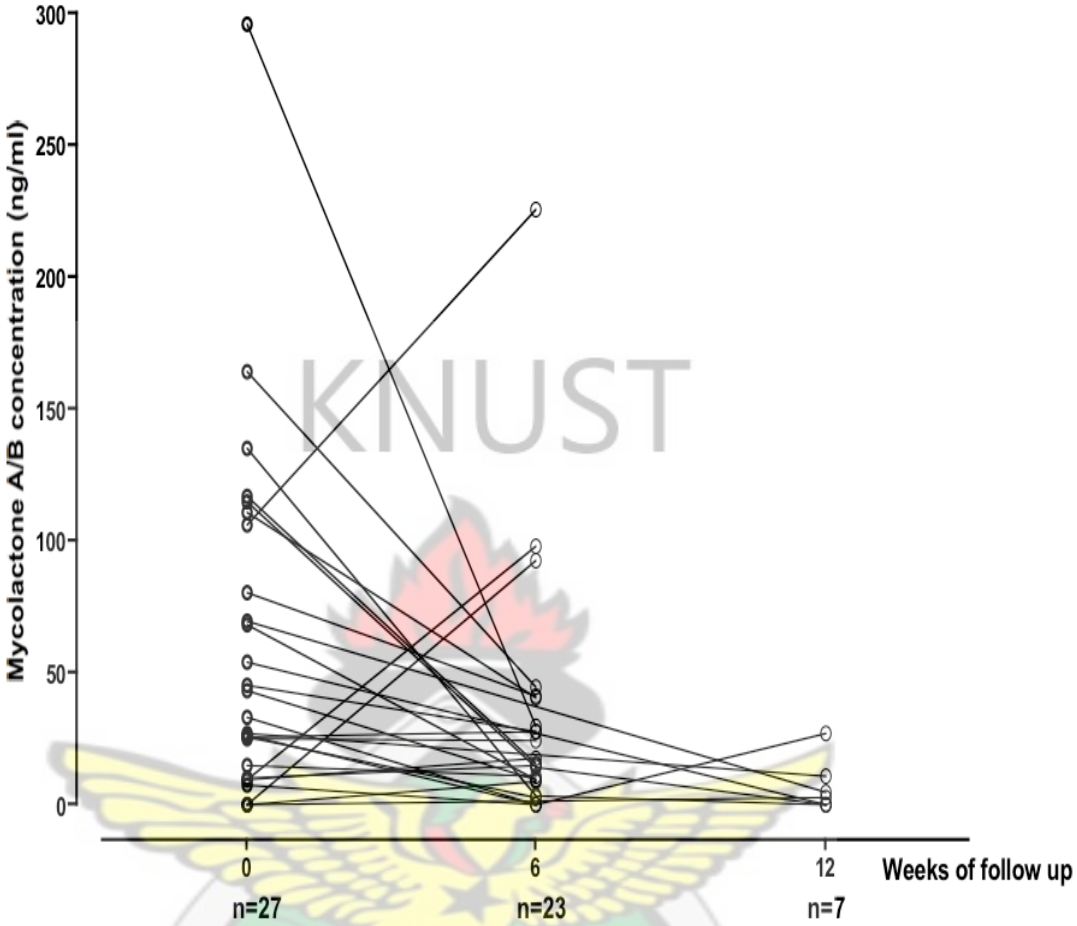


Figure 4.14. Tissue mycolactone concentration measured by mass spectrometry in serial biopsies during and after antibiotic treatment. Mycolactone A/B concentration in ASL measured by multiple reaction monitoring (MRM) methodology with liquid chromatographic separation coupled to a tandem mass spectrometer.

Correlation between healing time and mycolactone concentration at baseline:

There was a weak but significant correlation between mycolactone measured by mass spectrometry at baseline with time to complete healing (Pearson's correlation coefficient 0.24; p=0.05). However there was a stronger correlation for category I lesions and among nodular and category I ulcerative lesions in particular (Figures 4.15a-d). Nodules with mycolactone concentration below 64.9ng/ml (the median value) healed in 8 weeks (range 4-32) compared with 12 weeks (6-52) for those with mycolactone concentration above the median ($p<0.05$). For instance, a 5-year old male patient with a category 1 nodular lesion with the highest concentration of mycolactone at baseline (1,970ng/ml) (see Figure 4.12) took the longest time to complete healing (32 weeks) among patients with nodular lesions. This patient had both positive culture and microscopy results at initiation of therapy with RS2RC6 and still had positive culture after 6 weeks of treatment. However, no additional sample was available for mycolactone measurement at week 6. Similarly, the median time to complete healing of category I ulcers was 8 weeks (6-32) for those below 9.0ng/ml compared to 16 weeks (6-40) for those above that concentration. No significant correlations were observed between time to healing and mycolactone concentrations measured by cytotoxicity assay.

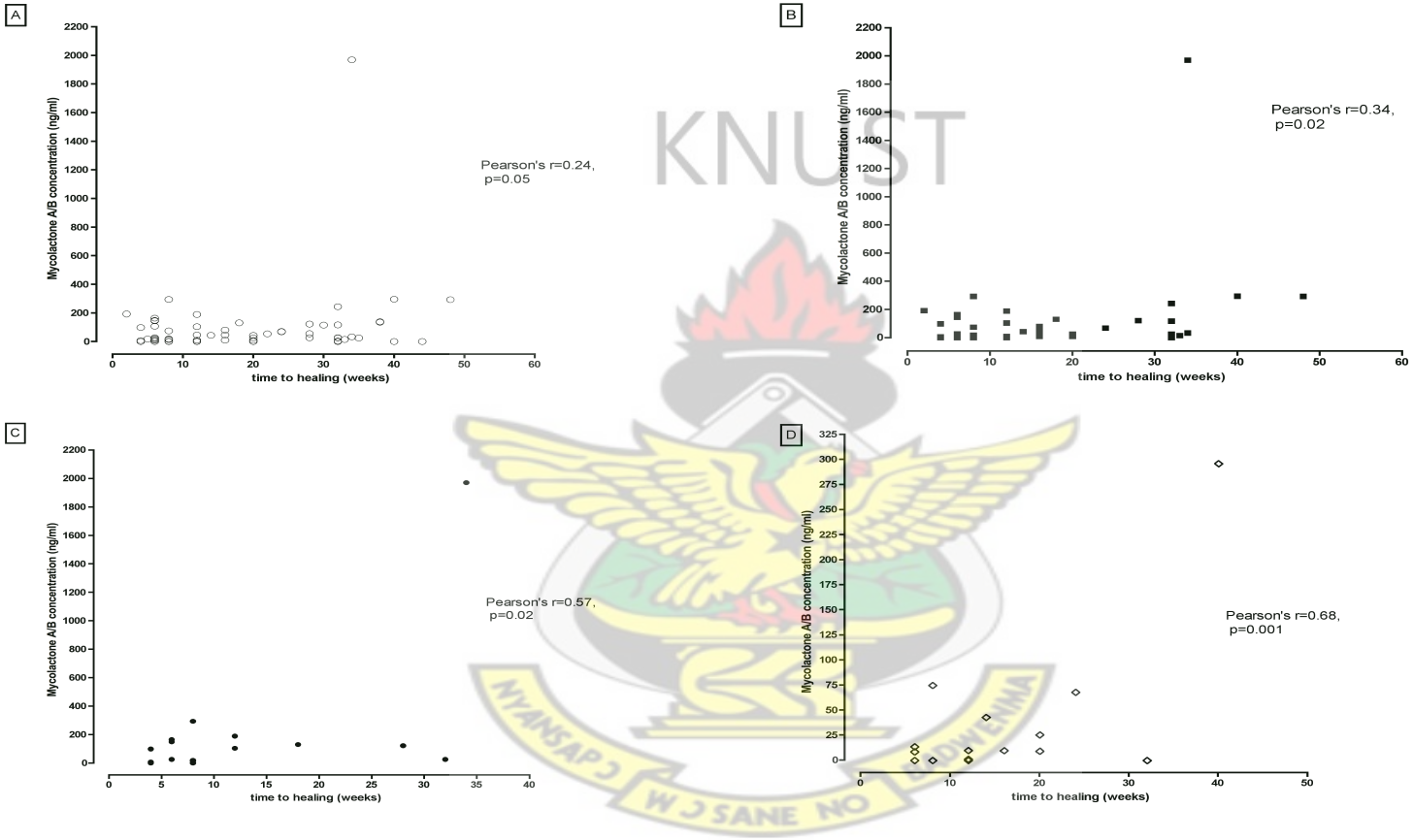


Figure 4.15. Correlation between baseline mycolactone A/B concentration (measured by mass spectrometry) and time to complete healing. 4.15a All categories and forms of buruli lesion. 4.15b All category I lesions. 4.15c Nodular lesions. 4.15d Category I ulcers (less than 5cm in widest diameter).

Mycolactone Detection In Lesions Sampled For *M. ulcerans* Culture

Samples were taken for culture of *M. ulcerans* from 60 patients before antibiotic treatment, 15 patients at week 6 during antibiotic therapy and 3 patients at week 12 (i.e. 4 weeks after completing antibiotic treatment). The results of culture and mycolactone detection at weeks 0, 6 and 12 are shown in table 4.7. Before antibiotic therapy, 66% of PCR-positive lesions were also culture-positive whilst mycolactone was detected in 87% of lesions by cytotoxicity assay and in 70% of lesions by mass spectrometry in this subset of 60 patients. Despite higher overall sensitivity, mycolactone was not detected in some samples that were culture-positive at week 0; 5 by cytotoxicity and 14 by mass spectrometry. At week 6, mycolactone was present in 7 culture negative samples using both the cytotoxicity assay and mass spectrometry. At week 12, mycolactone was detected by cytotoxicity in 2 culture positive lesions but mass spectrometry was negative. The overall sensitivity of mycolactone detection by cytotoxicity assay and mass spectrometry compared with *M. ulcerans* cultures were 90% and 68%, respectively.

Table 4.7. Mycolactone detection by mass spectrometry and cytotoxicity assay in patients with Mu disease sampled for *M. ulcerans* culture

Time	Week 0		Week 6		Week 12	
Mycolactone	**Culture result**		**Culture result**		**Culture result**	
detection	positive	negative	positive	negative	positive	negative
Cytotoxicity positive	35	17	7	7	2	0
Cytotoxicity negative	5	3	0	1	0	1
Total	**40**	**20**	**7**	**8**	**2**	**1**
MS positive	26	16	6	7	0	0
MS negative	14	4	0	1	2	1
Total	**40**	**20**	**6**	**8**	**2**	**1**

MS - Mass spectrometry

4.3. Kinetics of systemic interferon gamma and interleukin-5 before, during and after antibiotic therapy in humans with *M. ulcerans* disease.

Gamma interferon and IL-5 concentrations among lesion forms

Gamma interferon concentration in supernatants among lesion forms: Before antibiotic therapy the median (IQR) concentrations of gamma interferon in supernatants were significantly higher in patients with oedematous and ulcerative forms of the disease 1770 (1238 – 2669 pg/ml) and 753.3 (87.8 - 2377 pg/ml), respectively, compared with nodules 354.6 (55.8 – 1736pg/ml) and plaques 390.3 (136.1 – 2384pg/ml) as shown in figure 4.16.

IL-5 concentrations in supernatants among lesion forms: Before antibiotic therapy, the median (IQR) concentrations of IL-5 in supernatants were significantly higher in patients with oedematous and ulcerative forms of the disease 95.5 (42.0-482.9 pg/ml) and 32.7 (11.4-100.2 pg/ml) respectively compared with nodules and plaques 12.8 (7.2-23.4 pg/ml) and 25.7 (18.7-33.1 pg/ml) as shown in figure 4.17.

Figure 4.18 shows the moderate-to-strong positive correlation between baseline gamma interferon and IL-5 production (Pearson's correlation coefficient of 0.67, $p<0.001$) in supernatants after stimulation of whole blood of patients using *M. ulcerans* sonicate 1.

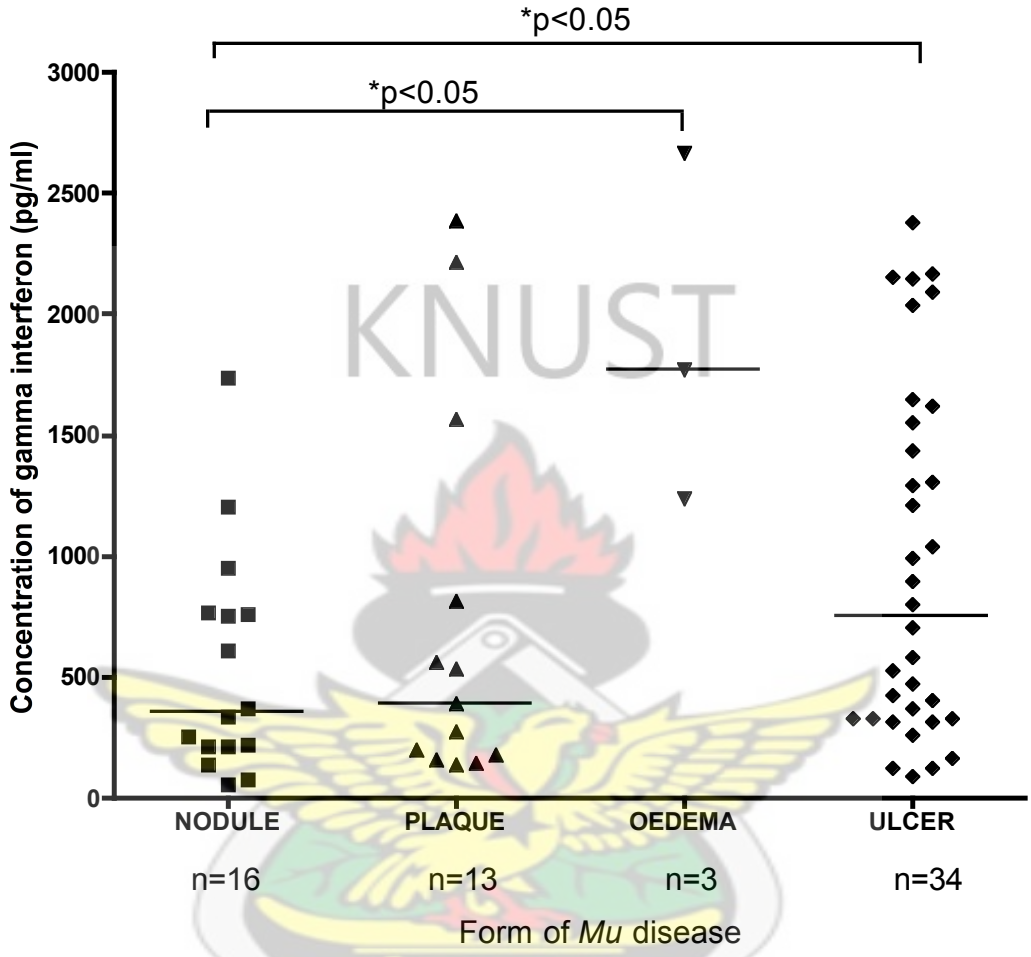


Figure 4.16. Gamma interferon production and forms of *M. ulcerans* disease. Gamma interferon production after stimulation with *M. ulcerans* 1 sonicate of whole blood from patients with Buruli lesions before antibiotic treatment. Each dot represents one subject, and horizontal lines represent the medians.

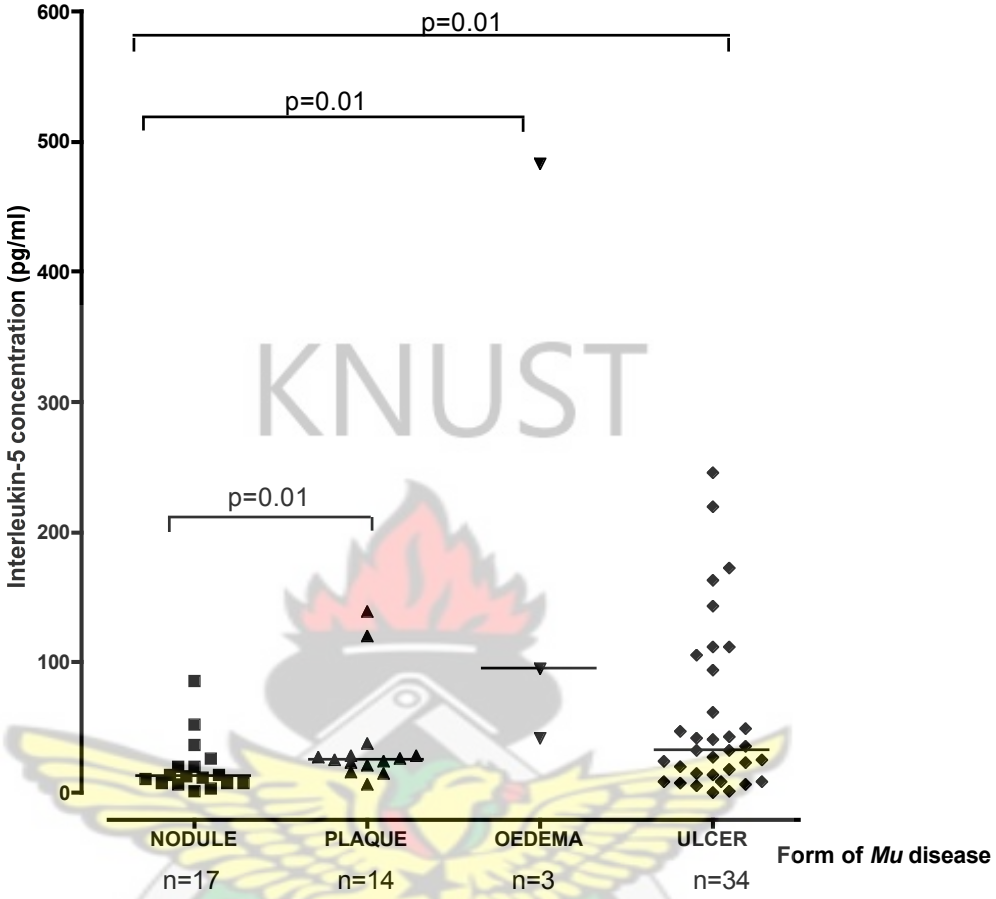


Figure 4.17. Interleukin-5 production and forms of *M. ulcerans* disease. Interleukin-5 production after stimulation with *M. ulcerans* 1 sonicate of whole blood from patients with Buruli lesions before antibiotic treatment. Each dot represents one subject, and horizontal lines represent the medians.

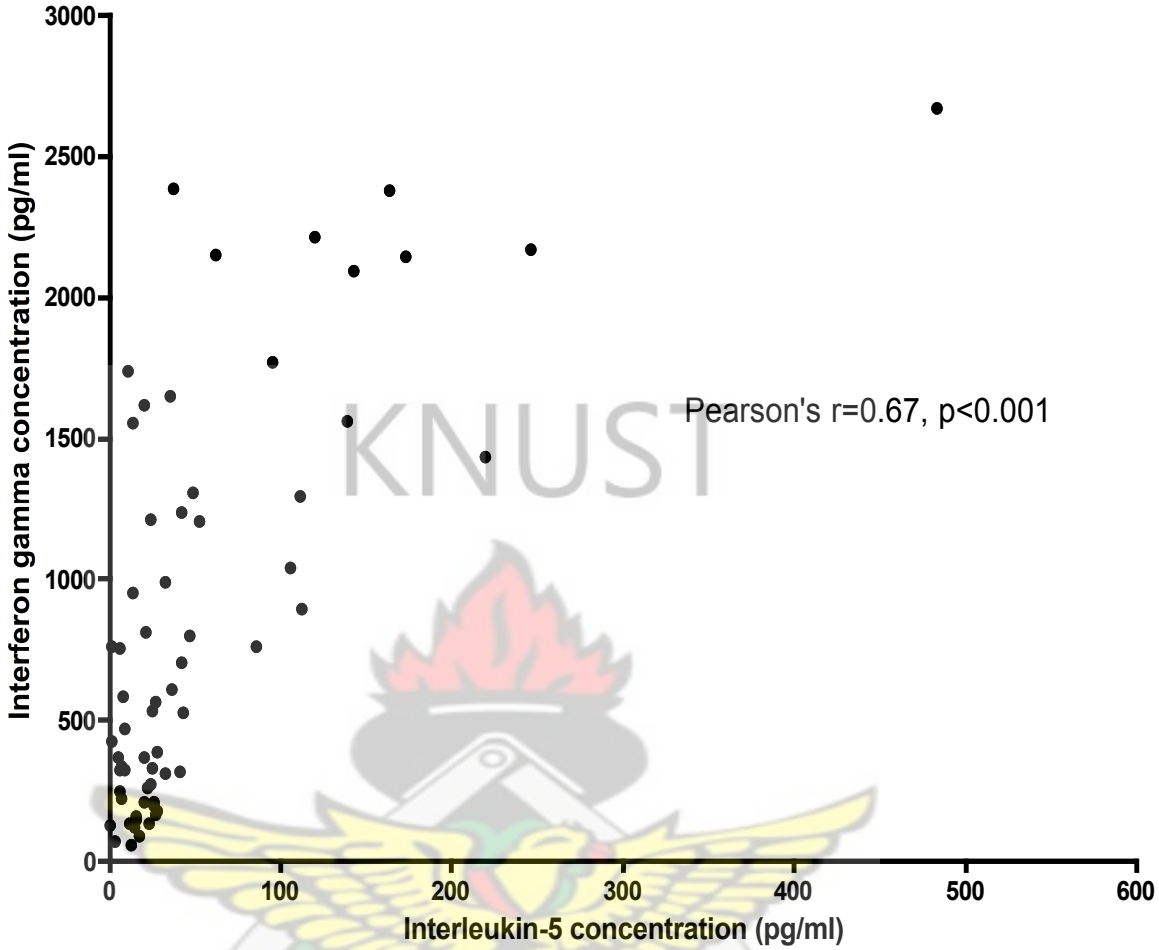


Figure 4.18. Correlation between interferon gamma and IL-5 secretion among patients with Buruli lesions before antibiotic treatment. Each dot represents a patient.

Kinetics of gamma interferon and IL-5 with antibiotic therapy

Kinetics of gamma interferon with antibiotic therapy

Curative antibiotic therapy was associated with recovery of gamma interferon secretion during treatment at 6 weeks: median (IQR) of 1071 (319.7 – 1951 pg/ml) compared to the baseline concentration of 572.7 (255.4 – 1372 pg/ml), $p<0.05$. This increment observed during treatment was further sustained for 4 weeks after completion of antibiotic therapy with a median (IQR) IFN-γ at 12 weeks of 1986 (1334 – 2811pg/ml), $p<0.001$ compared to baseline. At week 32 the gamma interferon concentration of 1411 (355.9 – 2172pg/ml) was still significantly higher than the baseline level, $p<0.05$ as shown in figure 4.19.

The median (IQR) concentrations of IFN-γ for patients randomised to receive standard SR8 regimen at baseline was 371.8 (255.4 – 1207pg/ml) at baseline, increased to 790 (203.4 – 2030pg/ml) at 6 weeks, then 1614 (483.1 – 2250pg/ml) at 12 weeks and 875 (300.6 – 2172pg/ml) at 32 weeks with no significant differences between the medians at the various time points. The median (IQR) concentrations of the comparator regimen of SR2CR6 were 754.1 (279.8 – 1634pg/ml), 1166 (594 – 1871pg/ml), 2335 (1873 – 2541pg/ml) and 1584 (558.5 – 2173pg/ml) at weeks 0, 6, 12 and 32 weeks respectively with $p<0.001$ at baseline vs 12 weeks and <0.05 at 6 vs 12 weeks.

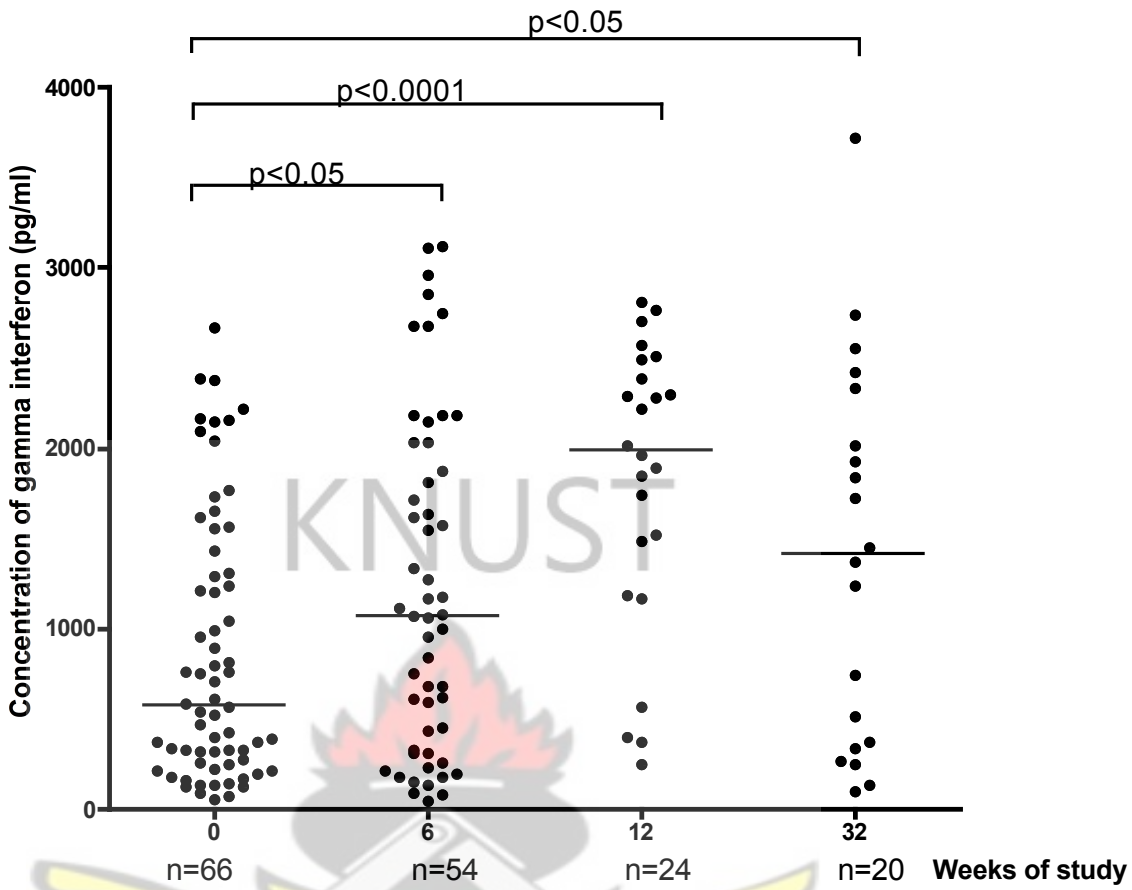


Figure 4.19. Kinetics of gamma interferon production during and after antibiotic therapy for *M. ulcerans* disease. Each dot represents one patient and horizontal lines are medians.

4.3.2.2. Kinetics of IL-5 with antibiotic therapy

The median (IQR) IL-5 concentration at baseline of 25.2 (12.5-48.1 pg/ml) was not significantly different from the median concentration at 6 weeks of antibiotic treatment of 32.1 (20.1-68.0 pg/ml). However, at 12 weeks, the median concentration of 68.2 (30.8-224.6 pg/ml) was significantly higher than at baseline as shown in figure 4.20. At week 32, the median concentration of IL-5 in supernatants of 66.7 (12.0-137.8 pg/ml) were still higher than baseline but not significantly so.

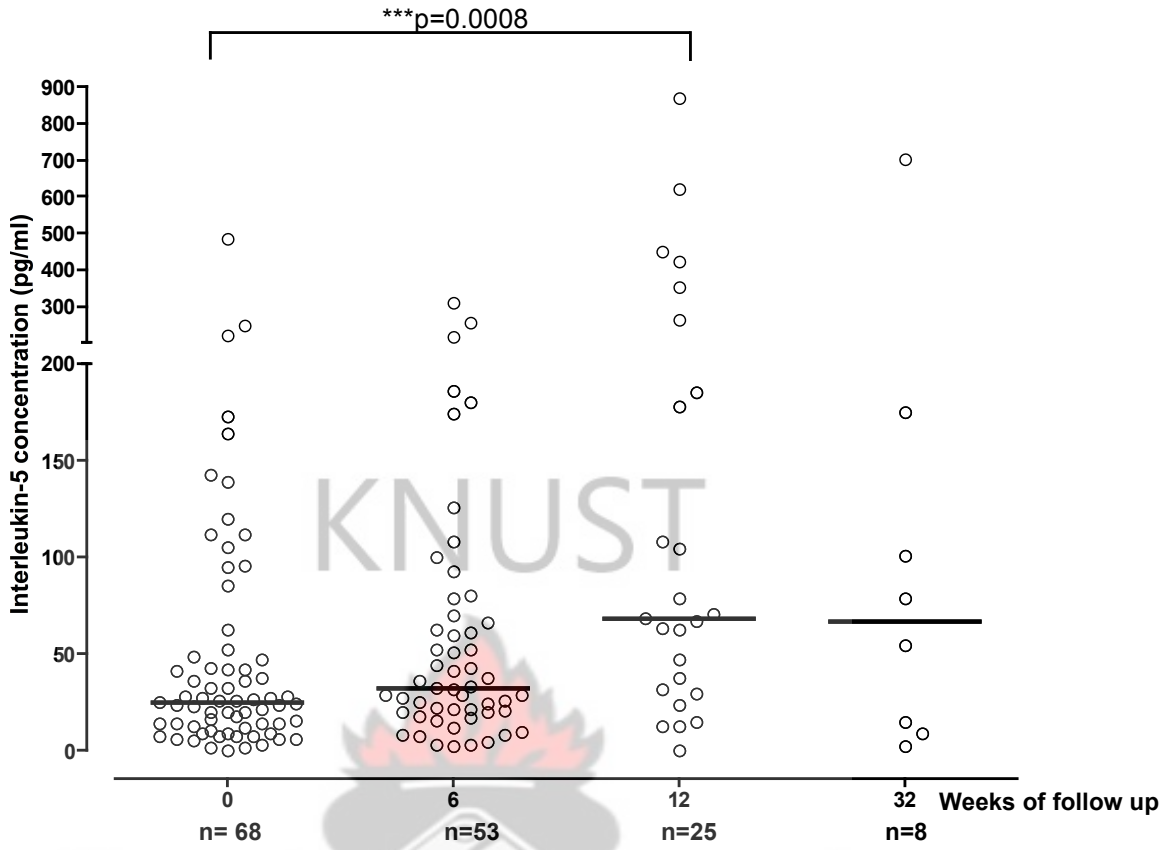


Figure 4.20. Kinetics of interleukin 5 production during and after antibiotic therapy for *M. ulcerans* disease. Each dot represents one patient and horizontal lines are medians.

Pathobiology of paradoxical reaction: a case report

A patient with a plaque lesion was started on antibiotic therapy with a combination of RS2RC6. Four weeks into his therapy the surface area of the lesion enlarged from a diameter of 7.0 cm at week 0 to 12.0 cm which was diagnosed as a paradoxical reaction by the clinicians managing the patient. As shown in figure 4.21, the concentration of gamma interferon also increased from 112.8 pg/ml at week 0 to 321.7 pg/ml representing a nearly 3-fold increase. At week 6, the diameter of the lesion had started to regress and measured 7.1 cm. At this time point, the concentration of gamma interferon had increased further to 622.9 pg/ml, IL-5 concentration had declined from a baseline value of 5.9 pg/ml to 5.2 pg/ml and mycolactone concentration measured by mass spectrometry had declined sharply from 115 ng/ml at week 0 to 14.6 ng/ml. The lesion continued to regress slowly such that at week 12, it measured 6.7 cm, with undetectable mycolactone by mass spectrometry but a high gamma interferon gamma concentration of 540.2 pg/ml. The lesion eventually healed by week 32 (not shown).

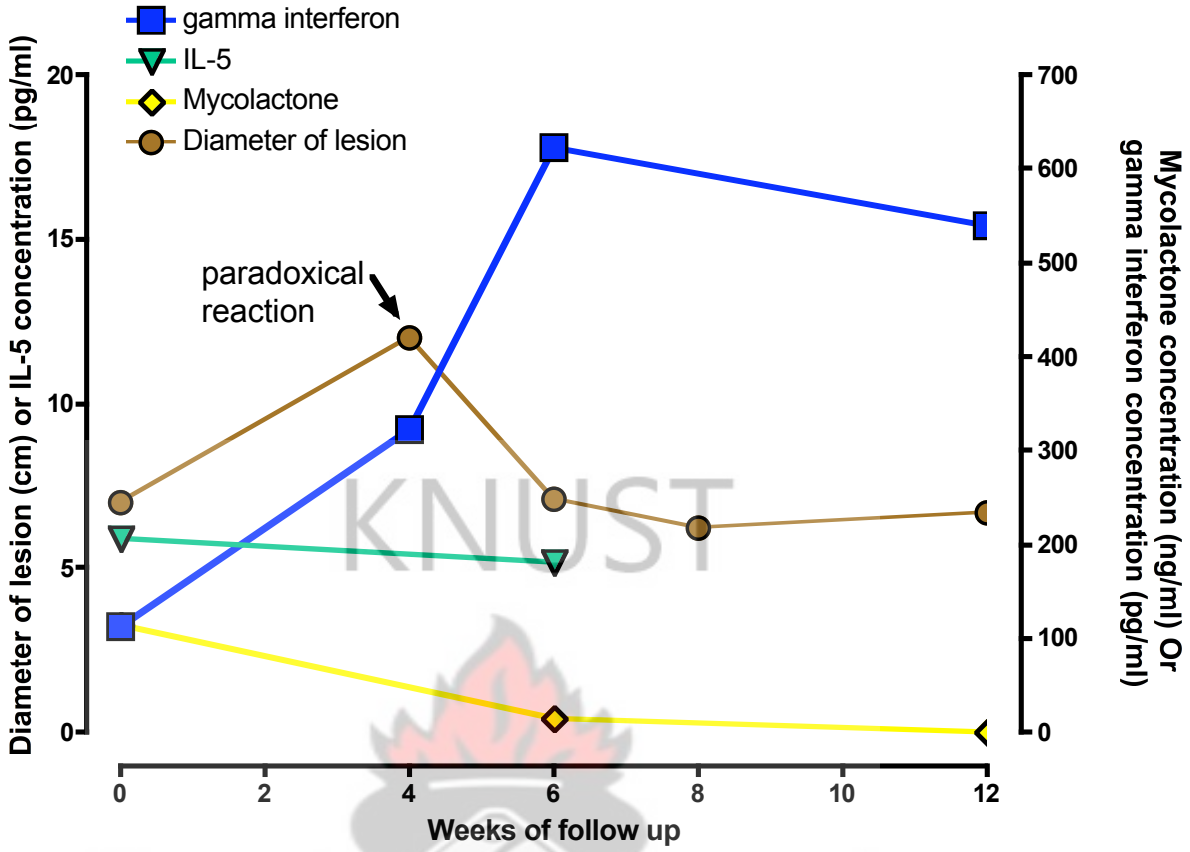


Figure 4.21. Pathobiology of paradoxical reaction. Kinetics of gamma interferon, IL-5, mycolactone concentrations and dimensions of a patient with a buruli plaque who had paradoxical reaction at week 4.

4.4. Microbiological, histological, immunological, and toxin response to antibiotic treatment in the mouse model of *Mycobacterium ulcerans* disease.

4.4.1. Footpad swelling and CFU counts before and after treatment

Three days after infection, mice harbored 3.29±0.33 $\log_{10}$ CFU in the infected footpads. Just before the onset of swelling, at day 20, the footpads contained 5.05±0.19 $\log_{10}$ CFU of *M. ulcerans*. Footpad swelling first appeared in some mice 3 weeks after infection and averaged grade 1 soon after the initiation of treatment at ~3.5 weeks after infection. At day 27, there were approximately 6.02±0.13 $\log_{10}$ CFU per footpad. Swelling continued to increase over the following 3 weeks (Figure 4.22) for an average of grade 3.42±0.43 in untreated mice whereas CFU counts remained essentially unchanged.

In contrast, both swelling and CFU counts declined markedly in the RS-treated mice (Figure 4.23). Swelling after 3 weeks of treatment averaged grade 0.14±0.18 and $\log_{10}$ CFU count per footpad was reduced to 0.52±0.45. By day 55 after infection, untreated mice had 6.37±0.32 $\log_{10}$ CFU whereas RS-treated mice, at this time point after 31 days of treatment, had only 0.20±0.35 $\log_{10}$ CFU (Figure 4.23). By day 63 (i.e., 39 days of treatment), the RS-treated mice were culture-negative. Swelling was essentially undetectable. Figure 4.23 also shows the contrast in *M. ulcerans* CFU counts between treated and untreated mice.

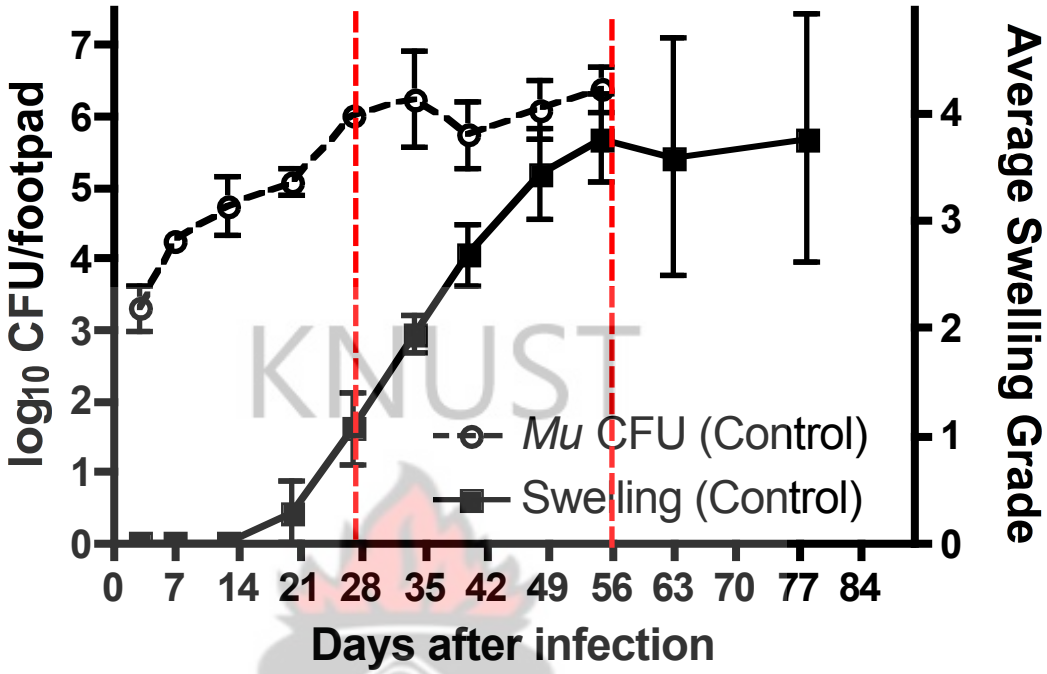


Figure 4.22. Footpad swelling and CFU counts without antibiotic treatment. After reaching plateau in bacillary numbers, *M. ulcerans*-infected mouse footpads continued to increase in swelling. Swelling first became apparent at day 20 after infection. For the first 3 weeks, the number of cultivable organisms increased from 3.29±0.33 $\log_{10}$ on day 3 to 4.26±0.08 $\log_{10}$ on day 7, 4.73±0.42 $\log_{10}$ on day 13, 5.05±0.19 $\log_{10}$ on day 20, and 6.02±0.13 $\log_{10}$ on day 24. CFU remained at approximately this level for the remainder of the experiment in untreated mice. Swelling averaged ~ grade 1 at day 27 after infection, nearly grade 2 at day 34, grade 2.67 at day 40, 3.42 at day 48, 3.75 at day 55, 3.58 at day 63, and 3.75 at day 78. Data based on 3 mice per group per time point. Red lines emphasize the period between the onset and augmentation of swelling and the plateau in CFU.

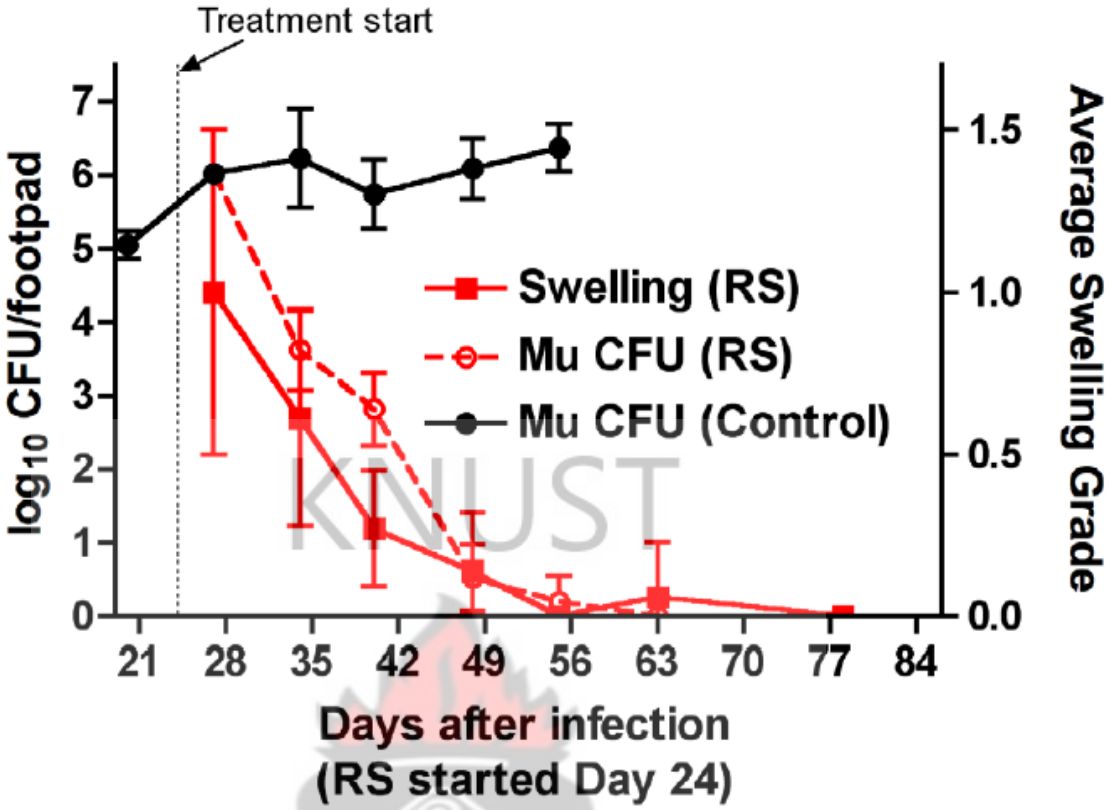
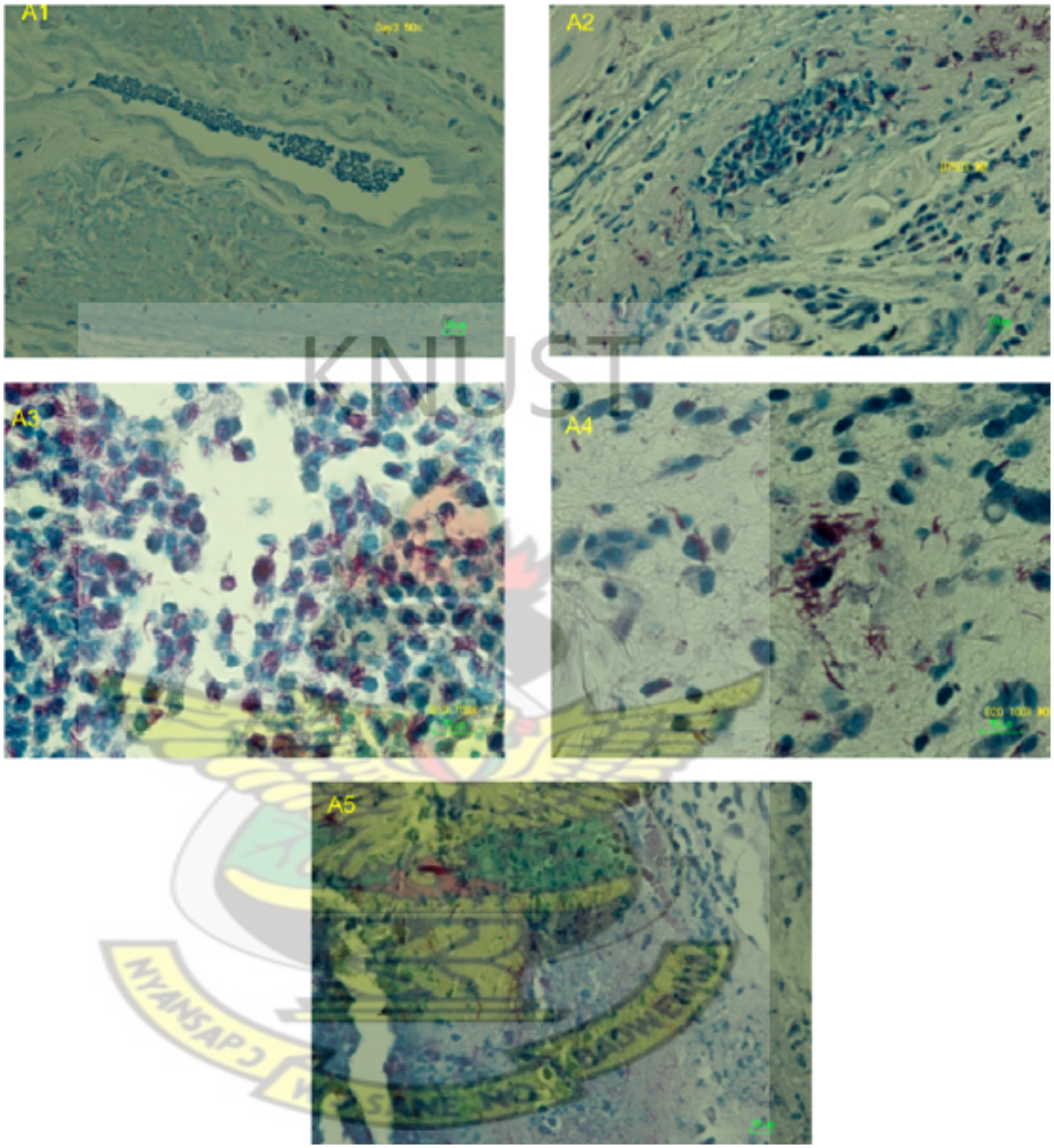


Figure 4.23. **Swelling and CFU Reduction after rifampicin-streptomycin treatment.** After 3 days of RIF-STR (RS) treatment (day 27 after infection), swelling averaged grade 1, and then declined to 0.61±0.33, 0.27±0.18, 0.14±0.18, 0, 0.06±0.17, and 0 on days 34, 40, 48, 44, 63 and 78, respectively. CFU also declined from 6.02±0.09 log_{10} on day 27, to 3.83±0.56 log_{10}, 2.82±0.49 log_{10}, 0.52±0.45 log_{10}, 0.2±0.35 log_{10}, 0, and 0 on days 34, 40, 48, 44, 63, and 78, respectively. For comparison, CFU per footpad for untreated controls (black line) and RS-treated (red-dashed line) show the impact of antibiotic treatment on bacterial burden. Data is based on 3 mice per group per time point.

4.4.2. Histopathological assessment of *M. ulcerans* in lesions before and after treatment

Histologically, increased numbers of AFB correlated with increased footpad swelling in untreated mice; bacilli were evident initially in the dermis and eventually in sub-epidermal zones and the epidermis with the onset of ulceration (Figure 4.24A.1-5). Inflammatory cell infiltrates appeared to be maintained in RS-treated mice but were disrupted and progressively disorganized in untreated, control mice (Figure 4.24B.1-4). After 2-3 weeks of treatment, AFB were still detectable but acquired a beaded appearance and this morphology persisted even after treatment completion. The beaded appearance of the bacilli correlated with reduced numbers of cultivable bacilli in treated mice (Figure 4.23) whereas the bacilli in untreated mice appeared solidly stained. Three months after treatment completion, bacilli were detectable in tissue by acid-fast staining but were not cultivable (Figure 4.24B.5).



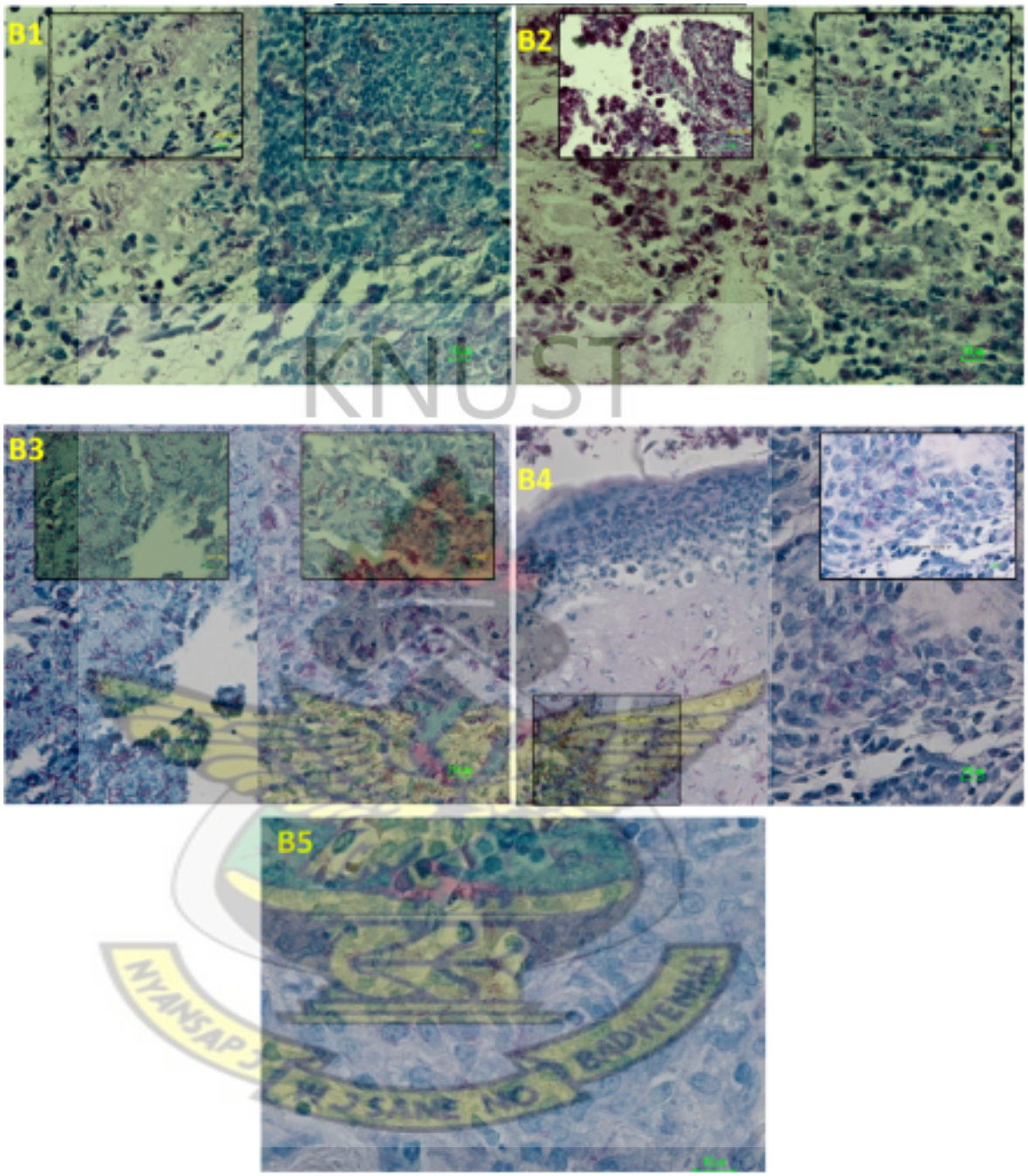
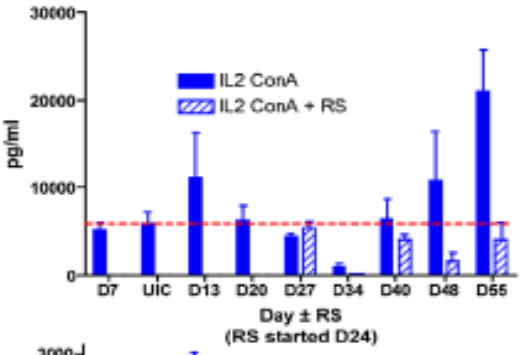
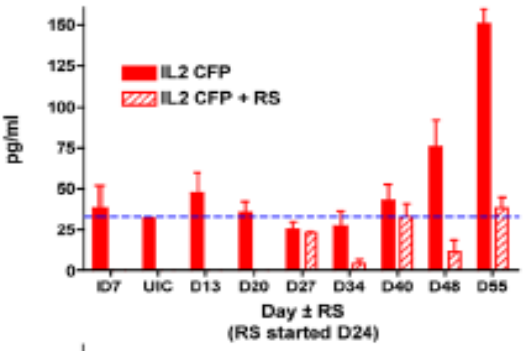


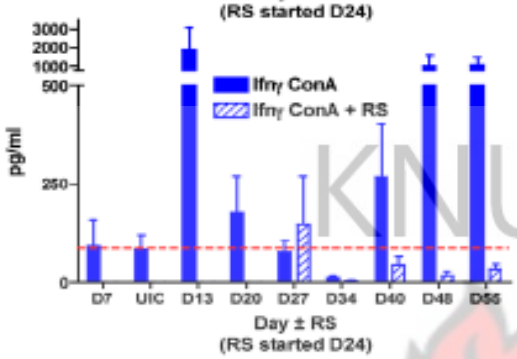
Figure 4.24. Progression of *M. ulcerans* infection in the mouse footpad, before (A, 1-5) and after (B, 1-5) treatment with Rifampin and Streptomycin. A1: AFB are detectable in the dermis 3 days after infection with little or no cellular infiltrate. A2: At day 7, the migration of inflammatory cells and phagocytosis has begun. A3: By day 13, AFB are primarily associated with inflammatory and phagocytic cells. A4: At day 20, the extracellular phase of *M. ulcerans* infection has begun, coinciding with the onset of footpad swelling. A5: At day 27, shortly after the initiation of antibiotic treatment, a mix of intracellular and extracellular AFB is observed. In panel B, untreated footpad lesions are shown on the left and treated footpads are shown on the right. B1: At day 34 after infection and after 10 days of treatment, *M. ulcerans* bacilli in treated mice are primarily associated with host cells whereas the bacilli are outside of cells in untreated mice; the cells are either not recruited or may have been destroyed by the mycolactone toxin. B2: At day 40, bacilli are found in extracellular masses in untreated mice but in mice treated for 16 days, the bacilli are still associated with host cells and stain less solidly. B3: At day 48, after 24 days of treatment, the bacilli have lost solid staining and have become beaded in appearance. B4: At day 55 after infection, *M. ulcerans* bacilli are apparent in the superficial dermis and epidermis and are being shed from the ulcerated lesions of untreated mice. B5: 3 months after treatment completion, the organisms are uncultivable but non-solid staining bacilli are still present in the footpads.

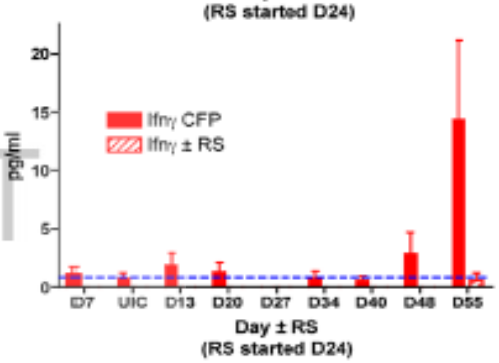
4.4.3. Evolution of immune responses during the course of *M. ulcerans* infection

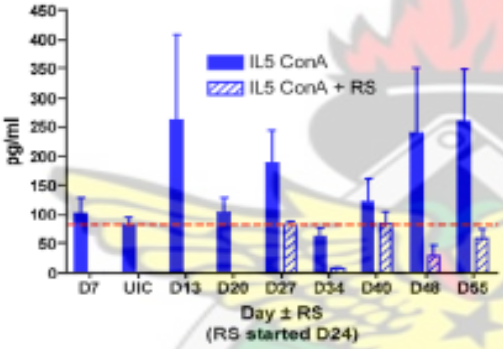
ConA and CFP of *M. tuberculosis* (Mtb) were used as a potent T-cell mitogen and a non-specific mycobacterial antigen, respectively, to both assess the functional capability of T-cells and the evolution of cellular immune responses by splenocytes to mycobacterial antigens during the course of *M. ulcerans* infection. There was a biphasic pattern to the kinetics of secretion of representative Th-1 (IFN-γ), Th-2 (IL-5) and Th-17 (IL-17) cytokines upon stimulation with the potent T-cell mitogen ConA (Figure 4.25, left column). There was an initial rise detected at week 2, then a decline during weeks 4 to 6 and then a later surge from week 7 onwards. The cellular responses to a culture filtrate protein of Mtb however was generally a slowly evolving one and gradually builds up (IL-2, IL-5, IFN-γ and TNF-α) over the course of infection with *M. ulcerans* (Figure 4.25, right column). Immune responses were, however, generally lower during the course of infection in mice treated with antibiotics compared with untreated mice (Figure 4.25). Although the amount of TNF-α detected in supernatants of splenocytes from mice treated with RS was much less after 10 days of treatment, it was statistically significant ($p<0.001$) only at day 55 by two-way ANOVA. A t-test analysis indicated borderline statistical significance ($p<0.05$) at day 48.

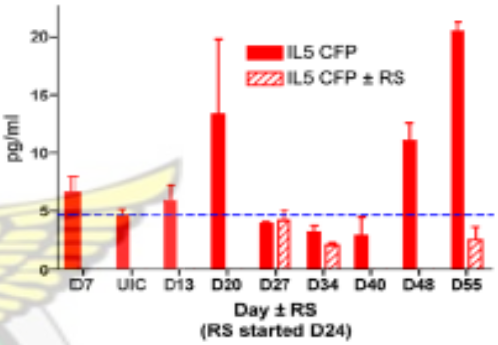

IL2 ConA
IL2 ConA + RS
pg/ml
D7 UIC D13 D20 D27 D34 D40 D48 D55
Day ± RS
(RS started D24)

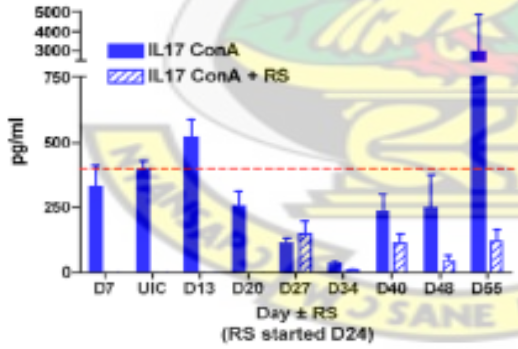

IL2 CFP
IL2 CFP + RS
pg/ml
D7 UIC D13 D20 D27 D34 D40 D48 D55
Day ± RS
(RS started D24)

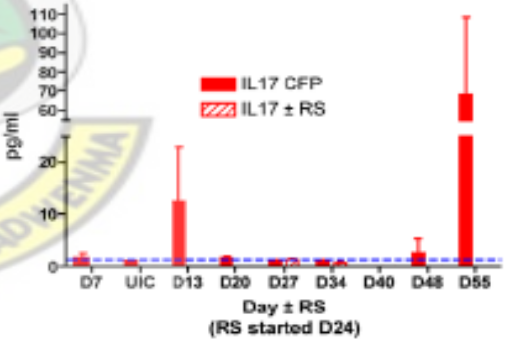

Ifnγ ConA
Ifnγ ConA + RS
pg/ml
D7 UIC D13 D20 D27 D34 D40 D48 D55
Day ± RS
(RS started D24)

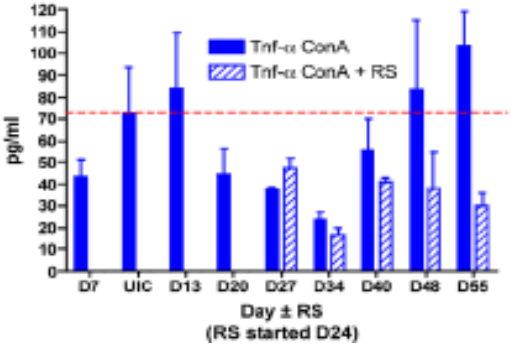

Ifnγ CFP
Ifnγ ± RS
pg/ml
D7 UIC D13 D20 D27 D34 D40 D48 D55
Day ± RS
(RS started D24)


IL5 ConA
IL5 ConA + RS
pg/ml
D7 UIC D13 D20 D27 D34 D40 D48 D55
Day ± RS
(RS started D24)


IL5 CFP
IL5 CFP ± RS
pg/ml
D7 UIC D13 D20 D27 D34 D40 D48 D55
Day ± RS
(RS started D24)


IL17 ConA
IL17 ConA + RS
pg/ml
D7 UIC D13 D20 D27 D34 D40 D48 D55
Day ± RS
(RS started D24)


IL17 CFP
IL17 ± RS
pg/ml
D7 UIC D13 D20 D27 D34 D40 D48 D55
Day ± RS
(RS started D24)


Tnf-α ConA
Tnf-α ConA + RS
pg/ml
D7 UIC D13 D20 D27 D34 D40 D48 D55
Day ± RS
(RS started D24)

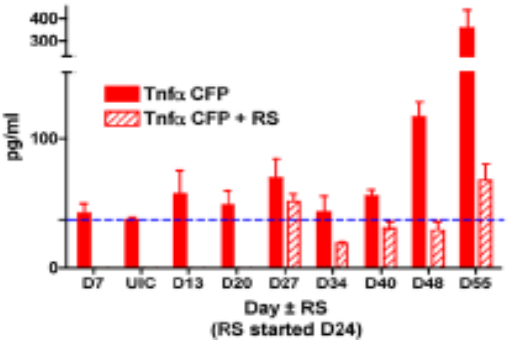

Tnfα CFP
Tnfα CFP + RS
pg/ml
D7 UIC D13 D20 D27 D34 D40 D48 D55
Day ± RS
(RS started D24)

Figure 4.25. Cytokine production responses to concanavalin A (Con A) or mycobacterial protein before and after Rifampicin-Streptomycin treatment. The dotted line indicates the levels in uninfected control (UIC) mice. Responses by untreated mice are indicated by solid bars and those by rifampicin-streptomycin (RS) treated mice are indicated by hatched bars. Responses of splenocytes stimulated with Con A are in the left column and with mycobacterial culture filtrate proteins (CFP) in the right column for representative cytokines, from top to bottom, IL-2, IFN-γ, IL-5, IL-17, and TNF-α. Data based on 3 mice per group per time point.

4.4.4. The presence of mycolactone in footpads before and after treatment

Mycolactone toxin production was assessed by three different methods: cytotoxicity against the HELF cell line, mass spectrometric analysis and thin layer chromatography.

Cytotoxicity assay of lipid extracts from mouse footpads

Lipid extracts from the right footpads into which *M. ulcerans* had been injected were increasingly cytotoxic from day 3 up to day 62. Surprisingly, lipid extracts from the left footpad of the same mice also demonstrated significant cytotoxicity (Figure 4.26). Lipid extracts from footpads of mice which had not been infected with *M. ulcerans* showed median cytotoxicity of 20%, suggesting that cytotoxicity observed in *M. ulcerans*-infected mice was due to the presence of a cytotoxic molecule with activity similar to, or targeting the same pathway as, mycolactone.

The mean concentration, as estimated in the cytotoxicity assay, of mycolactone in the right footpad (3 per group) increased from 286±105 ng/ml at day 3 to 948±215 ng/ml at day 7, plateaued between day 13 and 35 and progressively increased to 3603±478 ng/ml at day 62 (Figure 4.26). In the left footpad of mice infected on the right, the concentration also increased up to day 62 with a peak of 1524±607 ng/ml at day 27. In the right and left footpads of antibiotic-treated mice, there was a significant decline in estimated mycolactone concentration compared to untreated right footpads ($p<0.05$ at day 62). Likewise, there was a significant difference

($p<0.02$) in cytotoxicity between the right and the contralateral footpads obtained from untreated mice.

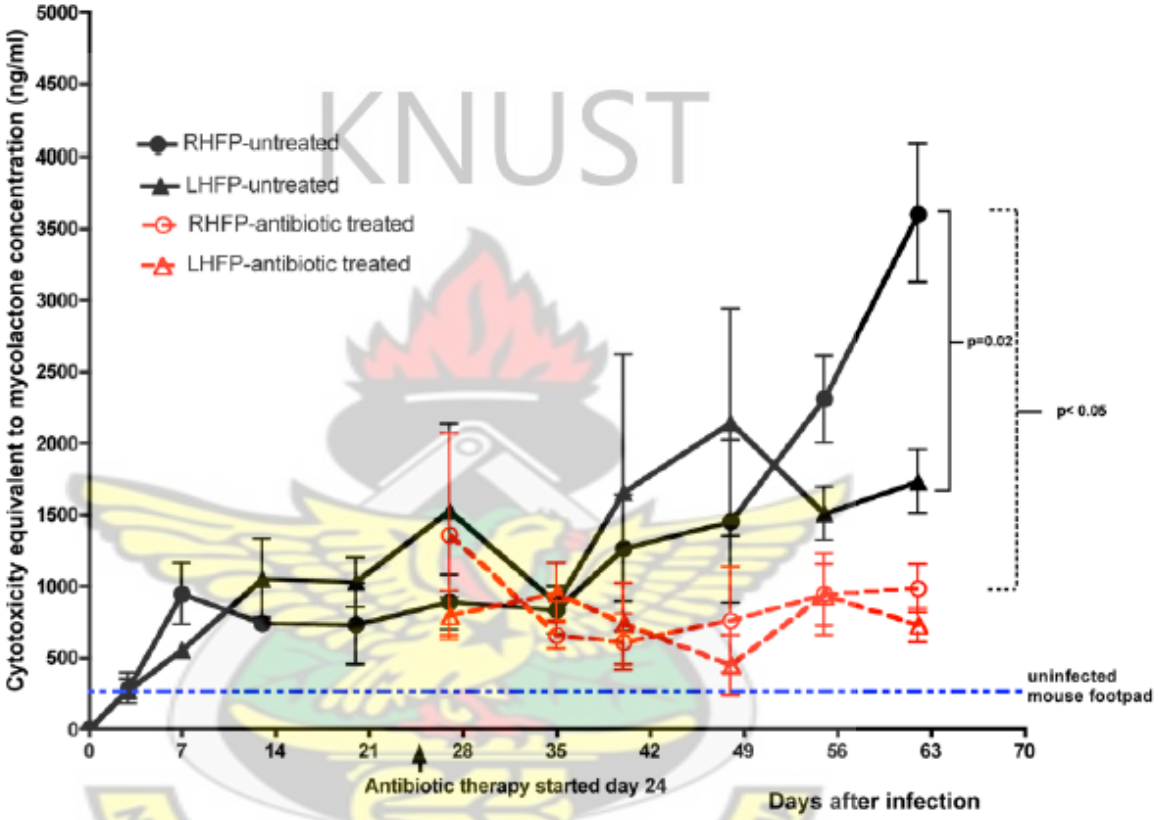


Figure 4.26. Cytotoxic activity of lipids extracted from footpads of mice without treatment or treated with RIF-STR. Cytotoxicity of lipid extracts was measured by the MTT assay after incubation of HELF cells for 48 hours. Cytotoxicity is expressed relative to that induced by synthetic mycolactone measured concurrently. Data based on 3 mice per group per time point. By day 63 there were statistically significant differences between the infected right hind footpad (RHFP, black solid circles) and the contralateral (LHFP, solid diamonds, $p<0.02$) and footpads of the

treated mice (RHFP, red open circles; LHFP, red open diamonds $p<0.05$). RHFP= Right hind footpad, LHFP= Left hind footpad.

Mass spectroscopic analysis

To establish whether cytotoxicity in infected mouse footpads was due to whole mycolactone molecules, liquid chromatography was coupled to mass spectroscopic detection of mycolactone A/B. Using an enhanced product ion (EPI) method, mycolactone A/B was detected as an ion with m/z of 765.7 which fragmented to give peaks at 429.5 (core lactone ring), 359.5 (polyketide side chain) and other minor peaks at 747.7, 659.6 and 565.6. Using this approach, intact mycolactone A/B was identified in the infected right footpad pools at day 13 onwards but not in the uninfected left footpads.

An approximate quantification of mycolactone A/B was achieved using multiple reaction monitoring (MRM) (Figure 4.27). In the infected right footpads, mycolactone A/B concentration increased from 1.6 ng/ml on day 13 to a peak of 195.6 ng/ml on day 62. In antibiotic-treated mice mycolactone concentration in the right footpads decreased from a peak of 23.7ng/ml on day 35 to 1.6 ng/ml on day 62. No mycolactone was detected in left footpads.

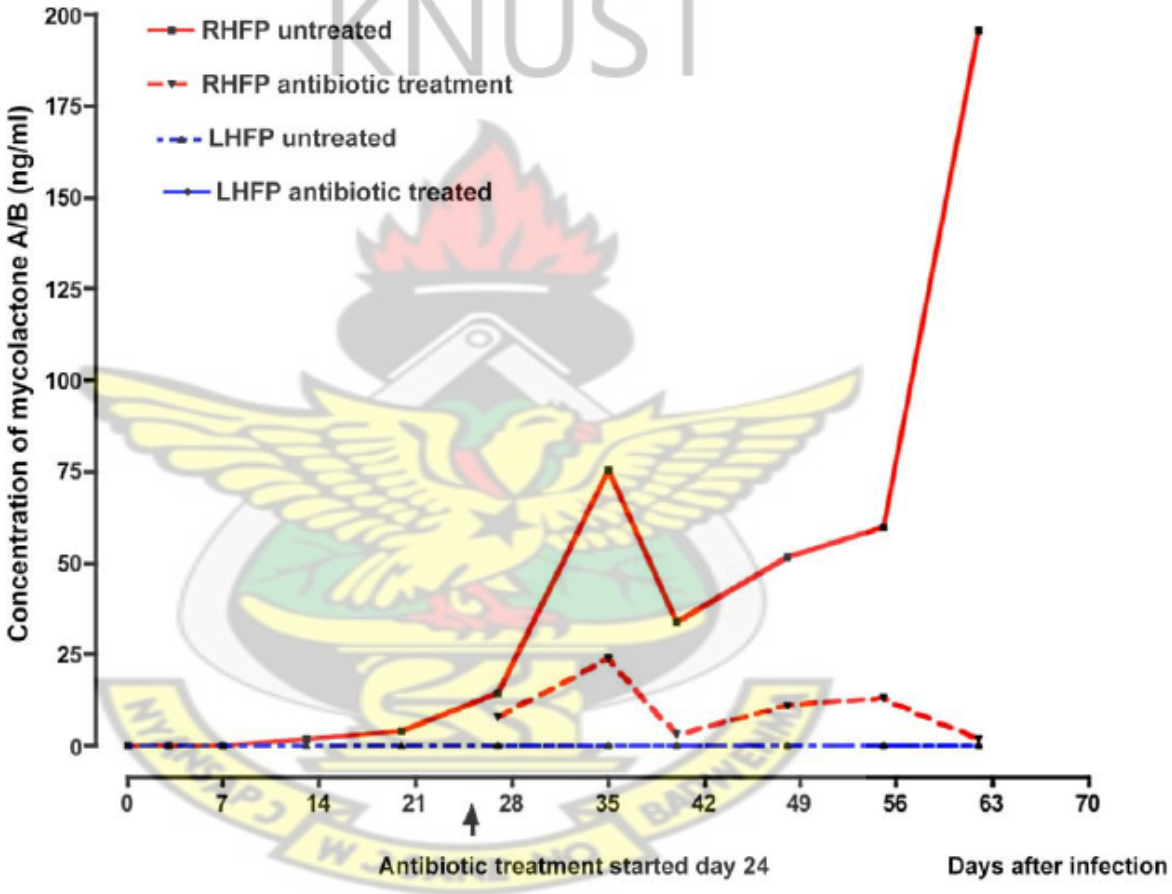


Figure 4.27. Mycolactone quantification by mass spectrometry in mouse footpads. Mycolactone (ML) was detectable as early as day 13 after infection and before the onset of visible swelling in mouse footpads. No ML was detectable in the contralateral footpad of either the RS-treated or the untreated control mice.

Mycolactone was identified using EPI (enhanced product ions) at m/z of 765.7 with ms:ms fragmentation. RHFP= Right hind footpad, LHFP= Left hind footpad.

Thin layer chromatography: TLC was not specific for mycolactone detection in lipid extracts from both right and left hind footpads as was found among lipid extracts from human samples (not shown).

Summary of findings from the murine study:

The results show that RS treatment of experimental *M. ulcerans* disease results not only in the reduction or elimination of viable bacteria but also in clinical and histopathological improvement of lesions, supporting results obtained in the analysis of human specimens (Schütte et al. 2007, 2008) and in RS-treated mice (Torrado, 2007, Martins, 2012, Ruf, 2012). The inflammatory response was also down-regulated with reduced production of cytokines such as TNF-α during treatment. By mass spectrometric analysis, mycolactone was detectable before the observation of swelling and increased markedly and continuously after swelling onset whereas *M. ulcerans* CFU counts peaked and remained at a plateau. RS treatment resulted in a parallel loss of cultivable *M. ulcerans* in the footpad lesions, a decline in footpad swelling and an abrupt decline in the production of mycolactone within days of treatment initiation.

CHAPTER 5

DISCUSSION

5.1. Efficacy of RS8 compared with RS2RC6.

In this study the clinical and microbiological response to standard rifampicin and streptomycin treatment for 8 weeks (RS8) was compared with the response to rifampicin and streptomycin for 2 weeks followed by rifampicin plus clarithromycin for 6 weeks (RS2RC6) in patients with small Buruli lesions. Overall, treatment was successful in 93% of patients in both treatment groups and there was no difference in healing rate using RS8 compared with RS2RC6. There were no recurrences in either treatment group within one year of follow up. On the basis of findings in this pilot study, a controlled trial would need to include about 350 patients to achieve a significant result with 80% power to show more rapid healing or lower recurrence rate with either treatment group. We recorded recovery of function with simple exercises in all patients who presented with functional limitation associated with Buruli ulcers but a functional limitation score was not applied (Stienstra et al., 2005).

One aim of this study was to investigate the microbiological response to treatment with RS2RC6 compared to the standard regimen of RS8. Experiments using the mouse footpad model of *M. ulcerans* infection showed that rifampicin streptomycin was the most powerful bactericidal combination (Bentoucha et al., 2001, Dega et al., 2002) but subsequent experiments in which bacteria were quantified after treatment for 8 weeks showed that the combination of rifampicin with clarithromycin was equally successful in sterilizing footpads after 8 weeks (Ji et

al., 2007) and in fact rifampicin alone was also successful (Ji et al., 2007, Ji et al., 2008). A recent trial in humans showed no difference in clinical outcome when rifampicin plus clarithromycin was substituted for RS in the second 4 weeks of treatment (Nienhuis et al., 2010). In the present study we demonstrated that out of 32 lesions cultured after antibiotic treatment for 6 weeks, 21 (66%) yielded a positive culture for *M. ulcerans* but there was no difference between RS8 and RS2RC6 in this respect. This was surprising after 6 weeks in the light of a previous study in which no positive cultures were obtained when early lesions were excised completely after treatment for 4, 8 or 12 weeks (Etuaful et al., 2005). More patients were included in the present study so the bacterial load at baseline may have been higher. Nevertheless, in the earlier study, the whole lesion was excised and 1 gm portions were minced and processed for culture whereas in the present study biopsies containing only about 120 mg of tissue were cultured so the chances of achieving a positive culture in the earlier study were higher. On the other hand, cultures were performed in Kumasi in this study whereas specimens were transported to Angers in France in the previous study. These results need to be confirmed by other studies.

Only 12 patients were sampled at 12 weeks in the present study but, of these, 3 in each treatment group yielded positive cultures. This means that 6 out of 60 patients from whom a culture sample was taken had a positive result, indicating that at least 10% of patients had a positive culture at this time point which is much higher than expected. All positive cultures were tested for the *IS*2404 repeat sequence of *M. ulcerans* so there was no doubt about their identity. Although all these lesions healed without further antibiotic treatment, it is possible that persistent infection together with mycolactone production slowed the rate of healing since, in low concentration,

mycolactone inhibits the action of some cytokines and chemokines known to act as growth factors in healing (Phillips et al., 2009). These findings raise the possibility that, in patients with a high initial bacterial load, a longer course of antibiotic treatment may be warranted. In countries such as Australia where there is good access to surgery, split skin grafting during or after antibiotic treatment is an alternative approach but this does not apply in many West African countries where Buruli ulcer is endemic. Two patients grafted in this study had poorly healing Buruli lesions that may be explained by persistence of viable organisms and mycolactone in the ulcer.

The incidence and optimal management of paradoxical reactions is not certain at present (O'Brien et al., 2007, O'Brien et al., 2009, Ruf et al., 2011, Nienhuis et al., 2012). Paradoxical reactions have been defined as new inflammatory disease within or close to a Buruli ulcer leading to extension of the existing ulcer or new ulceration, usually with pus formation. However diagnosis is challenging: change in size of ulcers on an uneven surface such as that of a limb is difficult to record and erythema is easily missed on black African skin. By analogy with other diseases such as tuberculosis, these reactions are thought to result from increasing inflammation as a result of enhancement of the immune response during treatment (Breen et al., 2004, Bloch et al., 2009, Wendel et al., 2001, Cheng et al., 2002). In the present study paradoxical reactions were observed in 8% of patients with all forms of Buruli ulcer disease from week 4 during antibiotic treatment up to week 32, 24 weeks after treatment was completed. *Mycobacterium ulcerans* was not cultured from reacting ulcers except in one that occurred at week 4 of antibiotic treatment. No additional antibiotics were administered to any of the patients and paradoxical lesions occurring

after antibiotic treatment were managed successfully by observation, wound care and aspiration of pus. Others gave either a second course of RS8 (Chauty et al., 2007) or corticosteroids (O'brien et al., 2009). Our observations did not suggest that these interventions were necessary since neither treatment had been investigated in a controlled trial. Reactions such as these may account for the perception in the past that antibiotic treatment had not been successful in some patients. For example, surgical intervention was justified on the basis of treatment failure 4 weeks after antibiotic initiation in a number of patients in a study (Kibadi et al., 2009). These large ulcers were not thought by the authors to have developed paradoxical reactions but in such circumstances rapid enlargement after a gradual reduction in size may not have been apparent.

A potential limitation of this study was its open-label design but masking was not possible in this case as one of the treatment arms included streptomycin that is administered by intramuscular route. In addition there was no formal data safety and monitoring board or external monitoring but one external expert (MWJ) was made to independently review all the patient data. We therefore believe the results of this study to be reliable.

The findings from this pilot study indicate that rifampicin combined with clarithromycin can replace rifampicin and streptomycin for the continuation phase after rifampicin streptomycin for 2 weeks without any apparent loss of efficacy. An implication is that a controlled trial of fully oral therapy using rifampicin and clarithromycin for 8 weeks (RC8) is justified. This is supported by evidence from 2 case reports (Gordon et al., 2010, Jenkin et al., 2002) as well as from a series of 30

patients in Benin with small, early lesions (<10cm diameter) all of which healed after RC8 with no recurrences (Chauty et al., 2011). Administration of RC8 would be less painful for children, avoiding the need for injections, and it would be easier to implement in rural West Africa. Neither this study nor our previous ones have shown any evidence that adherence was different among patients receiving oral therapy and those receiving injections of streptomycin (Etuaful et al., 2005).

5.2 Kinetics of mycolactone in human skin during antibiotic therapy for *Mycobacterium ulcerans* disease.

This study established the principle that detection of mycolactone or of cytotoxicity associated with its presence could be used as a diagnostic test for *M. ulcerans* disease. Before antibiotic treatment was started at week 0, mycolactone-associated cytotoxicity was detected in 92% of *IS2404* positive lesions. PCR for *IS2404*, currently regarded as the standard of care for diagnosis, has sensitivity of 95% (Phillips et al., 2005). Like PCR, a cytotoxicity assay would be a challenge to set up for routine use in West African countries and the same applies to mass spectrometry which is probably more specific than cytotoxicity but requires highly sophisticated and expensive equipment. The sensitivity of mass spectrometry was 77% despite a 17-fold difference in concentration estimated using the cytotoxicity assay by comparison with synthetic mycolactone. This difference suggested either that mass spectrometry failed to detect a proportion of the mycolactone present or that some of the cytotoxicity detected was caused by other molecules. The latter seems more likely since mass spectrometry using LC-MRM detected synthetic

mycolactone A/B at concentrations below 10 pg (10ng/ml) in the present studies. Cytotoxicity may be caused by other molecules such as minor cytotoxic congeners co-secreted with the core lactone ring of mycolactone but these are less potent than the complete mycolactone A/B molecule (George et al., 1999). Alternatively, mycolactone breakdown products or other cytotoxic lipids resulting from inflammation or necrosis may be present in *M. ulcerans* infected tissue. These findings indicate that interactions between *M. ulcerans* and the host at the site of infection could generate other bacterial and / or host lipids with associated cytotoxicity that is not completely accounted for by intact mycolactone A/B. It would be interesting to further characterize the nature of these cytotoxic lipids to elucidate their role in the pathogenesis of *M. ulcerans* disease in humans.

On TLC, the simplest method for mycolactone detection, mycolactone migrates at R_f 0.23 in silica gel. Strong bands corresponding with those of synthetic mycolactone A/B were seen at R_f 0.23 in 68% of 31 patients but probably this was not sufficiently specific for routine use. The recently published method for enhancing its sensitivity by boronic acid treatment of TLC plates (Spangenberg et al., 2010) was not consistently reliable. Perhaps modifications in clinical sample collection and preparation for mycolactone detection using the boronate-assisted fluorogenic chemosensor may enhance the sensitivity and specificity as well as the practicality of this approach for use in the field.

Using mass spectrometry the only significant difference between lesion types was a higher concentration of mycolactone in nodules and plaques, early forms of *M. ulcerans* disease, than in ulcers. There was considerable variation in mycolactone

concentration in these lesions (Figures 4.11 and 4.12) which may reflect differences in the duration of disease. For example, if the patient had been infected recently, the lesions would be expected to contain a lower load of bacilli secreting mycolactone. This notion is supported by the finding of a correlation between mycolactone concentration and time to complete healing of early lesions (Figures 4.15b and 4.15c). In the small number of oedematous lesions studied, there was a low concentration of mycolactone by mass spectrometry but high cytotoxicity. This is the least common but most aggressive form of early *M. ulcerans* disease in which oedema spreads rapidly to adjacent areas leading to formation of large ulcers. Before antibiotic treatment was available, extensive excision of skin was required to halt the process. The pathogenesis is unknown but the discrepancy between the concentration of mycolactone by mass spectrometry and the cytotoxicity assay suggests there was a high concentration of cytotoxic molecules other than intact mycolactone A/B in the tissue. This could represent a high turnover of mycolactone or an anomalous host response to *M. ulcerans* infection.

The centripetal gradient of mycolactone concentration from high in the centre to low in the periphery of pre-ulcerative lesions is compatible both with the clinical observation that lesions break down in the centre to form ulcers and with the histopathology of *M. ulcerans* lesions in which unevenly distributed clumps of organisms are surrounded by necrosis in some areas while other areas show acute or chronic inflammation but no visible *M. ulcerans*. However, variations in mycolactone concentration have to be interpreted with caution for a number of reasons such as sampling error and intra-assay variation.

After antibiotic treatment for 6 weeks, mycolactone was detected by both cytotoxicity and mass spectrometry in 7 patients whose culture samples were negative. Although not all patients were sampled at this or the 12-week time point, the findings strongly indicate that a proportion of patients with slowly healing lesions still had viable bacteria in the skin late in the course of treatment. It was expected that biopsies taken after antibiotic treatment for 6 weeks would be culture-negative on the basis of previous findings in patients with early Buruli lesions (nodules, plaques or small ulcers) treated with streptomycin and rifampicin in whom cultures of the excised lesion were negative from 4 weeks onwards (Etuaful et al., 2005). In the mouse footpad model of *M. ulcerans* infection discussed under section 5.4 below, it was also shown that cytotoxicity persisted upon completion of antibiotic therapy despite a decline of bacterial counts to undetectable levels.

A small number of lesions that had not healed 4 weeks after completion of antibiotic therapy were re-biopsied and 4 out of 8 still contained intact mycolactone on mass spectrometry while all 8 tested positive for cytotoxicity. Only 2 cultures were positive at this time point. The half life of mycolactone in human skin is unknown but these results indicate that viable organisms were still present in the skin of at least 2 subjects where the culture was positive and possibly another 6 in whom mass spectrometry or cytotoxicity tests were positive. It is also possible that mycolactone could persist in tissues even after the demise of bacilli. The lesions healed in all 8 patients, albeit slowly, without further antibiotic treatment so the immune response was sufficiently strong to keep *M. ulcerans* under control by that time. The presence of mycolactone or its breakdown products may have interfered

with healing since some of the cytokines and chemokines inhibited by low concentrations of mycolactone also serve as growth factors for wound healing (Barrientos et al., 2008). Importantly, mycolactone has also been shown to be cytotoxic to keratinocytes (Gronberg et al., 2010) which are crucial for wound healing. Recent evidence suggests that mycolactone binds to Wiskott-Aldrich syndrome protein (WASp) in epithelial cells and provokes uncontrolled activation of Arp2/3 thereby disrupting assembly of actin in the cytoplasm and leading to defective cell adhesion (Guenin-Macé and Veyron-Churlet et al., 2013). These findings indicate that persistence of mycolactone within an ulcer bed after completion of antibiotic therapy could delay wound healing. Compounds that inactivate mycolactone might play an adjunctive role in promoting healing of Buruli ulcers.

Detection of viable organisms may be an important tool for determining the optimal duration of antibiotic treatment and the present studies indicate for the first time the potential of mycolactone measurement for this purpose. There is a real possibility that early lesions containing low numbers of viable organisms could be treated with antibiotics for less than the standard 8 weeks and, on the other hand, that ulcers still containing mycolactone at 12 weeks require more prolonged treatment. Table 4.7 shows that mycolactone detection was usually superior to culture although there were some culture-positive, mycolactone-negative cases presumably due to sampling error. However, it would be premature to conclude that a positive cytotoxicity assay or the presence of mycolactone A/B detected by mass spectrometry directly correlates with viable organisms in lesions since it remains possible that mycolactone detected during and after antibiotic therapy was in the cell

wall of dead bacilli or free in tissue. Recently a specific assay for 16S rRNA of *M. ulcerans* has been described (Beissner et al., 2012) and studies are required to compare this with mycolactone detection as a marker of viable bacilli.

In conclusion, measurement of tissue mycolactone is a promising new method of monitoring the clinical response to antibiotic therapy which may lead to new insights into the pathogenesis of *M. ulcerans* disease as well as guiding treatment developments. The diagnostic potential of mycolactone also warrants further refinement.

5.3. Kinetics of systemic interferon gamma and interleukin-5 with antibiotic therapy in humans with *M. ulcerans* disease.

This study showed that the administration of curative antibiotic therapy for *M. ulcerans* disease was associated with a recovery of secretion of gamma interferon and interleukin-5, which indicated a restitution of both Th-1 and Th-2 arms of the cellular immune responses. However, recovery of gamma interferon secretion appeared to have proceeded more vigorously by week 6 compared with IL-5 response (figures 4.19 and 4.20). A previous study by Sarfo et al. in 2009 showed that during antibiotic therapy, IFN-gamma increased without corresponding significant changes in IL-10 concentrations and notably IFN-gamma concentrations were not correlated with IL-10 concentrations. It was subsequently shown that T-cells from patients with *M. ulcerans* disease compared with healthy controls demonstrated impaired ability to secrete Th-1, Th-2 and Th-17 cytokines upon stimulation with PHA, a potent T-cell mitogen (Phillips et al., 2009). Again, the

observation that gamma interferon responses in whole blood assays improved after surgical excision of *M. ulcerans* infected lesions (Yeboah-Manu et al., 2005) suggests that the presence of *M. ulcerans* in skin tissues remotely modulates the systemic immune response to establish chronic infections in humans.

Like *M. ulcerans*, two other human mycobacterial pathogens, namely *M. tuberculosis* and *M. leprae* have adopted strategies in evading the host immune responses to establish latent and subsequently overt disease. In common, these pathogenic mycobacteria are thought to deploy lipids in their cell envelopes as virulence factors at the host-pathogen interface. For instance, the lipoarabinomannans and phenolic glycolipids of *M. tuberculosis*, have been shown *in vitro* to potently impair the production of pro-inflammatory effector cytokines by antigen-presenting cells (Nigou et al., 2002, Reed et al., 2004). Also, the T-cell anergy characteristic of advanced stages of leprosy have been shown to be mediated by phenolic glycolipid and mannose-capped lipoarabinomannan, which preferentially curtails T-cell receptor/CD28-triggered upstream cell signaling events leading to reduced IL-2 secretion and T-cell blastogenesis (Dagur et al., 2012). Mycolactone thus represents a corresponding lipid virulence factor for *M. ulcerans* with potent immunosuppressive properties. In the present study, concentrations of mycolactone in skin tissues were shown to decline significantly during antibiotic therapy at 6 weeks and this observation coincided with the recovery of the secretion of gamma interferon and interleukin-5. This might support the notion that mycolactone interferes with the generation of an adaptive immune response to *M. ulcerans* infection in humans.

The outcomes of mycobacterial infections are determined predominantly by the interactions between the adaptive immune response and the virulence mechanisms of the mycobacteria. It has been demonstrated, for instance, that during the early stages of *M. tuberculosis* infection, a cell-mediated immune response dominated by a Th-1 gamma interferon profile is mounted, but with disease progression, there is a shift in dominance from Th1 to Th2, with associated anergy of cellular responses and development of humoral responses (Dlugovitzky et al., 2000; Boussiotis et al., 2000). For an obligate intracellular pathogen such as *M. tuberculosis*, a Th-2 predominant pattern of adaptive immune response, which is preferably evoked by the host towards extracellular pathogens would not suffice in controlling the infection. However, *M. ulcerans* differs from *M. tuberculosis* in that even though a transient intracellular phase in macrophages has been observed, *M. ulcerans* elaborates mycolactone which kills macrophages and becomes resident predominantly in the extracellular compartment. It is therefore conceivable that the immune response mounted by the host towards *M. ulcerans*, would be directed towards a pathogen that spends part of its time either in the intracellular or extracellular compartments.

Indeed the data from this study showed that both gamma interferon (a representative Th-1 cytokine) and interleukin-5 (a representative Th-2 cytokine) concentrations were significantly higher among patients with ulcerative forms of the disease than those with pre-ulcerative forms of the disease (Figures 4.16 and 4.17) before commencement of antibiotic therapy. There was a wide variation in the concentrations of both gamma interferon and IL-5 production among the lesion forms at baseline which could be reflective of differences in duration of disease at

presentation. A positive correlation between baseline concentrations of gamma interferon and interleukin-5 (Pearson's correlation r=0.67, p<0.001) in supernates after whole blood stimulation with *M. ulcerans* sonicate was observed, an indication that Th-1 and Th-2 cytokine responses were correspondingly related in individual patients. Also, following antibiotic therapy, recovery of both gamma interferon and IL-5 were noted. Taken together, this data supports the notion that the pattern of adaptive immune response to *M. ulcerans* is suppressed by mycolactone and when the secretion of this toxin is attenuated by antibiotic therapy, the profile of T-cell responses that emerges is an admixture of both Th-1 and Th-2 (Phillips et al., 2006). Hence there is no evidence of counteraction of gamma interferon (Th-1) secretion by interleukin-5 (Th-2) in *M. ulcerans* infection on the basis of the evidence from this study.

Evidence from histopathological analysis of skin tissues harvested after completion of antibiotic therapy showed that administration of antibiotics was accompanied by an active inflammatory process at the site of infection which was characterised by massive infiltration of activated T and B cells (Schütte et al., 2007). IL-5 is an important T-cell cytokine that stimulates both activated B and T cell proliferation and differentiation of helper $CD4^+$ T cells into Th2 cells. Thus the resurgence of gamma interferon and IL-5 responses in whole blood assays of patients reflected the reversal of mycolactone-mediated local immunosuppression and restoration of active humoral and cellular immune responses. However in this study, the local immune response during antibiotic therapy was not investigated mainly due to the limits imposed by the number of biopsies that could be obtained

from an individual patient (after obtaining biopsies for culture and mycolactone studies).

Perhaps, the balance between the recovery of the host immune response during antibiotic therapy and the decline in tissue mycolactone concentration is finely tuned to prevent an exuberant inflammatory response in tissues which would appear to worsen the clinical appearance of lesions to constitute what is now referred to as the paradoxical reaction. In the case report of a patient with a plaque shown in figure 4.21, the paradoxical reaction occurred at week 4, and it was accompanied by a profound increase in gamma interferon production and a sharp decline in mycolactone concentration. Concurrently, the dynamics in IL-5 concentrations were not profound at week 6 compared to the baseline value, although a decline was observed. This would indicate that the rate of decline of mycolactone from tissues together with the balance between the Th-1 and Th-2 profile may be important to explore to further our understanding of the pathophysiology of paradoxical reactions. Clearly, the production of anti-inflammatory cytokines such as IL-5, 1L-10 and transforming growth factor beta, in response to *M. tuberculosis* has been shown to down-regulate the immune response to limit tissue injury provoked by uncontrolled production of pro-inflammatory cytokines such as interferon gamma and TNF-alpha (Sharma et al., 2001). Further studies are needed to elucidate the pathophysiology of this phenomenon. In conclusion, this study showed that following curative antibiotic therapy for *M. ulcerans* disease, there is a restitution of Th-1 and Th-2 arms of the adaptive immune response. The clinical implications of these findings for monitoring patients on therapy remain to be elucidated in further studies.

5.4. Microbiological, histological, immunological, and toxin response to antibiotic treatment in the mouse model of *Mycobacterium ulcerans* disease.

The initial clinical response to infection with *M. ulcerans* is similar to the course of infection with *M. marinum* (Connor et al., 1965; Connor et al., 1966; Connor et al., 1976; Ramakrishnan 1997). A nodule develops in both cases but, with *M. ulcerans*, there is an absence of tenderness. The absence of pain and an inflammatory response is attributed to the production of mycolactone inhibiting the immune response (Adusumilli et al., 2005), damaging nerves (Goto et al., 2006; Goto et al., 2008) and/or destroying infiltrating inflammatory cells (Oliveira et al., 2005, Torrado et al., 2007). At which point in the course of infection sufficient mycolactone is present to mediate these effects has not been ascertained in humans but can be investigated in the mouse model. The mouse model is also suitable to monitor the impact of antibiotic treatment on mycolactone production, which is thought to block mycolactone production and to reverse local immunosuppression leading to the restoration of an active inflammatory process (Peduzzi et al., 2007).

In the experiment reported here, mycolactone was detectable 2 to 3 weeks after inoculating approximately 4.6 $\log_{10}$ CFU of *M. ulcerans* into mouse footpads and before detectable swelling of the footpads. The numbers of bacilli at the beginning of a human infection are unknown but presumably considerably less. A sensitive assay that would be appropriate for use in endemic settings should be able to detect infection before the nodular phase or confirm the diagnosis of *M. ulcerans* infection in nodular or plaque lesions before the onset of ulceration. Treatment with the current gold standard of rifampicin and streptomycin appeared to result in

inhibition of mycolactone production. Whether the inhibition blocks the pathway of enzymes encoded by the *M. ulcerans* giant plasmid or has an impact on the plasmid itself has not been assessed here but it could be investigated using samples where there are still cultivable bacilli but negligible mycolactone production. Mechanistically, streptomycin and rifampicin inhibit protein synthesis by different mechanisms but they both result in a decrease in bacterial viability and, directly or indirectly, in a decrease of mycolactone production.

By sampling mouse footpads on a weekly basis we were able to confirm the early phagocytic, intracellular phase of *M. ulcerans* infection followed by a later extracellular phase (Torrado et al., 2007). One week after infection (Fig. 4.24A2) there was moderate infiltration of host cells and bacilli were predominantly intracellular, an observation made by Ruf et al., (2012). The extracellular phase of bacillary growth appeared to coincide with the onset of footpad swelling, beginning about 3 weeks after infection in this experiment. Over the next two weeks, AFBs were increasingly found outside of cells. The shift to an extracellular growth was blocked by treatment with rifampicin and streptomycin, consistent with the notion that mycolactone production and phagocyte destruction are inhibited by antibiotic treatment (Schütte et al.,2008; Ruf et al.,2012; Martins et al.,2012). In the absence of treatment, the location of bacterial clusters also changed from relatively deep in the dermis to sub-epidermal and epidermal zones to the skin surface with the onset of footpad ulceration. In contrast, in mice treated with antibiotics, *M. ulcerans* was found in a few clusters associated with phagocytic cells within the dermis. The appearance of the bacteria became beaded with less intense acid-fast staining (Schütte et al.,2008, Ruf et al.,2012, Martins et al.,2012), comparable to the changes

observed in the morphological index of leprosy bacilli after the onset of treatment. At 3 months after the completion of 8 weeks of daily treatment, pale-staining bacilli were still detectable and associated with host cells in the dermis but remained uncultivable, suggesting that the residual antigenic components may have helped maintain an inflammatory infiltrate. Overall, from literature (Coutanceau et al., 2005, Oliveira et al., 2005, Ruf et al., 2012), we find that early after infection, there is a dynamic picture of infiltrating host cells and bacillary localization within phagocytic cells. When the same time points were examined in mouse footpads on day 7 (this study, Ruf et al., 2012) and day 13 (this study, Oliveira et al., 2005), the histological impressions were strikingly similar.

In this experiment, we observed increases in *M. ulcerans* CFU counts starting at day 3 (3.29 ± 0.33 $\log_{10}$) and continuing to day 27 (6.02 ± 0.13 $\log_{10}$) after infection. The CFU counts remained at approximately 6 $\log_{10}$ for the duration of the experiment through day 55, after which time the footpads started to ulcerate and could not be readily decontaminated for further analysis, given the prolonged culture time for this organism (8-12 weeks). Swelling was not apparent until day 20 in some mice, but by days 24 and 27, it averaged grade 1. Swelling continued to increase during this time, while CFU counts were stable, and peaked only at day 55 at an average of swelling grade 3.75. The fact that mycolactone concentration continued to rise after the plateau in cultivable organisms suggests that there was an equilibrium between living and dying *M. ulcerans* cells with mycolactone being released by both populations. Alternatively, or in addition, mycolactone may be induced by a quorum-sensing mechanism as bacterial numbers reach the plateau stage. Concurrently, or slightly before the manifestation of swelling, mycolactone could be

detected by mass spectrometric analysis at day 13 (1.6 ng/ml of homogenized footpad extract) and 4.18 ng/ml on day 20. In untreated mice the concentration of mycolactone in infected footpads continued to rise throughout the experiment. In mice treated with antibiotics from day 24, mycolactone concentration tailed off after day 35 but it did not become undetectable by MS until day 63. This suggests either that viable organisms persisted for more than 5 weeks after antibiotics were started or that mycolactone could still be detected some time after *M. ulcerans* had been killed. The latter is supported by the observation that no viable *M. ulcerans* were detected in mouse footpads after antibiotic treatment similar to that used here followed by a course of corticosteroid therapy (Martins et al., 2012) but the recently described 16S rRNA assay was not used to determine if viable organisms were still present (Beissner et al., 2012).

Using the cytotoxicity assay, biological activity associated with mycolactone was detectable as early as day 3 and persisted even at day 63 despite antibiotic treatment. Although it is possible that this assay is more sensitive than MS, experiments *in vitro* using synthetic mycolactone as a standard show that MS can detect mycolactone at a concentration of less than 10pg so it is more likely that some cytotoxicity was caused by other lipid molecules generated during the course of *M. ulcerans* infection. Nevertheless this evidence that mycolactone may have persisted in mouse tissue well after antibiotics had started to reduce the burden of infection when footpad swelling was diminishing is important in the context of human infection in which slow healing has been observed in some cases without an obvious reason.

Mycolactone has profound effects on the production of cytokines and chemokines *in vitro* even at sub-cytotoxic concentration and this may well influence the rate of wound healing. In the present experiments for example, TNF-α production by splenocytes increased in response to mycobacterial antigens during *M. ulcerans* infection but this reverted to normal during antibiotic treatment. A similar but delayed and relatively attenuated response was observed for other cytokines such as IFN-γ, IL-5, and IL-17. ConA, a potent mitogen was used to assess the capacity of splenocytes to produce cytokines upon stimulation while the Mtb CFP elicited responses to mycobacterial antigens but is not specific for *M. ulcerans*. The initial rise in the secretion of cytokines coincided with infiltration of host inflammatory cells and the intracellular stage of *M. ulcerans* when mycolactone concentrations were low. Subsequently as mycolactone concentrations started to build up at the site of infection, *M. ulcerans* assumed an extracellular localization and prevented antigen-presenting cells from processing mycobacterial antigens for a systemic immune response (Fraga et al, 2011). Again Hong et al, (2008) showed that mycolactone A/B could be detected in PBMC's of mice infected with *M. ulcerans*. Recently mycolactone was detected in sera of some humans infected with *M. ulcerans* (Sarfo et al., 2011).

The later recovery of systemic cytokine secretion in the light of increasing concentrations of mycolactone locally in the footpads is difficult to explain. In the proximal phases of *M. ulcerans* infection, this initial exposure may be inhibited by increasing bacillary load and the production of mycolactone. Subsequently, the splenocytes could encounter and process mycobacterial antigens released into the circulation or conveyed from the site of infection in the later stages of *M. ulcerans*

infection from degradation of defunct *M. ulcerans* in mice treated with bactericidal antibiotics (Figure 4.25, both columns). Some patients are able to control *M. ulcerans* infections at the early nodular stage without antibiotics and these data may provide some insight into why this might be the case. It would seem that those with robust immune responses, a lower burden of *M. ulcerans* and thus mycolactone secretion initially may be able to contain the *M. ulcerans* infection. Further studies are however required to elucidate the correlations between local and systemic mycolactone kinetics in tandem with immune responses in these two compartments in this model.

The finding that cytotoxicity increased in contra-lateral footpads well away from the site of infection was difficult to explain. Mycolactone molecules were not detected by MS but the possibility that cytotoxic breakdown products of mycolactone, or other molecules, circulated to these footpads cannot be ruled out. Other studies have shown that mycolactone itself was detectable in peripheral blood white cells during mouse infection with *M. ulcerans* (Hong et al., 2008).

Further studies should focus on the determination of cytokines in the milieu of the footpad lesion in mice before and after treatment with the current regimen or a new all oral regimen, such as one replacing streptomycin with clarithromycin, as well as the impact of vaccination on mycolactone production and local immunity after footpad challenge. Refinements in calculating the amount of mycolactone obtained after extraction may be made by spiking different concentrations of synthetic mycolactone into uninfected footpads and determining the linearity of the yield.

5.5. Concluding discussions

The principal objective of this study was to explore and elucidate the role of mycolactone on the pathogenesis of *M. ulcerans* disease. To answer this question techniques for extraction, detection and quantification of mycolactone from tissues were successfully developed and optimized for the first time. This represents an advance in the field of Buruli ulcer research because mycolactone is thought to be central to the pathogenesis of *M. ulcerans* infection and a potential biomarker for diagnosis and monitoring of clinical response to therapy. These methods were used to examine the kinetics of mycolactone in *M. ulcerans*-infected tissue under the influence of antibiotic therapy in humans and mice. In both models of *M. ulcerans* infection, mycolactone declined with curative antibiotic therapy and this was accompanied by reduction in colony forming units from bacteriological cultures with attendant restoration of a cell-mediated immune response and resolution of clinical lesions with healing. The principal conclusions from this study have been schematically represented in figures 5.1 and 5.2 below.

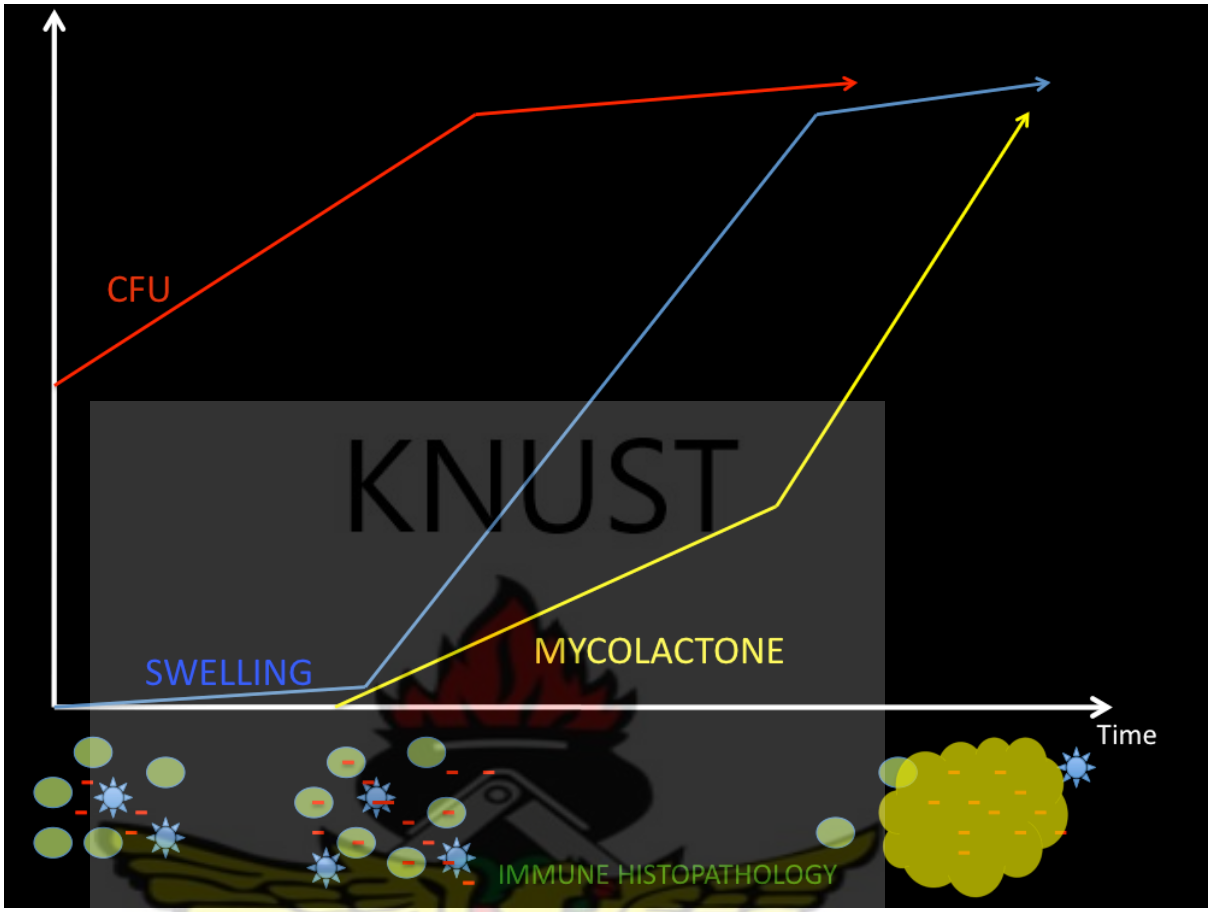


Figure 5.1. Hypothetical scheme of the pathogenesis of *M. ulcerans* disease proposed from murine model. Infection with *M. ulcerans* is followed by proliferation of bacilli in tissues after which there is a plateau. Footpad swelling tracks along with the elaboration of mycolactone and the demise of the immune cells. Bacilli are predominantly extracellular. CFU is colony forming units, green circles and blue stars are immune cells (monocytes, neutrophils, dendritic cells), yellow cloud represents mycolactone and red lines represent *M. ulcerans*.

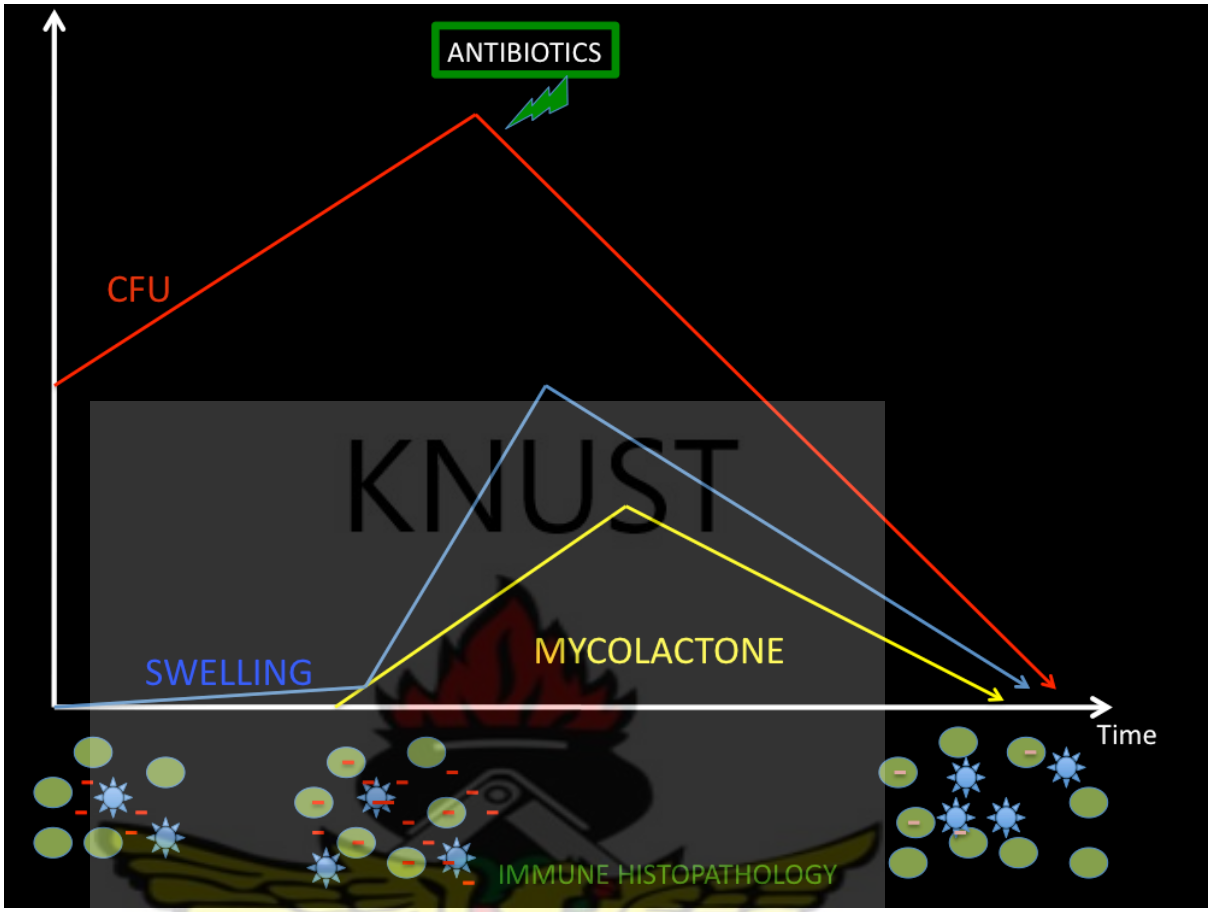


Figure 5.2. Hypothetical scheme of the impact of antibiotic therapy on the pathogenesis of *M. ulcerans* infection. Antibiotic therapy kills bacilli in tissues and is accompanied by decline in mycolactone concentration and regression of footpad swelling. There is restoration of the local immune response with defunct bacilli found inside immune cells. CFU is colony forming units, green circles and blue stars are immune cells (monocytes, neutrophils, dendritic cells), yellow cloud represents mycolactone and red lines represent *M. ulcerans*.

Even though the murine model allowed for more frequent sampling and hence allowed the kinetics of mycolactone, bacteriological, histological and clinical parameters to be closely monitored, one important differentiation of this model from human lesions should be noted. *M. ulcerans* disease in humans is characterised by varied pre-ulcerative forms namely nodules, plaques and oedemas. Particularly, the oedematous forms of *M. ulcerans* disease are not observed in mice. What accounts for these different pre-ulcerative forms of the disease is not clearly known. A major strength of this study is that a large number of pre-ulcerative forms of the disease as well as ulcerative disease forms were studied thus permitting some discussions into plausible reasons for these observations.

In humans, the events that precede the clinical manifestations of *M. ulcerans* disease are not detected; hence has not been studied. Insights from the murine study suggest that this pre-clinical stage is characterised by proliferation of bacilli within immune cell infiltrates and the transient development of a cell-mediated immune response. This occurs because there is no mycolactone secretion in this proximal phase of the infection.

The appearance of footpad swelling (clinical stage) is heralded by the secretion of mycolactone, the demise of inflammatory cells, extracellular localization of bacilli and attenuation of systemic immune responses. This early clinical stages of the disease in mice would correspond to the presentation of nodules and plaques in humans where mycolactone concentrations measured by mass spectrometry was highest in the early nodules and plaques and was accompanied by lower gamma interferon and IL-5 concentrations. Among the patients with oedematous disease,

mycolactone concentration by mass spectrometry was the lowest amongst the pre-ulcerative forms of the disease with higher gamma interferon and IL-5 secretion. Interestingly, cytotoxicity trended higher among oedematous lesions compared with nodules and plaques. It would therefore appear that the oedematous form of the disease is associated with a high turnover mycolactone synthesis and breakdown and perhaps a vigorous systemic immune response to *M. ulcerans*. However, further studies are required to resolve the pathogenesis of Buruli oedemas since only four lesions were examined in the present study.

The late ulcerative stages of the disease in mice were associated with profound swelling and ulceration, and bacilli counts had reached a plateau even though mycolactone secretion continued in earnest and a recovery of the systemic immune responses were observed. In humans, the ulcerative stage was, however, associated with lower mycolactone concentrations, in contrast to that found in mice. An explanation for the disparity between mycolactone concentrations in ulcerative lesions in mice and humans may be due to the fact that, mycolactone was measured in whole footpads of mice whereas in humans they were measured from a single biopsy obtained from the edge of an ulcer. Higher gamma interferon and IL-5 concentrations were found in patients with ulcers compared with patients with pre-ulcerative lesions and is similar to the later recovery of the systemic immune response in mice. These findings would accord with the notion that as Buruli ulcer progresses in humans, mycolactone secretion may decline allowing an immune response to develop and could explain why some lesions heal spontaneously, albeit slowly without any treatment. Antibiotic therapy therefore appears to hasten the

process of recovery by killing bacilli and abrogating mycolactone secretion to permit an immune response to emerge to accelerate healing.

5.6 Recommendations

This study has provided useful proof-of-concept data that mycolactone is detectable in infected tissue in a high proportion of patients with PCR confirmed Buruli ulcer disease. The main methods employed included mass spectrometry and cytotoxicity-based bioassay. Unfortunately, thin layer chromatography, the simplest of the techniques for detection was not specific enough. It remains to be determined whether other novel approaches such as aptamers specific for mycolactone could be used to detect mycolactone with high sensitivity and specificity to be used as a field-friendly, low-cost diagnostic test for Buruli ulcer disease. Further studies are needed to develop simpler methods for mycolactone detection.

Further studies are required to ascertain the association between mycolactone concentration in infected tissues and the local immune response as well as the systemic immune response. This will be an important study to undertake to understand how the toxin influences these two levels of immune response to *M. ulcerans* because it is a necessary requirement for vaccine immunology. The main challenge with mycolactone detection within tissues currently is that, punch biopsies were used in the present studies to undertake lipid extractions for mycolactone assays. It would be ideal to optimize a minimally invasive approach such as fine needle aspiration techniques for mycolactone detection.

In spite of the excellent clinical efficacy of the combination rifampicin and streptomycin for the treatment of all forms and categories of Buruli ulcers, this treatment is beset with the challenge of the discomfort of daily painful streptomycin injections for 8 weeks. This has prompted a desire in the Buruli ulcer community for a replacement of streptomycin with an oral alternative. The findings from this study showed that treating patients with 2 weeks of streptomycin-rifampicin followed by 6 weeks of clarithromycin-rifampicin produced comparable clinical and bacteriological efficacy to 8 weeks of streptomycin-rifampicin. It is important to note that this trial was not powered to demonstrate superiority, equivalence nor non-inferiority, hence it can not at this stage be used for making recommendations for a change in the protocol of Buruli ulcer management. However, it does provide reasonable scientific evidence that a randomized controlled trial of 8 weeks oral clarithromycin-rifampicin combination compared with streptomycin-rifampicin for 8 weeks is justified.

Finally, the question of how long should antibiotic for treating Buruli ulcer disease be administered requires some consideration. Currently, 8 weeks of treatment is recommended for all categories (sizes) and forms of the disease. As this and other studies have shown, the time to complete healing of lesions has varied considerably from as early as two weeks for small lesions to over 52 weeks for some large lesions which sometimes require adjuvant surgical intervention. This study shows that in human lesions, cultures are occasionally positive even up to 4 weeks after completing antibiotic therapy, a finding that needs confirmation in other studies

involving slow healing lesions. Specifically, studies are urgently needed to understand what factors account for delayed healing of lesions with antibiotic therapy in order to optimize this modality of treatment.

5.7 Limitations

1. Mycolactone concentration measured in skin biopsies obtained from human samples may not be representative of mycolactone present in the entire lesion.

2. The local immune response in human subjects was not studied in the present studies.

3. A small number of patients with oedematous form of *M. ulcerans* disease were studied, hence findings from this sub-group of patients are preliminary.

4. The pilot study on the efficacy of RS8 versus RS2RC6 was not sufficiently powered to provide evidence to change clinical practise.

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