KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOOLOGY

COLLEGE OF HEALTH SCIENCES

SCHOOL OF MEDICAL SCIENCES

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CELLULAR IMMUNE RESPONSE TO *MYCOBACTERIUM ULCERANS* INFECTION

A THESIS SUBMITTED IN FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

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In the

Department of Molecular Medicine

by

MICHAEL FRIMPONG

DECLARATION

The experimental work described in this thesis was carried out by me at the Department of Molecular Medicine, KNUST. This work has not been submitted for any other degree.

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DEDICATION

This work is dedicated to my parents Mr. Martin Kwadwo Frimpong and Madam Mary Adwoa Adusa-Poku, and my lovely wife Mrs. Rita Frimpong for their guidance and support. I will forever be grateful to you.

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ABSTRACT

Background: *M. ulcerans* infection (Buruli ulcer) is a disease of the skin and soft tissue endemic in sub-Sahara Africa, with the major burden in West Africa. It belongs to a large group of environmental mycobacteria. Generally, protection against mycobacterial infection is thought to be based on cell-mediated immunity, specifically Th-1 type cellular immune responses are essential for control of mycobacterial infections, while humoral response have little benefit. The reasons why only some individuals in endemic areas who are exposed to *M. ulcerans* develop lesions are not known but are likely to reflect individual differences in the immune response to infections with this mycobacterium.

Aim of study: These study aims at describing the cellular immune response to *Mycobacterium ulcerans* infection (Buruli ulcer) associated with protection and determine the efficacy of two subunit proteins (MUL 4978 and MUL 3720) as potential candidates for vaccine against Buruli ulcer. And finally investigate the effect of co-infection with *M. perstans* on BU susceptibility.

Methods: All clinically suspected cases of Buruli ulcer were confirmed by standard PCR and ZN microscopy. Interferon gamma (IFN-γ) secretion following stimulation with mycobacterial antigens of peripheral blood mononuclear cells (PBMC) and whole blood from Buruli ulcer confirmed patients and household contacts were investigated using IFN-γ ELISA. The effectiveness of two subunit protein vaccines (MUL 3720 and MUL 4978) as potential vaccine candidates was tested using the method mention above. Also CD4+, CD8+ T-cell profiles and CD19 + cell populations in patients with Buruli ulcer were determined by flow cytometry analysis. A case control of 66 *M. ulcerans (Mu)* disease patients and 30 household contacts were investigated for *Mansonella perstans (Mp)* co-infection. Patients confirmed to have *Mu* disease

by PCR were given the WHO recommended combination of rifampicin 10mg/kg and streptomycin 15mg/kg for 8 weeks. Ivermectin 150ug/kg and doxycycline 200mg were administered for 6 weeks as treatment of *Mp* infection when present.

Results: The results showed that following stimulation with *M. ulcerans* antigens, PBMC from Buruli ulcer patients and their household contacts mounted high IFN-γ response. Also the Buruli ulcer patients with ulcerative lesions produced more IFN-γ than those with pre-ulcerative lesions ($p = 0.026$). IFN-γ secretion increased with treatment, with significant difference ($p = 0.0078$) at 6 weeks compared to baseline (pre-treatment), corresponding to a decrease in patients' lesion sizes. Patients with severe forms of Buruli lesions (Category II and III) had a significantly decreased CD4+ T-cell population compared with healthy contacts ($p = 0.0395$). There were no statistically significant differences in the populations of CD8+ T-cell and B cell (CD3-CD19+) populations. There were high IFN-γ responses to subunit protein vaccines and IFN-γ levels in both candidates vaccine antigens (MUL 3720 and MUL 4978) were significantly high after 6 weeks of treatment compared to before treatment ($p = 0.03$ and 0.005) respectively. Fifteen out of 66 (23%) patients with *Mu* disease were co-infected with *Mp* while 4 out of 30 (13%) of the household contacts had infection with *Mp*. While filarial infection was more common among Buruli ulcer patients than household contacts it did not influence healing time of Buruli lesions ($p = 0.93$) or predispose patients to more severe forms of the disease.

Conclusions: These findings suggest that T helper cell-1 (Th-1) immune response to *M. ulcerans* may play a protective role in the control of the disease. Also patients with severe forms of Buruli ulcer had depleted CD4+ T-lymphocyte populations. Furthermore, the results indicate that subunit protein vaccines *MUL 3720* and *MUL 4978* are immunogenic in human ex-vivo assays. This study also provided clear evidence of *M. perstans* co-infection in Buruli ulcer patients.

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LIST OF ABBREVIATIONS

AFB	acid fast bacilli
Ag	antigen
ANOVA	analysis of variance
APC	antigen presenting cells
APC	allophycocyanin
BCG	Bacille Calmette Guerin
BU	Buruli ulcer
CD	cluster of differentiation
C.I	confidence interval
CLS	cell lysis solution
DC	dendritic cells
DNA	deoxyribonucleic acid
DLN	draining lymph node
DRB	dry reagent based
DTH	delayed type hypersensitivity
ESAT	early secretory antigenic target
FACS	fluorescent activated cell sorting

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FITC	fluorescein isothiocyanate
FNA	fine needle aspiration
IFN	interferon
Ig	immunoglobulin
IL	interleukin
Io	ionomycin
IS	insertion sequence
Mp	*Mansonella perstans*
Mu	*Mycobacterium ulcerans*
MUCF	*Mycobacterium ulcerans* culture filtrate
NK	natural killer
PANTA	Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Axlocilin
PBMC	peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PMA	phorbol myristate acetate
PPD	purified protein derivate
PPS	protein precipitate solution
RT	room temperature

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TB	tuberculosis
TH	T helper
W H O	World Health Organization
ZN	Ziehl Neelsen

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CHAPTER 1

INTRODUCTION

1.0 Background

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Mycobacterium ulcerans (*M. ulcerans*) as the causative agent for Buruli ulcer was first reported in 1948 by MacCallum and colleague in Australian patients who each had a single ulcerative lesion on an arm or leg (MacCallum and Tolhurst, 1948). These ulcers had undermined edges, and one patient had a positive tuberculin skin-test result, although no evidence of tuberculosis was found. Acid-fast bacilli were seen in histological specimen and when the *Mycobacterium* sp. present in the subcutaneous lesions of these patients was inoculated into animals, progressive ulceration was seen. They provisionally named this mycobacterium the Bairnsdale bacillus, after the region where five of the six patients lived. It was subsequently renamed *Mycobacterium ulcerans*. However, there is evidence that the disease had been described in patients from Uganda as far back as at 1897 by Sir Albert Cook (Ward, 1970).

The first international conference on Buruli ulcer control and research was held during July 6-8, 1998, in Yamoussoukro, Cote d'Ivoire. The disease was declared by the World Health Organization in 1998, as an emerging skin disease of public health concern (World Health Organization. Office of Health Communications and Public Relations., 1997). The Yamoussoukro declaration on Buruli ulcer expressed concern that little was known about the disease, and called on the international community to support control and research efforts.

Buruli ulcer (BU) disease as at the time was the third most often reported mycobacterium infection in the world after tuberculosis and leprosy (van der Werf et al., 1999). The disease has been reported in at least 30, mostly tropical countries (Johnson et al., 2005). The major burden is in West Africa (Amofah et al., 2002) where the clinical lesion starts as a painless subcutaneous nodule, plaque, or edema that subsequently ulcerates with characteristic undermined edges. Bones can also be involved (van der Werf et al., 1999).

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Mycobacterium ulcerans belong to the large group of environmental mycobacteria. It is a slowly growing acid-fast and alcohol-fast microorganism that is best cultured in egg-yolk-enriched Lowenstein-Jensen medium at a temperature between 31-33^{o}C (pH 5.4-7.4, pO_2 <2.5 kPa) (Palomino and Portaels, 1998). DNA sequences specific for *M. ulcerans* have been amplified by polymerase chain reaction (PCR) targeting two different sequences to detect *M. ulcerans* in clinical and environmental samples, namely IS*2404* and IS*2606* (Stinear et al., 1999).

M. ulcerans produces a polyketide-like toxin, mycolactone, which is responsible for the extracellular phase of the infection (Coutanceau et al., 2005) and for the deep necrotizing skin lesions typical for the advanced stages of the disease (George et al., 1999). Endemic zones are located in rural areas in tropical and sub-tropical regions where BU occurs in cluster villages near swamps and slow flowing water sites. The reservoir of *M. ulcerans* is unknown, however there are compeling evidence to suggest that it is environmental (Sizaire et al., 2006; WHO, 2003; van der Werf et al., 2005). The frequency of BU varies widely from village to village, even within the same district (Johnson et al., 2005) which presumably reflects differences in environmental exposure to *M. ulcerans*. Studies of risk factors for disease must take into account

this variation in exposure. Prevention of BU is complicated by the fact that while most infected people live near lakes, rivers and swamps, where the bacteria are commonly seen in disease endemic areas (Fyfe et al., 2010), the route of transmission is largely unknown. Person-to-person transmission is very rare (Debacker et al., 2004). Until the transmission route is fully understood or an effective vaccine is developed, it will be difficult to prevent BU. For this reason, the fight against BU is primarily by spreading information through outreach programs for early case detection and treating patients. The bacteria mostly infect the limbs where the skin is colder than the trunks, as the optimum temperature for *M. ulcerans* is 30-33^{0}C. Once the infection is established, the disease appears as a painless nodule, plaque or edema in the skin which can be treated with antibiotics (Einarsdottir and Huygen, 2011). Since BU mostly affects rural populations with limited geographic, financial, and cultural access to health services, patients usually present late with severe clinical forms of the disease, including extensive skin destruction, multiple lesions, secondary infection and/or bone involvement. Even with adequate treatment, permanent disabilities can remain.

The two mycobacteria of clinical interest which are best understood in terms of their immune response are *Mycobacteria tuberculosis* and *Mycobacteria leprae*, the causative agents of tuberculosis (TB) and leprosy respectively, are intracellular pathogens. TH1-type cellular immune responses are essential for control of both infections, while humoral response have little benefit and may even be detrimental to the host (Demkow et al., 2005, Touw et al., 1982). Even though the immune response to *M. ulcerans* is not fully understood, TH 1-type cellular immune responses appear to be important for control of *M. ulcerans* (Phillips et al., 2006).

Additional studies to identify risk factors for infection and disease are needed in the absence of effective vaccine or antitoxin. However, prevention of infection and disease through immunization of populations at high risk will ultimately be the best strategy for controlling this emerging infection.

1.1 Rationale for study

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Buruli ulcer is the third most common mycobacteriosis of humans following tuberculosis and leprosy (van der Werf et al., 1999). The emergence of the disease in West African countries over the past decade has been dramatic. The disease is more severe in poor inhabitants of remote rural areas with limited access to health facilities. Mortality due to the disease is low, but morbidity is high. Complications include contracture deformities and amputation of limbs and even blindness due to extensive scarring. The current economic and social burden imposed by the BU is enormous. In Ghana, the average cost of treatment per patient is estimated to be one-third of affected household income (Grietens et al., 2008). A more effective *M. ulcerans* vaccine would help to control this debilitating disease that affects mostly children. Data available shows that about 70% of those affected are children under the age of 15 years (Walsh et al., 2011).

Control strategies against Buruli ulcer disease are limited because there is poor understanding of the exact mode of transmission and the absence of a specific vaccine. Hence it is important to understand the immune response to *M. ulcerans* associated with protection, in order to develop effective vaccine candidates. It has been shown that peripheral blood mononuclear cells (PBMC)

from Australian subjects with current *M. ulcerans* disease produce less interferon gamma (IFN-γ) after stimulation with live *M. ulcerans* or live *Mycobacteria bovis* than PBMC from healthy tuberculin-positive individuals. This suggests T-cell anergy to mycobacterial antigens (Gooding et al., 2001). Using reverse transcription and PCR, they showed that, after stimulation with live *M. ulcerans* or *M. bovis*, PBMC of Buruli ulcer patients expressed mainly the TH-2 cytokines, interleukin-4 (IL-4), IL-5, IL-6 and IL-10, whereas unaffected contacts responded with TH-1 cytokines IFN-γ and IL-12 (Gooding et al., 2002). In a similar study conducted in French Guyana, patients with nodular forms of the disease had predominantly TH-1 cytokine profile, while those with ulcerative forms of the disease had TH-2 cytokine profile (Prevot et al., 2004). More recently, Phillips et al. showed that in early stages of *M. ulcerans* disease there was a mixed TH-1 and TH-2 cytokine response, but the TH-1 emerged the dominant type when they stimulated whole blood with culture filtrate antigens (Phillips et al., 2006). Several other reports indicate TH-1 response via IFN-γ play an important role in the protective immune response against mycobacterial infections (Gooding et al., 2002, Gooding et al., 2003, Phillips et al., 2006, Prevot et al., 2004, Westenbrink et al., 2005).

Residents of regions where BU is endemic are frequently exposed to a variety of infections including parasites. In Ghana, lymphatic filariasis due to *Wuchereria bancrofti* co-exists in several regions highly endemic for BU such as the Upper Denkyira District in the Central region of Ghana but its prevalence is unknown (Hoerauf et al., 2008). The presence of filarial worms in the blood of patients with BU has never been reported. This it raises questions regarding the impact of human filariasis on the immunological polarization and clinical presentation of *M. ulcerans* disease as well as opening up a new avenue for investigation of the transmission of *M.*

ulcerans to humans. This study will clarify further the immune response to *M. ulcerans* associated with protection and severe disease for BU patients since the immune mechanisms associated with BU is largely unknown.

1.2 Study Hypothesis

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We hypothesize that patients with BU are capable of mounting an appropriate TH-1 type immune response demonstrable by interferon gamma production to control *M. ulcerans* infection, supporting evidence of potential spontaneous healing earlier reported. We also hypothesize that vaccination against *M. ulcerans* infection is possible.

1.3 Research questions

This thesis addresses the following research questions.

1. Can *M. ulcerans* infected patients mount an appropriate immune response capable of controlling the infection?
2. Is there alteration in the immune cell populations of Buruli ulcer patients contributing to observed immune responses and susceptibility to *M. ulcerans* infection?
3. Will the subunit protein vaccine candidates that have shown great promise in mouse models, have similar effect in human in vitro assays?

4. What is the effect of co-morbidity with helminthic nematodes on the immune response to *M. ulcerans* infection?

1.4 Main objective

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To describe the host immune response to *M. ulcerans* infection associated with protection and severity of disease.

1.4.1 Specific objectives

1. Laboratory confirm all suspected cases BU by standard Polymerase Chain Reaction and to assess the usefulness of Ziehl-Neelsen staining technique and microscopy as a diagnostic tool.
2. Describe the immune status and T cell cytokine expression pattern of BUD patients under therapy and healthy contacts.
3. To investigate the changes in the lymphocyte subpopulations in Buruli ulcer patients.
4. To evaluate the immunological reactivity of subunit protein vaccines with BUD patients' lymphocytes
5. To evaluate the effect of co-infection with *Mansonella perstans* on the host immune response

CHAPTER 2

LITERATURE REVIEW

2.1 Epidemiology

2.1.1 Geographical distribution

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Buruli ulcer is endemic in rural wetlands of tropical countries of Africa, America, Asia and Australia. Cases have been reported in about thirty-three (33) countries around the world (see Plate 1 and Table 1) (World Health Organization., 2013).

Table 1: Regions and countries with reported cases of Buruli ulcer disease

Region	Countries
West Africa	Benin, Burkina Faso, Cameroon, Côte d'Ivoire, Ghana, Guinea, Liberia, Nigeria, Sierra Leone, Togo
Other Parts of Africa	Angola, Congo, Democratic Republic of Congo, Gabon, Sudan, Uganda
Western Pacific	Australia, Papua New Guinea
Asia	China, India, Indonesia, Japan, Malaysia
Americas	Bolivia, French Guyana, Mexico, Peru, Suriname

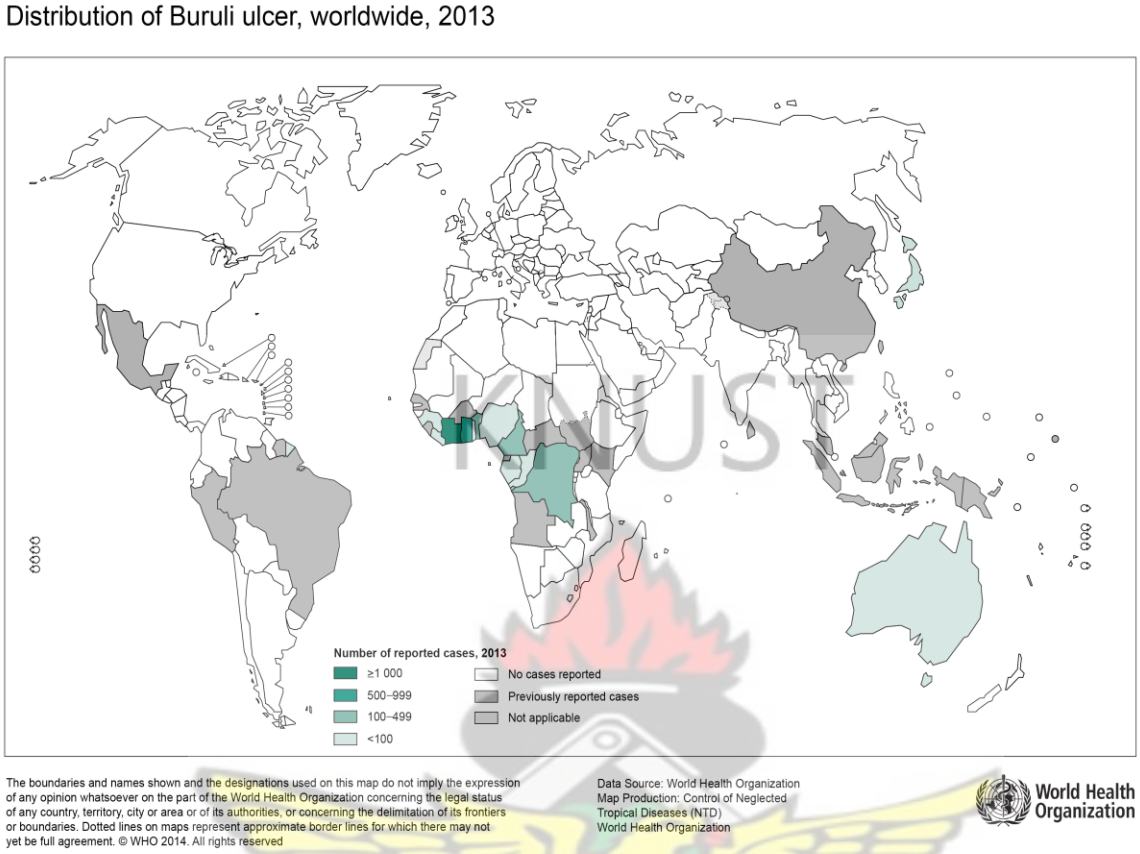

Plate 1: Countries reporting Buruli ulcer (World Health Organization., 2013).

2.1.2 Incidence and Prevalence

The global burden of Buruli ulcer (BU) is not clear; this is due to the lack of efficient reporting system in most endemic countries (Johnson et al., 2005). Nevertheless, it is now known that BU is endemic in at least thirty-three tropical countries of Africa, Western Pacific, Asia, the Indian Ocean and Latin America (World Health Organization., 2010) (Plate 1). The worst affected region is within countries lying along the Gulf of Guinea in West Africa, where BU has replaced leprosy as the second most common mycobacterial disease, after tuberculosis. Cases have been

detected in all the countries with Ghana, Ivory-Coast, Togo, Cameroon and Benin recording the highest number of cases (Fukunishi, 1999, Kanga and Kacou, 2001, Meyers et al., 1996). The prevalence of BU in some of the villages in this area is higher than that of tuberculosis and can affect more than 20% of the inhabitants. In Ivory-Coast, more than 15,000 (Kanga and Kacou, 2001) cases were reported between 1978 and 1999 while nearly 2,000 cases were reported within a 4-year period in one hospital in Benin (Debacker et al., 2005). Very few cases have been reported in non-endemic areas in Europe and North America (Ezzedine et al., 2009, Ezzedine et al., 2010, McGann et al., 2009). Although, BU affects all age groups in both sexes, it has been reported to affect mainly children 15 years of age and below in Africa (Vincent et al., 2014).

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2.1.3 Mode of Transmission

The exact mode(s) of transmission from environment and the ultimate natural reservoir(s) of infection remain unknown. There are suggestions that humans probably become infected by traumatic introduction of *M. ulcerans* into the skin from overlying *M. ulcerans*-contaminated surface (Huygen et al., 2009). Some studies suggest that aquatic invertebrates serve as reservoirs for *M. ulcerans*, although transmission pathways remain unknown (Williamson et al., 2012, Williamson et al., 2008). Work with aquatic insects showed that *M. ulcerans* organisms inoculated through the skin can establish infection in mice (Marsollier et al., 2002) but there have been recent arguments against the hypothesis that humans may be infected in a similar manner (Benbow et al., 2008). In Southeast Australia, evidence has been found linking infected mosquitoes, *Aedes camptorhynchus* with human cases using PCR (Johnson et al., 2007, Lavender et al., 2011) but there is currently no proof that their bites can transmit the infection.

2.1.4 Risk factors associated with *Mycobacterium ulcerans* infection

Jacobson and Padgett systematically reviewed the risk factors associated with *M. ulcerans* infection around the world and indicated that poor wound care, failure to wear protective clothing and living or working near riverine areas were commonly identified factors in most studies (Jacobsen and Padgett, 2010). A number of epidemiological studies have identified other potential risk factors such as arsenic-enriched drinking water in mining communities (Duker et al., 2004), living near cocoa plantation or woods (Pouillot et al., 2007) and even exposure to mosquitoes (Quek et al., 2007). However, some immunological studies have shown that, household contacts of BU patients in endemic areas have antibodies to *M. ulcerans*, an indication to exposure (Diaz et al., 2006, Gooding et al., 2002). Therefore host susceptibility factors need to be explored to understand factors explaining the development of BUD once an individual has been infected with *M. ulcerans*. These factors could be genetically or environmentally determined. One of such environmental factors could be helminthic co-infection, which could drive the host immune response towards a predominantly TH-2 pattern, away from TH-1 preponderant protection against mycobacterial infection (Stienstra et al., 2001).

2.2 Buruli ulcer disease in Ghana

In Ghana, the first documented case of Buruli ulcer was a patient from Amasaman at Korle-Bu Teaching Hospital in 1971 (Bayley, 1971). In 1989, van der Werf et al, published a series of 96 cases in the Afram valley at Agogo in the Ashanti Akim North district of Ashanti Region (van

der Werf et al., 1989). Amofa et al, described a major endemic focus in the Amansie west District in the Ashanti Region (Amofah et al., 1993).

The National Buruli Ulcer Control Programme (NBUCP) has since its establishment in 2000 been challenged to create awareness of the disease among both the medical and general community for early detection and prompt treatment. With an initial 6 endemic districts; (Amansie West, Asante Akim North, Ga West, Akwapim South, Upper Denkyira and Suhum Kraboa Coaltar), over the years the number of endemic districts have increased significantly to over 30 (shown in plate 2). In 1999, a national survey reported over 6000 cases from 90 out of the then 110 districts. Since 2005, 35 districts from 6 regions (Ashanti, Brong Ahafo, Central, Eastern, Greater Accra and Western) have consistently reported a national average of about 1000 cases annually (NBUCP report, 2008).

2.2.1 National Surveillance

In 2007, the NBUCP stopped collection of district summary cases and begun data collection on a line list for patients. This accorded the programme the opportunity to map communities reporting and as well monitor and follow up on patients. The line list routinely collects data on patient using the BU02. Non-standardized forms.

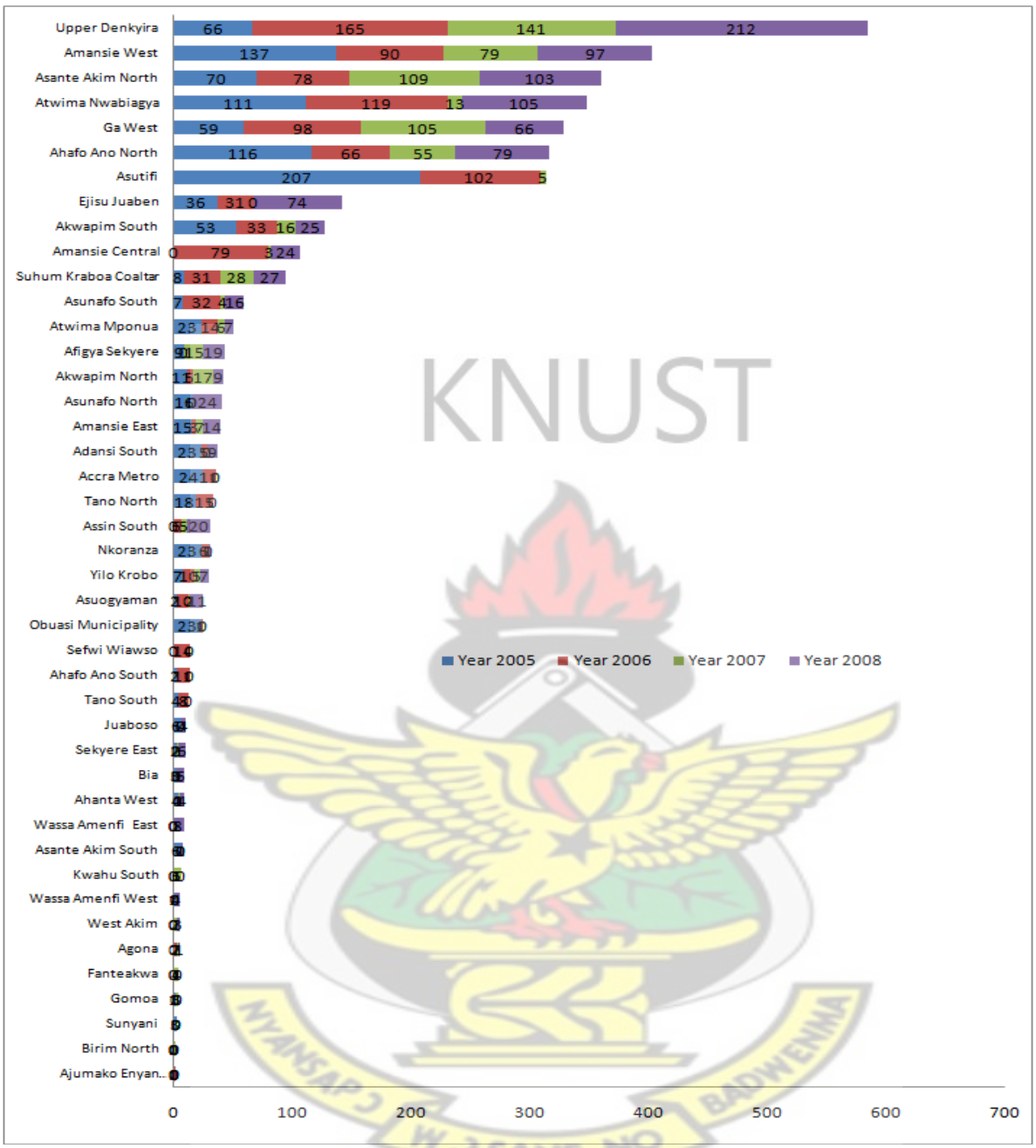

Plate 2: District trend of reported new cases of Buruli ulcer 2005-2008 (Source: NBUCP Report, 2008)

2.3 Clinical Manifestation of Buruli ulcer

Mycobacterium ulcerans may enter the skin by traumatic inoculation and biting insects may be involved (Meyers et al., 1974). After the organism has successfully entered the host, it confines itself to the subcutaneous tissue and the overlaying skin, where it multiples. A nodule which extend from the skin into subcutaneous tissue usually indicates the first stage of disease, but pre-ulcerative lesions also include plaques, edema and in some geographical areas papule (van der Werf et al., 1999). After few weeks, the nodule gradually enlarges and erodes through the skin surface, leaving a well demarcated ulcer with necrotic slough in the base and undermined edges (Portaels et al., 2009). Analysis by Meyers and colleagues of large numbers of cases suggested that in some cases, infections spread rapidly, bypassing the localized nodular-ulcerative stage. This results in edemas that if untreated, leads to large ulcers (Abalos et al., 2000). Another presentation of *M. ulcerans* infection, although rare is osteomyelitis (Portaels et al., 2009).

2.3.1 Clinical Forms of BUD according to WHO definitions

I. **Papule**: This is defined as a painless, raised skin lesion, less than 1 cm in diameter. The surrounding skin is reddened. This form is commonly seen in Australia.

II. **Nodule**: Is a lesion that extends from the skin into the subcutaneous tissue. It is 1-2 cm in diameter. It is usually painless but may be itchy and the surrounding skin may be discoloured compared to adjacent areas (Plate 3A). This form of disease is commonly seen in Africa.

III. **Plaque**: This is a firm, painless, elevated, well-demarcated lesion more than 2cm in diameter with irregular edges. The skin over the lesion is often reddened or otherwise discoloured (Plate 3C).

IV. **Edema**: There is a diffuse, extensive swelling. The affected area has ill-defined margins, is firm and painless and involves part or all of the part of the body. There may be colour changes over the affected region (Plate 3B)

V. **Ulcer**

Ulcers usually develop from the non-ulcerative forms. When fully developed, the ulcer has undermined edges and is indurated peripherally. The floor of the ulcer may have a white cotton wool-like appearance from the necrotic slough (Plate 3D).

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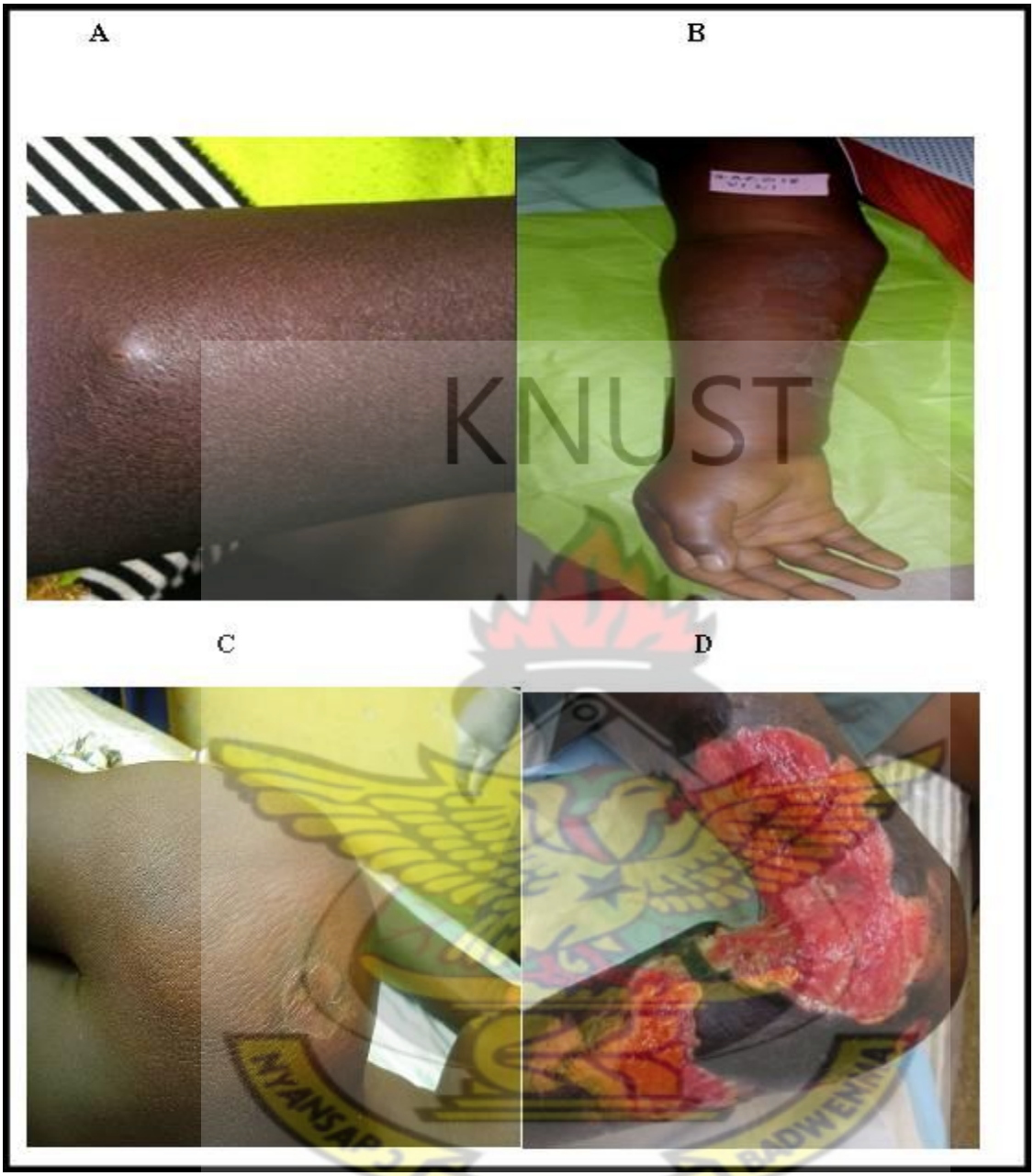

Plate 3: Clinical forms of Buruli ulcer disease (A: Nodule B: Edema C: Plaque D: Extensive ulcer).

2.4 Laboratory Diagnosis

Clinical suspicion of Buruli ulcer disease needs to be laboratory confirmed for the following reasons as stated by Huygen and colleagues (Huygen et al., 2009).

1. To confirm the precise prevalence and incidence of BU in a given area;
2. To confirm new foci, especially where health care workers lack experience with BU;
3. To help manage the disease by surgical and/or antimycobacterial treatment;
4. To confirm and differentiate relapse and reinfection after treatment.

Currently there are four major laboratory tests available to confirm the diagnosis of BU (Bretzel et al., 2007). Direct smear examination for acid-fast bacilli by Ziehl-Neelsen or auramine stain, in vitro culture, IS*2404* Polymerase chain reaction technique (PCR) and histopathological examination. PCR due to its high sensitivity and specificity is regarded as the gold standard.

2.5 Treatment of Buruli ulcer disease

Surgical excision with/without skin grafting was for a long time the recommended therapy for BU. Recurrence rates after surgery vary between 6 and 28% (Debacker et al., 2005, Huygen et al., 2009). Since 2005, the use of combination antimycobacterial therapy consisting of oral rifampicin 10mg/kg and intramuscular streptomycin 15mg/kg daily for 8 weeks (RS8) has been recommended by the WHO, (World Health Organization., 2012). Several centers in Africa have followed the recommendation of WHO and initiated therapy with antibiotics according to the WHO guidelines (World Health Organization., 2012). A study showed that following drug

therapy for 8 weeks, 73% who successfully completed antibiotic treatment had healed lesions without surgery. A recurrence rate within the year following the end of drug therapy was 1.44% (Chauty et al., 2007). However, because the disease is most endemic among rural poor with restricted access to health services and due to the painless nature of the lesions, patients often seek treatment late. Thus, treatment is usually delayed, causing frequent and severe complications, leading to prolonged and expensive hospitalization. Wide surgical excision of infected tissue is no longer necessary to achieve microbiological cure: antibiotics have shown to be fully effective (World Health Organization., 2012); however, in some cases debridement and skin grafting may be needed to aid healing and minimize scarring that might limit movement.

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2.6 Pathogenesis and Immunity

Molecular studies suggest a very close genetic relationship between *Mycobacterium ulcerans* and *Mycobacterium marinum* (Stinear et al., 2000), an intracellular pathogen that triggers inflammatory responses and cell-mediated immunity (CMI) (Mor et al., 1981). However, these organisms are phenotypically distinct and cause diseases with different pathologies. *M. ulcerans* produces a family of toxic macrolides identified as mycolactone, which are required for virulence (Demangel et al., 2009, George et al., 1999).

Mycolactone when secreted, diffuse into infected tissues and surrounding areas, but the amount and precise distribution of the toxin in the lesions are not known. This toxin is known to have cytotoxic activity that induces apoptosis and necrosis of several cell types (Adusumilli et al., 2005, Hong et al., 2008, Oliveira et al., 2005).

2.6.1 *Mycobacterium ulcerans* lifestyle in the host

Immunological and molecular tools have given us more insight into the mechanisms of *M. ulcerans* infection, the progression to BU and the immune responses involved. However, a key question with respect to the development of an effective vaccine remains to be answered: is *M. ulcerans* an intracellular or extracellular parasite? If intracellular, antibodies are likely to play a very little role in protection and vaccination should focus on the stimulation of cellular immune responses, particularly those conferred by the TH 1 type T cell population (Huygen et al., 2009). If the latter, a vaccine aimed at stimulating specific antibody production would be a more obvious option. Perhaps most probable possibility is that both arms of immune response are required for optimal protection.

M. tuberculosis and *M. marinum* which are closely related to *M. ulcerans* are labeled as intracellular parasites based on the following characteristics; they grow in vitro (Ramakrishnan and Falkow, 1994) and in vivo (Cosma et al., 2003, Russell, 2001) within macrophages and have genes to promote their entry, survival and multiplication within host cell (El-Etr et al., 2004). They also elicit CMI, DTH response and a granulomatous tissue reaction (Cooper and Flynn, 1995, Flynn and Chan, 2001).

Mycolactone produced by *M. ulcerans* inhibits phagocytosis by macrophages in vitro and this is assumed to interfere with the in vivo phagocytosis of *M. ulcerans*, promoting extracellular localization of bacilli (Coutanceau et al., 2005, Pimsler et al., 1988). However, it has been observed that this inhibition only happen when high concentrations of toxin are present

(Adusumilli et al., 2005), and the actual concentration of the toxin in infection sites is still unknown. Torrado et al. showed that Mycolactone producing *M. ulcerans* strains are phagocytosed in vivo by macrophages at similar rates as *M. tuberculosis* and *M. bovis* BCG when a low multiplicity of infection was used (Torrado et al., 2007). The presence of intraphagocytic bacilli in active untreated human and experimental *M. ulcerans* infections suggest that even if some inhibition of phagocytosis is induced, substantial in vivo uptake of the pathogen by macrophages does happen. The interpretation that the causes of *M. ulcerans* has the essential hallmark of intracellular pathogens like other pathogenic mycobacteria is supported by the observation that *M. ulcerans* is phagocytosed in vitro by macrophages and has the ability to grow within these phagocytes (Torrado et al., 2007).

Extracellular multiplication in vivo happens during specific phases of the infection processes of several intracellular parasites (Casadevall and Pirofski, 1999). The presence of extracellular bacilli in large clumps in necrotic areas suggests that extracellular multiplication of *M. ulcerans* would take place in active, advance disease (Mac et al., 1948, Fenner, 1956). However, the contribution of extracellular multiplication to the pathogenesis of infection with *M. ulcerans* in necrotic areas, including production of mycolactone has not been fully evaluated. Extracellular *M. ulcerans* might be of relevance in the context of antibody mediated immunity, since several studies reported *M. ulcerans* specific antibodies in mice and in patients with Buruli ulcer (Gooding et al., 2001, Gooding et al., 2002, Okenu et al., 2004) but a possible protective activity of these antibodies is unknown.

2.6.2 Importance of Mycolactone for pathogenesis

M. ulcerans is thought to have evolved from *Mycobacterium marinum* (Yip et al., 2007) and this is evidenced by over 98% nucleotide sequence identity between the two species (Stinear et al., 2000). However, over the course of its evolution *M. ulcerans* acquired a giant virulence plasmid, pMUM001, responsible for the synthesis of the exotoxin mycolactone (Stinear et al., 2004). The pathological manifestation of *M. ulcerans* infection is thought to be mediated by this toxin(s) production. Mycolactones are unusual among bacterial exotoxins because they are poorly immunogenic polypeptide-derived macrolides (Hong et al., 2008, Pimsler et al., 1988). Mycolactone A/B is the most active and widespread, and it is characteristic of *M. ulcerans* strains from Africa (Mve-Obiang et al., 2003). Experiments with externally added mycolactone A/B show that it has intense cytotoxic activity in vitro, affecting monocytes, macrophages, neutrophils, lymphocytes, fibroblasts, epithelial and adipose cells (Oswald et al., 2005, Simmonds et al., 2009). In addition mycolactone causes apoptosis, tissue necrosis and immunosuppression and cytotoxicity (George et al., 2000, Hong et al., 2008, Oswald et al., 2005). This action of mycolactone has been assumed to be the basis of tissue destruction typical of infections with *M. ulcerans*. All this makes mycolactone a key factor in the virulence of *M. ulcerans* (George et al., 1999), but the actual size of its effect on pathogenesis is not clear.

2.6.3 Role of mycolactone on the immune response

In vitro studies have shown that non-toxic doses of mycolactone are immunosuppressive on professional antigen presenting cells. Mycolactone blocked the migration of mouse-skin

dendritic cells (DCs) to draining lymph nodes, as well as their maturation in vivo. In human peripheral blood-derived DCs, mycolactone inhibited the ability to activate allogeneic T cell priming and to produce inflammatory molecules (Coutanceau et al., 2007).

The most striking finding is the complete inhibition of Tumour necrosis factor (TNF) production by monocytes and macrophages following infection with *M. ulcerans* or incubation with exogenous mycolactone. These findings are supported by histopathology of BU and the idea that local production of mycolactone prevents the trafficking of inflammatory cells to ulcerative lesions, hence less granuloma formation in early BU lesions. However, some studies of intra-lesional mRNA contradict immunosuppression at the local level due to mycolactone, they showed clearly that the innate immune system is activated at the site of BU lesions, indicated by high mRNA levels of cytokines interferon gamma (IFN-γ), interleukins 6, 10, 12, 15 (IL-6, IL-10, IL-12, IL-15) and TNF (Peduzzi et al., 2007, Phillips et al., 2006, Prevot et al., 2004).

2.6.4 Cellular immune response to *Mycobacterium ulcerans* infection

The ability of the host to resist *M. ulcerans* is dependent on the development of TH 1 type response (Gooding et al., 2002, Gooding et al., 2003, Prevot et al., 2004, Westenbrink et al., 2005) and as BUD progresses to healing, granuloma formation has been reported (Hayman, 1993, Kiszewski et al., 2006). With healing, the DTH burulin test (Stanford et al., 1975) tends to change from negative to positive (Dobos et al., 2000, Marston et al., 1995). However, some published work on *M. ulcerans* infections seem to suggest that, by contrast with other pathogenic

mycobacteria, *M ulcerans* is an extracellular pathogen that induces infections associated with limited or no inflammation (Cosma et al., 2003, Torrado et al., 2007).

As reported in tuberculosis and leprosy, IFN-γ seems to play a pivotal role in the control of *M. ulcerans* infection, and peripheral blood mononuclear cells (PBMC) or whole blood from Buruli ulcer patients show reduced capacity to produce this cytokine upon in vitro stimulation with whole *M. ulcerans* bacilli and M. ulcerans sonicate preparation (Gooding et al., 2001, Phillips et al., 2006).

2.6.5 Role of different T helper cell subsets in immune protection against Intracellular mycobacteria

Until recently, effector CD4 T helper cells were classified into T helper 1 (TH-1) and T helper 2 (TH-2) effectors (Mosmann and Coffman, 1989) based on the specific cytokines that they produce. TH-1 effector cells produce the cytokine Interferon-gamma (IFN-γ) and are known to regulate immunity against intracellular infections, whereas TH-2 cells produce the cytokines IL-4, IL-5 and IL-13 and known to mediate humoral immunity against parasite infections (Khader and Gopal, 2010). Recent evidence has changed the TH-1/TH-2 cell dichotomy to include a new T cell subset referred to as T helper 17 (TH-17) cells (Harrington et al., 2005, Park et al., 2005).

The actual role of TH-17 cells in the adaptive immune response remains unclear. Some studies suggest that the IL-23/TH-17 pathway has evolved to confer protective immunity against

extracellular bacterial infections (Aujla et al., 2008, Happel et al., 2005, Zhang et al., 2008). Other scientists more recently have demonstrated that TH-17/IL 23 pathway may play a crucial role in protective immunity against other intracellular pathogens by regulating the innate and the adaptive immune response (Khader and Gopal, 2010).

2.6.6 Role of cytokines in *Mycobacterium ulcerans* infection

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The host defense mechanisms against *M. ulcerans* (MU) infection still remains poorly understood, but cytokines have been firmly established to have a major role in determining the outcome of infection with *M. ulcerans* (Phillips et al., 2006, Yeboah-Manu et al., 2006) as in other pathogenic mycobacteria (Cooper and Khader, 2008). These evidences are derived from both studies in experimental models and observations in patients recovering from BUD and those on antimycobacterial therapy (Sarfo et al., 2009, Zavattaro et al., 2010). Of particular note is interferon gamma (IFN-γ) signaling the functions of which in host resistance to *M. ulcerans* and *M. tuberculosis* have been documented in both mouse models and infected humans (Cooper and Khader, 2008, Huygen et al., 2009, Zhang et al., 2008).

Cytokines are known to have an important role in the adaptive immune response as both effectors and regulators of mycobacterial immunity, and their expression profile in CD 4+ T cells clearly shows the dominant TH-1-like response that is associated with control of infection (Cooper et al., 2011). Cytokines equally have an important function in the innate defense against mycobacterial infection and in determining the subsequent T- cell response.

Various studies on BU patients investigating their cytokines have come to a common observation that, BU patients initially have low levels of IFN-γ and high levels of TH-2 cytokines interleukin 4 (IL-4), IL-5, IL-6 and IL-10 (Gooding et al., 2002, Westenbrink et al., 2005, Zavattaro et al., 2010). They further suggested that low levels of TH-1 cytokines (i.e. IFN-γ) may be due to down regulation by dominant TH-2 cytokines (Prevot et al., 2004, Westenbrink et al., 2005). But in similar study conducted in Ghana where whole blood from BU patients were stimulated with MU specific antigen, patients with nodular disease did show low IFN-γ responses in the presence of higher IL-10 response, but patients with ulcerative disease had the highest IL-10 responses and, at the same time showed high IFN-γ levels. There was no inverse correlation between IFN-γ and IL-10, suggesting that IFN-γ production in BU was not suppressed by IL-10 (Phillips et al., 2006) contrary to earlier suggestion.

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2.6.7 Antibody Response

In studies with *M. ulcerans* culture filtrates (MUCF), highly reactive immunoglobin G (Ig G) antibody responses were observed among patients with BUD, and also in healthy endemic controls and tuberculosis patients from areas where BUD is not endemic (Diaz et al., 2006, Okenu et al., 2004). By contrast, patients' Ig M antibody responses to MUCF proteins were more distinct than those of healthy family members living in the same village (Okenu et al., 2004). This suggests B-cell stimulation in patients with disease.

Diaz and colleagues used highly immunogenic *M. ulcerans* 18kD small heat shock proteins, which has no homologs in *M. bovis* and *M. tuberculosis* to monitor *M. ulcerans* specific Ig G responses in BUD patients and house-hold contacts from Ghana. They reported that 75% of the patients, independent of the disease stage or form and 38% of contacts showed reactivity. Interestingly, samples from Europeans and non-exposed Africans showed no reactivity (Diaz et al., 2006). These data suggest that, specific humoral responses against *M. ulcerans* develop in exposed individuals. Similar immune responses in healthy contacts have also been described by immunoblot analysis in Australian samples (Gooding et al., 2002).

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The control of *M. ulcerans* infection may be dependent on cell-mediated immunity involving macrophages, T-cells and TH-1 type cytokines as is suggested to be the case in *M. tuberculosis* and *M. leprae* infection (Huygen et al., 2009). However, antibodies could provide additional protective mechanisms against the largely extracellular *M. ulcerans*. Opsonization might improve phagocytosis and killing by infiltrating neutrophils, increase intracellular killing by macrophages or improve antigen presentation and induction of protective T-cell responses.

2.7 Prevention of Buruli ulcer

Buruli ulcer (BU) still remains a major economic and social burden for developing countries (Grietens et al., 2008), although significant progress has been made in the management of the disease in endemic countries. Prevention of the disease is difficult because the exact mode of transmission is unknown and no specific antigens have been identified for vaccine development.

Epidemiological studies found farming activities close to riverine areas as a risk factor for developing *M. ulcerans* disease (Jacobsen and Padgett, 2010), but this is difficult to advice against because in most areas it remains the major source of economic activity.

The most appropriate prevention method for the disease will be vaccination against *M. ulcerans*. BCG vaccination was found to have only short-term benefit but the studies were inconclusive (Anonymous, 1969, Smith et al., 1976). A DNA vaccine encoding antigen 85A from *M. bovis* has been tested in mice. Comparison of the genes from this antigen in BCG and *M. ulcerans* showed 91% conservation and there was 48% sequence identity in the amino acids of the antigens, so it might be expected to give some protection against *M. ulcerans* infection. When mouse footpads were infected with *M. ulcerans* after vaccination, the number of organisms cultured from the lesions was significantly reduced (Tanghe et al., 2001). These results are encouraging but should not be over interpreted, since tuberculosis research has shown that development of a protective immune response does not necessarily indicate that there will be clinical benefit.

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CHAPTER 3

MATERIALS AND METHODS

3.1 Study Design

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This was a cohort study of patients more 5 years old presenting with Buruli ulcer disease. It was estimated that 250 patients would be recruited from two treatment centres at Tepa Government Hospital and Agogo Presbyterian Hospital based on previous annual reports on the number of cases treated at these hospitals. The standard Buruli ulcer treatment form (BU01) (Appendix III) together with specially designed data forms (Appendix III) were used for collecting demographic data. A careful history to establish when early lesions (nodules, plaques and ulcers) were first observed and the type and dimensions of lesions were documented together with digital photographs and tracings onto acetate sheets. For oedematous lesions only digital photographs were obtained. Patients clinically diagnosed with *M. ulcerans* disease, had swabs or fine needle aspirates taken for laboratory confirmation. Subjects determined to be eligible, based on the inclusion and exclusion criteria described in sections 3.3.1 and 3.3.2 were enrolled in the study. After providing written informed consent, blood samples from subjects were taken for immunological assays.

Blood samples were obtained before onset of antibiotic treatment, for the peripheral blood mononuclear cell (PBMC) isolation, whole blood assay, flow cytometry analysis and enzyme linked immunosorbent assay (ELISA). Patients were reviewed at 2 weekly intervals during

standard antibiotic treatment (rifampicin 10mg/kg and streptomycin 15mg/kg) with further recordings of clinical data as was done for all routine patients. These measurements were used to calculate the rate of healing and healing time in relation to lesion size and type. One hundred household contacts of patients were contacted for comparison. A case control study was also designed to investigate the effect of filarial nematode co-infection with *M. ulcerans* to determine the effect of co-morbidity on the immune response of BU patients.

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3.2 Ethical considerations

Ethical approval for this study was obtained from the Committee on Human Research, Publications and Ethics (CHRPE), School of Medical Sciences (SMS), Kwame Nkrumah University of Science & Technology (KNUST)/Komfo Anokye Teaching Hospital (KATH), Kumasi, Ghana. Permission to conduct the study in the Agogo Presbyterian Hospital and Tepa District Hospital was sought from the hospital management and the districts health directorates. Consent was read in the appropriate local language. All collected information was treated as confidential. Subjects who agreed to be part of the study signed an informed consent form. Clinical procedures like fine needle aspiration and venesection were performed by a qualified physician or a nurse. Patients had the right to withdraw consent to participate in part or in full at any time during the study without giving reasons. Their withdrawal of consent did in no way negatively affect their further management. Consent was sought from household contacts of patients (i.e. those who did not have BU) to be recruited as controls.

3.3 Study setting and population

Subjects were from Ashanti Akim North and Ahafo Ano North districts in Ghana, which are endemic for *Mycobacterium ulcerans* disease. Recruitment was done at the Agogo Presbyterian Hospital and Tepa Government Hospital where patients were being managed. Agogo Presbysterian Hospital is located at 87 km east of Kumasi in the Asante Akim North district. The district covers 1,160 km²; district population is estimated at 140,694 (Ghana statistical service., 2010). Farming is the predominant economic activity. The Ahafo Ano North district covers an area of 571 square kilometers and has a population of 94,285, of which 80% live in small rural agricultural communities (Ghana statistical service., 2010).

3.3.1 Inclusion criteria for enrolment of subjects

I. Males and females from 5 to 55 years.
II. Good general health without any clinical condition requiring long-term medication.
III. Subjects not previously treated for Buruli ulcer disease.
IV. Contacts should be age matched to cases and with no evidence of BU.
V. Willingness to participate in the study as evidenced by signing of the informed consent document or guardian consent in the case of minors (subjects less than 18 years).

3.3.2 Exclusion criteria for enrolment of subjects

I. Subjects below the age of 5 years and above the age of 55 years.

II. Subjects who are pregnant, lactating or breast feeding.

III. Subjects with a history of other mycobacterial infection (like tuberculosis) or immunosuppressive disease (HIV/AIDS).

IV. Evidence of clinically significant neurological, cardiac, pulmonary, hepatic, or renal disease by history, physical examination and/or laboratory tests.

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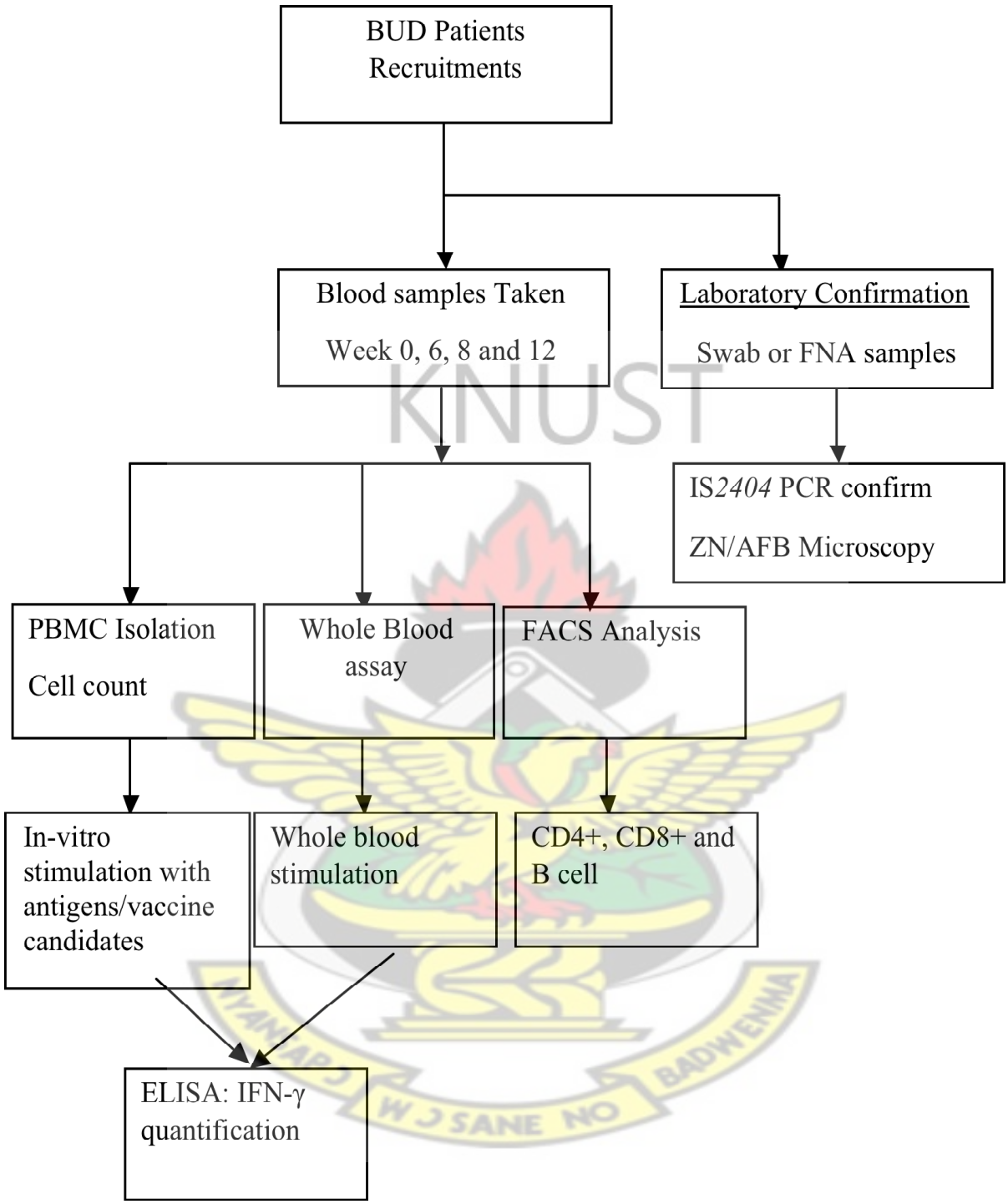

Figure 1: Flowchart of study procedure

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3.4 Laboratory confirmation of Buruli ulcer disease

Depending on the type of Buruli ulcer lesion presented, swabs (in the case of ulcers) or fine-needle aspirates (in the case of non-ulcers) were obtained from patients. This was done by microscopy after staining by the Ziehl Neelsen's technique, and a modified PCR for the insertion sequence IS*2404*. The different confirmation tests were evaluated for their sensitivity, specificity and predictive values.

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3.4.1 Subjects and sample processing

Two hundred and thirty subjects diagnosed by clinical criteria as BU disease were evaluated by microscopy for acid-fast bacilli (AFB) and polymerase chain reaction (PCR). Microscopy and PCR were performed at the Kumasi Centre for Collaborative Research in Tropical Medicine at KNUST in Ghana.

In order to standardize specimen collection and to provide optimal conditions for storage and transport, specimens were kept in media to prevent degradation of diagnostic material as cooling facilities in most hospitals were limited. Table 2 shows the type of diagnostic specimen and the respective transport/storage media used for sample preservation.

Table 2: Type of diagnostic specimen collected from patients, transport media and diagnostic tests

Specimen	Transport media	Diagnostic test	Transport container
Swab	4 ml PANTA	Microscopy/culture	
Swab	700 µl Cell lysis solution (CLS)	DRB-PCR	
FNA	200 µl CLS	DRB-PCR	
Swab/FNA	None	Microscopy	

CLS: Cell lysis solution; DRB-PCR: Dry reagent based Polymerase Chain Reaction; PANTA: Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, Azlocillin

3.4.2 Microscopic examination for acid-fast bacilli by Ziehl-Neelsen's technique

Analysis of swab and FNA specimen for the presence of acid-fast bacilli by microscopy comprised 2 steps (WHO, 2001):

1) Preparation and staining of slides

2) Microscopic examination and analysis of slides

3.4.3.1 Preparation, staining of slides and microscopy examination

Smears were prepared by ejecting aspirate from non ulcerative lesions or applying cotton swab soaked in ulcerative lesion directly onto a glass slide. The frosted ends of the slides were labelled with the patient identification using a pencil. The mycobacteria were fixed on slides by heating on a Bunsen burner. Slides were placed on staining rack, flooded with Ziehl Neelsen carbol-fuchsin that had been filtered through a funnel with a filter–paper prior to use. Slides were heated using an ignited cotton swab soaked in a few drops of 70% ethanol until steaming for 5 min. They were rinsed gently under clean running water until free of stain, flooded with decolorizing solution (3ml HCl in 97ml 70% ethanol) for 3 min, and rinsed thoroughly with water. After flooding with 0.1% methylene blue chloride, slides were left for 45 seconds, rinsed thoroughly with water and air dried ready for microscopy. The quantification scale for reporting is summarized in Table 3. Slides were examined under the x100 oil immersion objective. Slides positive for acid-fast bacilli show red coloured rod-like bacilli.

Table 3: Grading scale for Ziehl-Neelsen microscopy

Result	Grading
More than 10 AFB / field for at least 20 fields	+++
1 – 10 AFB / field	++
10 – 99 AFB / 100 fields	+
1-9 AFB / 100 fields	marked with exact number
No AFB in at least 100 fields	- (negative)

3.4.4.1 Extraction of mycobacteria DNA from clinical Specimen

Cell lysis was achieved by adding 10 µl proteinase K (20 mg/ml) to 700 µl CLS buffer containing sample, incubated at 55^{o}C in the thermomixer for 4 hours (or overnight) until complete lysis. The proteinase K was inactivated by incubation at 80^{o}C in the thermomixer. The samples were then cool down to room temperature and 15µl lysozyme (10 mg/ml) was added and incubated again at 37^{o}C in the thermomixer for 1 hour. The samples were then cooled on ice for 5 minutes. Hundred or 230 µl Protein Precipitation Solution (PPS) (Qiagen, USA) for FNA or swab specimen respectively was subsequently added to the samples and vortexed vigorously at 13000 rpm for 20 seconds to mix the PPS uniformly with the cell lysate (in CLS). Samples were afterward placed on ice for 5 minutes and then centrifuge at 13000 rpm for 5 minutes. During the centrifugation a respective number of 2 ml reaction tubes containing 700 µl Isopropanol and 2µl glycogen (Qiagen, USA) were prepared. The supernatant containing the DNA (leaving behind the precipitated protein pellet) was poured into the 2 ml reaction tube containing 700 µl Isopropanol and 2µl glycogen. The content of the new tube was mixed by inverting gently 10 times and then centrifuged at 13000 rpm for 5 minutes. The supernatant was poured off and 700 µl 70% Ethanol added. The tube was inverted gently several times to wash the DNA pellet and centrifuged again at 13000 rpm for 5 minutes and the ethanol poured off afterwards. The tubes containing the DNA pellet were drain on a clean absorbent paper towel and allowed to air dry for I hour. The DNA are then hydrated by adding 50 or 200 µl DNA Hydration Solution (Qiagen, USA) for FNA or swab specimen respectively, carefully pipetting up- and down about 20 times and then incubated in a thermomixer for 1 hour at 65 ^{0}C. Hydrated DNA was then kept at 2-8 ^{0}C until PCR was setup.

3.4.4.2 Dry-Reagent Based PCR for IS*2404*

As a prerequisite for the DRB-PCR, primers MU5 (5' agcgaccccagtggattggt 3') and MU6 (5' cggtgatcaagcgttcacga 3') (Stinear et al., 1999) were lyophylized in 200 µl reaction tubes. Lyophilisation is carried out in a RVC 2-25 vacuum concentrator, (Christ, Osterode, Germany).

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PuReTaq Ready-To-Go PCR beads (Amersham Biosciences, UK) were added into the reaction tube containing the lyophilized primers and dissolved in 22.5µl DNAse free water. A total of 2.5 µl extracted patients DNA were then added. For quality control purposes the PCR included a negative extraction control and positive, negative and inhibition controls. The thermal cycling protocol were as follows: 94˚C for 10 min, followed by 40 cycles at 94 ^{0}C for 10 s, 58 ^{0}C for 10 s, and 72 ^{0}C for 30 s, with a final cycle at 72 ^{0}C for 15 min. The amplification products were held at 4 ^{0}C until they were analyzed and detected by 1.5% agarose gel electrophoresis (Siegmund et al., 2007).

3.4.4.3 Agarose gel electrophoresis

To visualize and interpret the amplification, the PCR reaction products were separated by gel electrophoresis. A 1.5% agarose gel was prepared by heating 1.8g of agarose in 120 ml of 1X TBE buffer (108g Tris, 55g boric acid, 40ml 0.5M EDTA pH 8.0 in 1 litre water) in a 500ml conical flask placed in a microwave oven for 4 min at 750 Watt. Zero point five (0.5) µg /ml ethidium bromide was added, mixed and cooled in a 60°C water bath for 30min. Autoclave tape was placed on the gel casting tray and the gel poured after placing the combs. The combs were

removed after the gel had solidified. The running tray with the gel in the electrophoresis tank was immersed in 800ml of TBE. Fifteen µl PCR product was loaded after addition of 5µl of loading buffer. Six µl of a 100 base pair DNA molecular weight marker (invitrogen, USA) was first loaded in the first slot after which the negative, positive and inhibition controls were also loaded. The gel tray was then connected to a power supply for 45 minutes at 100 volts. The agarose gel was photographed using the video camera integration on 1.50s, UV 100%, exposure time 1/8 sec, zoom 12.5 and focus 1 meter under UV illumination. The band size of 492 bp corresponding to each patient sample are compared with that of the positive control band and reported. Samples are reported positive, negative or inhibited.

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3.5 Assessment of clinical response to treatment

Patients were categorized into two major groups, those with ulcers and those with non-ulcerative lesions such as nodules, plaques and oedemas. The non-ulcers were considered early lesions and ulcers as late since almost all the patients could not provide the exact duration of their lesions before seeking treatment. Small lesions, less than 10 cm in diameter were traced onto acetate sheet (as shown in plate 4), and their surface area calculated by approximation to a circle. It was impractical to measure large lesions >10 cm in diameter. Multiple lesions and those on uneven body surfaces, were monitored by serial photography until healing occurred as described previously (Sarfo et al. 2009). The sizes of lesions were assessed before treatment, bi-weekly up to 12 weeks and monthly thereafter up to week 48.

Plate 4: Tracing a patient's lesion onto acetate sheet for lesion size monitoring

3.6 Description of the immune status of *M. ulcerans* patients and controls

3.6.1 Blood samples for immunological techniques

Blood samples were obtained by venepuncture using the BD vacutaner safety-Lok blood collection set (BD Franklin Lakes, NJ USA). Blood was collected into preservative-free heparin tubes (BD Vacutaner Systems, Belliver Industrial Estate, Plymouth, UK). The samples were kept at room temperature for not more than 4 hours before use. Ten millilitre of whole blood was

obtained from subjects above 10 years and 6 ml from those below 10 years before, during and after treatment for all patients. Same amount of blood was taken from controls.

3.6.1.1 Peripheral Blood Mononuclear Cells Isolation from heparinized blood

To each whole blood, an equal volume of sterile phosphate buffer solution (1X PBS) was added to the total blood volume (1:1 dilution). Carefully and slowly 10 ml Ficoll hypaque solution was layered with blood/PBS mixture in a sterile 50ml centrifuge tube. The tubes in upright positions were gently transferred into a centrifuge. Centrifugation was carried out at 480 xg (1600 rpm) for 30 minutes at room temperature with no brakes. During centrifugation, a new set of labeled sterile 50 ml tubes was placed on ice. The initial tubes were carefully removed after centrifugation and together with its content placed on ice. The tubes were then inspected for hemolysis or small visible clots at the cell interface and any anomaly documented. There were 4 layers after successful separation made up of the top plasma layer, the cloudy PBMC layer, the ficoll solution and the bottom layer containing polynuclear cells and red blood cells. Using a new sterile pipette for each sample, the upper yellowish, plasma-PBS fraction were removed down to within approximately 1 to 2 cm of the cloudy white PBMC band located at the interface between the plasma-PBS (yellowish) fraction and the clear separation medium solution. The plasma-PBS fraction was discarded into a waste bottle. Using another sterile pipette, all the cells at the cloudy white interface were collected. The collected cells from one tube were transferred into a corresponding, pre-labeled tube. 30 ml ice cold 1X PBS was added to each tube containing cells, mixed gently and centrifuged at 270 x g (1200rpm) for 20 minutes at 4 ^{0}C (brake ON). The supernatant were removed and discarded without disturbing the cell pellet. Tubes were held at an

angle to remove residual supernatant with 1000 µl pipette. The pellet was resuspended in a 1ml of ice cold 1X PBS, followed by addition of 9ml ice cold 1X PBS. This was mixed thoroughly into a homogenous cell suspension and then centrifuged at 1200 rpm for 10 minutes at 4 ^{0}C (brake ON). The supernatant was removed and PBMC resuspended in 1 ml of 1X PBS.

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3.6.1.2 PBMC Count

Cell numbers were determined with trypan blue; 10 µl cell suspension in 190 µl trypan blue. Ten µl of the cell/trypan blue mix was pipetted in a Neubauer chamber. A 4 x 16 quadrants were counted and the cell number computed (cell number per ml was determined by multiplying counted cells; chamber factor (10^4) and dilution factor (1:20). Cells were split into two fractions:

Fraction 1): for direct stimulation (approximately 5 x 10^6 cells).

Fraction 2): for storage in liquid nitrogen (residual cells)

The cells for direct stimulation were re-suspended at 2 x 10^6 cells/ml in X-VIVO medium (1% penicillin/streptomycin).

3.6.2 Antigens selection for in-vitro cell stimulation

3.6.2.1 *Mycobacterium ulcerans* lysates

M. ulcerans lysates 1 and 2 antigens were prepared from *M. ulcerans* isolates of African origin by sonification (Phillips et al., 2006) as follows; A loopful of *M. ulcerans* colonies cultivated on Lowenstein Jensen slopes was transferred into 10 ml of Sauton's medium (2 mM MgSO4, 10 mM citric acid, 3 mM K2HPO4, 30 mM asparagine, 0.005% ferric ammonium citrate, 520 mM glycerol, pH adjusted to 7.2 with ammonia, autoclaved for 20 min at 121°C) and incubated at 30°C. The 10-ml *M. ulcerans* culture was passaged into 50 ml and subsequently into 500 ml of Sauton's medium with shaking (speed, 100 rpm; New Brunswick Scientific Co., Inc., Edison, N.J.). After 4 weeks, the bacterial pellet, obtained after centrifugation of *M. ulcerans* 1 cultures at 4°C for 30 min at 18,000 x *g*, was washed twice with 1X PBS in 500 ml polycarbonate tubes and centrifuged at 4°C for 30 min at 18,000 x*g* (10,500 rpm) in a Sorvall Plus centrifuge. The pellet was suspended in 35 ml sterile water and sonicated with a Branson 250 Sonifier at 50% duty cycle using a small probe in a cup-horn container: four cycles of 15 min with continuous cooling, interspersed with 5-min breaks cooling on ice. The *M. ulcerans* sonicate was aliquoted in 2-ml portions and lyophilized.

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3.6.2.2 Recombinant Antigen 85A Proteins

Ag85A tub (from *Mycobacterium tuberculosis*), Ag85A para (from *Mycobacterium Subsp. paratuberculosis*) and *Ag85A ulcerans* (from *Mycobacterium ulcerans*) are recombinant proteins provided by Prof. Kris Huygen (WIV-ISP Site Ukkel; service immunology; Brussels, Belgium). The process used to obtain the recombinant proteins was as described previously (Tanghe et al., 2008) as follows; Hexa-histidine tagged Ag85A protein from *M. tuberculosis* was purified from recombinant *E. coli*. The gene encoding the mature Ag85A protein from *M. ulcerans* was amplified by PCR from V1J.ns.tPA-85A vector. The primers used were 59-CGCGGATCCGCGTTTTCGCGGCCGGGCCTGCCGTGGAA- 39 (forward) and 59 CCCAAGCTTGGGCTAGGCGCCCTGGGTGTCACCG- 39 (reverse) with respectively BamHI and Hind III restriction sites. Ag85A gene was amplified without its mycobacterial signal sequence. Cloning in expression vector pQE-80L (Qiagen, USA), containing an NH2-terminal histidine tag coding sequence and purification were performed. Briefly, positive clones were screened on LB-ampicillin medium after ligation of the gene in a vector and transformation in *E. coli* DH5a cells. For expression, Top-10F' E. coli (Invitrogen, USA) cells were transformed with plasmid encoding the 85A sequence. Recombinant protein was purified by immobilized metal affinity chromatography (IMAC) using gravity flow. The endotoxin level measured with the Limolyl Amoebocyte Lysate kinetic chromogenic assay was inferior to 10 EU/ml (endotoxin units per millilitre) or 0.03 EU/mg of purified protein (Cambrex Bioscience, New Jersey, America).

3.6.2.3 Other Antigens

ESAT6 (Early secretory antigenic target 6) and CFP10 (Culture filtrate protein 10) are recombinant proteins kindly supplied by Lionex. PPDtub (from *Mycobacterium tuberculosis*), PPDsens (from *Mycobacterium avium* an environmental mycobacterium): both are purified protein derivates from the Staten Serum Institute, Copenhagen Denmark.

PMA (Phorbol myristate acetate)/IO: Ionomycin is an ionophore produced by the bacterium *Streptomyces conglobatus* (Sigma, germany*);* it allows PMA to enter the cell. PMA is a potent mitogen, activation is independent of antigen specificity. It was used alongside aCD3/aCD28: polyclonal T cell activator, anti-CD3/anti-CD28 antibody coupled to beads.

Selected subunit vaccine candidates (i.e. MUL3720 = hypothetical protein with lectin domain, 207aa and MUL4987 = mycolyl transferase 85A of *Mycobacterium ulcerans*) were provided kindly by Prof. Pluschke (Swiss Tropical Institute, Switzerland).

3.6.3 PBMC stimulation Assay

The PBMC suspension (2 x 10^5 cells/well in 100 µl x-VIVO medium) for each subject was stimulated with anti-CD3/anti-CD28 dynabeads, PMA+IO at a final concentration of 0.2 µg/ml and 10ng/ml respectively in separate wells. Extra 11 wells were also stimulated with PPDtub (tuberculin), PPDsens (Sensitin), ESAT 6/CFP 10, Ag85A tuberculosis, Ag85A paratuberculosis, Ag85A ulcerans, *Mu lysate* 1 and *Mu lysate* 2 antigens all at a final concentration of 5 µg per ml. One well for each subject was unstimulated to serve as background control. Plates were

subsequently incubated at 37 ^{o}C with 5% CO_2 for 5 days. The assay was performed in the immunology laboratory at KCCR.

3.6.4 Whole-Blood stimulation Assay

The whole-blood assay was performed under a safety hood. One ml of whole blood was aseptically distributed in duplicate in 24-well tissue culture plates (Falcon; BD, UK). Cell cultures were incubated with antigens; *M. ulcerans* lysate 1 and 2, Ag85A from *M. ulcerans* and *M. tuberculosis* at a final concentration of 5 µg/ml. One well was unstimulated and another stimulated with PMA+IO to serve as background control and positive control respectively. The plates were gently swirled 10 times clockwise and 10 anticlockwise on a flat surface and incubated at 37^{0}C with 5% CO_2 for 18-20 hours. Plasma supernatants (200 to 300 µl) were collected and stored at -20^{0}C.

3.6.5 IFN-gamma Quantification by Enzyme Linked Immunosorbent Assay

Cytokine ELISAs were performed using the Mabtech IFN-γ ELISA kits (Mabtech AB, Sweden). Recommended buffers and solutions necessary to develop enzyme-linked immunosorbent assays were obtained from BD Biosciences, Pharmingen (San Diego, Calif. USA). Results were presented as mean OD measurements in duplicate wells, converted into cytokine measurements (pg/ml) according to the standard curve. A positive cytokine measurement in the unstimulated culture supernatants, if detected, was subtracted from measurements in the test wells.

Table 4: Recommended buffers and solutions for ELISA

Catalogue number 550534	Components
coating buffer	0.1 M carbonate, pH 9.5
assay diluent	phosphate buffered saline with 10% fetal bovine serum
wash buffer	phosphate buffered saline with 0.05% Tween-20
substrate solution	hydrogen peroxide (solution A) 3'3'5'5'tetramethylbenzidine (TMB) (solution B)

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3.6.5.1 Antigen coating

Each assay was performed in a high protein binding ELISA plate. The plate was coated with monoclonal antibody 1-D1K, diluted to 2 µg/ml in coating buffer (indicated in table 4), pH 7.4, by adding 100 µl/well. These were incubated overnight at 4-8 ^{0}C.

3.6.5.2 Blocking of unspecific binding sites

The well contents were aspirated and washed twice with PBS (200µl/well). The wells were blocked by adding 200 µl/well of assay diluent (indicated in table 4). The assay diluent was added to block any unspecific sites on the antibodies that had adhered to the wells. This was incubated for 1 hour at room temperature.

3.6.5.3 Incubation of culture supernatants

Plates were washed 5 times with washing solution (indicated in table 4). Recombinant human IFN-γ standard (Mabtech AB, Sweden) was prepared by reconstituting contents in 1 ml 1X PBS with 1% BSA to a concentration of 1 µg/ml. The standard was diluted in assay diluent serially starting at a concentration of 1000pg/ml. Hundred µl/well of standard in duplicates, supernatants and blank were added and incubated for 2 hours at room temperature.

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3.6.5.4 Antibody detection

The well contents were aspirated and the wells washed 5 times with 200 µl/well of washing solution. Hundred µl/well of biotinylated monoclonal antibody 7-B6-1-biotin (Mabtech AB, Sweden) at 1 µg/ml in incubation buffer was added and incubated for 1 hour at room temperature (RT). Contents of plates were aspirated and washed for 5 times. Hundred µl/well of streptavidin-HRP diluted 1:250 in assay diluent was then added and incubated again for 1 hour at RT.

3.6.5.5 ELISA reading and calculation

The content of wells was discarded and washed 5 times with washing solution. Plates were placed on a white background and 100 µl of substrate solution (indicated in table 4) added to each well. After a developing time of 5-10 minutes, the reaction was stopped with 25 µl/well of 2M H_2SO_4. The optical densities of standard and samples were measured using TECAN Sunrise

ELISA reader (Tecan Group Ltd. Switzerland). The mean absorbance of duplicate standards, samples, and controls were calculated for each plate, and the mean zero standard absorbance subtracted. Results were analyzed with GraphPad Prism 5 software (GraphPad Software, Inc.) and a standard (best-fit) curve plotted. Values for unstimulated cultures were subtracted from those for stimulated cultures. Results were validated by a significant response to anti-CD3/anti-CD28 and Phorbol-Myristat-Acetat (PMA)/Ionomycin (IO) stimulation.

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3.7 Complete blood count and flow cytometric analysis of peripheral blood lymphocytes

3.7.1 Cases and Controls Recruitment

In this case-control study, 37 newly diagnosed with Buruli ulcer patients from the Agogo Presbysterian Hospital, Agogo and Tepa District Hospitals were recruited as cases. Twenty-one healthy age-matched household contacts of these patients were also recruited as controls. Clinical diagnosis of BU was confirmed by a positive IS*2404* PCR for *M. ulcerans* (described in section 3.4.3).

3.7.2 Sample collection

Whole blood (2 ml) was aseptically collected from patients and controls by venipuncture into sterile EDTA Vacutainer blood collection tube (BD Vacutainer Systems, Belliver Industrial

Estate, Plymouth, UK) after informed consent had been obtained. Samples were stored at room temperature until immunoflourescent staining was done at KCCR immunology laboratory. Absolute counts of neutrophils, monocytes, lymphocytes and eosinophils in fresh peripheral blood were determined with a hematology analyzer.

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3.7.3 Direct Immunofluorescence staining of Whole Blood

Becton Dickinson (BD) protocol for direct immunofluorescence staining of whole blood was adopted and modified for use. Twenty µL of the fluorochrome-conjugated monoclonal antibodies (CD3+-APC, CD 4+-FITC, CD 8+-FITC, CD 19-FITC) was added to 100 µL of whole blood in three separate 12 x 75-mm tubes (BD Biosciences, USA). Tubes were vortexed gently and incubated for 15 minutes in the dark at room temperature. Afterwards 2 ml of 1X FACS lysing solution (BD Biosciences, USA) was added to lyse erythrocytes under gentle hypotonic conditions while preserving the leucocytes. Samples were vortexed gently and incubated for 15 minutes in the dark at room temperature. Samples were then centrifuged at 300 x g for 5 minutes and supernatant decanted. Samples were washed with 2 ml of 1X PBS by centrifugation at 300 x g for 5 minutes. Supernatant was decanted, and 500 µL of 1X PBS added to samples, mixed thoroughly and analyzed on the FACSCalibur (BD San Jose, CA USA).

3.7.4 Sample Acquisition and Analysis

Using the BD CellQuest Pro Software, acquisition and analysis templates were set up for acquiring and analyzing all patients and controls data. The machine was calibrated to achieve the

right instrument settings before acquisition.

3.8 Co-infection with *Mycobacterium ulcerans* and *Mansonella perstans* among Buruli ulcer patients in Ghana

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3.8.1 Identification of filarial worms (*M. perstans*)

Peripheral blood mononuclear cells (PBMC) were separated from heparinized whole blood using Biocoll separating solution (Biochrom Ag, Germany) density gradient centrifugation as described in section 3.6.1.1. Cells were resuspended in culture medium (X-VIVO 15 Lonza, Belgium) at a concentration of $2.0x10^5$ cells/well and stimulated with different antigens. Cells were incubated at 37^0C in 5% CO_2 overnight and examined under X10 magnification (Carl Zeiss, Axiovert 25) for morphological changes and for the presence of microfilariae.

3.8.2 Microscopy Examination and characterization of *M. perstans*

For participants with filarial worms in their tissue culture 1 ml of whole blood was obtained for full blood count (FBC) analysis and confirmation of filarial worms. The sediment prepared by Knott technique (Denham, 1975) was stained with Giemsa and Delafield's hematoxylin. The *Mansonella perstans* stained preparation was examined under X10 and X40 objectives for microfilariae. *M. perstans* was distinguished from *L. loa* and *W. bancrofti* by its small size and the absence of a sheath microscopically.

3.9 Statistical Analysis

Statistical analyses were done using Microsoft Excel 2010 and GraphPad Prism 5 software programmes. The raw data was entered using Microsoft Excel. GraphPad Prism 5 software was used for drawing standard curves for ELISA plates and plotting graphs. Descriptive statistics were used to obtain general descriptive information such as the mean and standard deviation from the data. One sample analysis (Fisher's exact test) was used to compare two proportions or groups. One-way ANOVA (Kruskal-Wallis test) was used to compare group means. Descriptive results of cytokines levels were expressed as medians and ranges. Cytokine levels were evaluated for an association with disease status (early-versus late stage BUD or control) with Kruskal-Wallis test. The variables category of lesion, use of chemotherapy, BCG status, gender, and age were evaluated for association or correlation with measured cytokine responses with the Spearman r for categorical data and the Mann-Whitney U test for numerical data; $P < 0.05$ was considered statistically significant. The GraphPad Prism software was again used for box-and-whisker plot representation, with outlier cut-off determined by Tukey's test. Outliers were kept in all statistical analyses and were represented by dots in box-and-whisker plots.

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CHAPTER 4

RESULTS

4.1 Laboratory Confirmation of Clinical suspected Cases of *M. ulcerans* infection

4.1.1 Patients Characteristics

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Table 5 shows the characteristics of 236 suspected patients with Buruli ulcer lesions of which there were 111 pre-ulcerative and 125 ulcerative forms. There were 49 (44%) nodules, 41 (37%) plaques and 21 (19%) oedema forms. The median age of those with pre-ulcerative lesions was 13 years (range 2-68), which was significantly different from 16 years (range 2-80) for those with ulcerative lesions (p=0.03 Mann Whitney test).

Agogo Presbyterian Hospital treated 86 (77%) of pre-ulcerative and 57 (46%) ulcerative lesions, which was significantly different from 25 (23%) pre-ulcerative and 68 (54%) ulcerative lesions at Tepa Government Hospital (p<0001 Fisher's exact test).

Table 5: Characteristics of participants sampled for laboratory confirmation

	No. (%) of pre-ulcerative lesion (n= 111)	No. (%) of ulcerative lesion (n= 125)	No. (%) of total lesions (n=236)	*P* value
Sex				
Male	44 (40)	60 (48)	104 (44)	0.2372*
Female	67 (60)	65 (52)	132 (56)	
Age in years				
median (range)	13 (2-68)	16 (2-80)	14 (2-80)	0.0304
Treatment centre				
Agogo	86 (77)	57 (46)	143 (61)	
Tepa	25 (23)	68 (54)	93 (39)	<0.0001*
Lesion type				
nodule	49 (44)	0 (0)	49 (21)	
plaque	41 (37)	0 (0)	41 (17)	
edema	21 (19)	0 (0)	21 (9)	N/A
ulcer	0 (0)	125 (100)	125 (53)	
Lesion category				
I	64 (58)	57 (45)	121 (51)	
II	25 (22)	41 (33)	66 (28)	0.1369
III	22 (20)	27 (22)	49 (21)	

*Fisher's exact test NB: Median comparison was done by Mann-Whitney test and lesion categorization comparison by chi-square.

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4.1.2 Results of PCR and ZN Microscopy by standard Procedure

Using standard procedures, 96 (86%) pre-ulcerative and 109 (87%) ulcerative lesions were IS*2404* PCR positive while 55 (50%) pre-ulcerative and 63 (50%) ulcerative lesions were positive for AFB by microscopy. When the pre-ulcerative lesions were sub-grouped into nodules, plaques and edemas, 41 (84%) nodules, 36 (88%) plaques and 19 (91%) edemas were IS*2404* PCR positive while 29 (41%) nodules, 23 (56%) plaques and 12 (57%) edemas were positive for AFB by microscopy. Results by category are summarized in (Table 6).

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Table 6: Laboratory confirmation results by PCR and microscopy by standard procedure

	No. (%) positive samples	
	PCR	**Microscopy**
Pre-ulcerative (n=111)	96 (86)	55 (50)
Nodule (n=49)	41 (84)	20 (41)
Plaque (n=41)	36 (88)	23 (56)
Edema (n=21)	19 (91)	12 (57)
Ulcerative (n=125)	109 (87)	63 (50)
category I (n=121)	103 (85)	61 (50)
category II (n=66)	57 (86)	36 (55)
category III (n=49)	45 (92)	21 (43)

4.1.3 Improve Sensitivity of ZN microscopy by preparing two smears on-site

As improving the transport of samples may further improve detection rate this study evaluated the usefulness of preparing two direct smear slides at the treatment centres before transport to the laboratory for microscopy. This was a means to improve the sensitivity of AFB detection in swab samples and fine-needle aspirate (FNA) samples in a large cohort of clinically diagnosed BU patients. Table 7 shows that when two smears were prepared at the clinic (on-site) from FNA samples of 111 patients. The sensitivity of AFB detection was 52% (41-62, 95% CI) for the first slide increasing to 55% (45-66, 95% CI) when a second slide was examined. Similarly when swab samples from 125 patients were examined, the sensitivity of microscopy was 51% (42-61, 95% CI) but increased to 57% (48-67, 95% CI) when a second sample was examined. These observations were confirmed by an independent external scientist from the LMU.

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Table 7: Sensitivity of immediate slide smear microscopy in the districts improves detection of *M. ulcerans* when a second slide smear is examined

Sample type	One slide ZN Microscopy results	Average of two slides ZN microscopy results
	Sensitivity (% [95% CI)]	Sensitivity (% [95% CI)]
Pre ulcerative		
FNA (n=111)	52 (41-62)	55 (45-66)
Ulcerative		
Swab (n=125)	51 (42-61)	57 (48-67)
Pre-ulcerative		
nodule	43 (27-59)	48 (32-64)
plaque	56 (38-73)	62 (44-78)
edema	63 (38-84)	63 (38-84)

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The result of examining an immediate slide smear by microscopy for AFB was further evaluated and correlated with the result of PCR as the gold standard confirmatory test for BU. Table 8 shows that for FNA samples, sensitivity for detection of *M. ulcerans* was 55% (45-65, 95% Cl) and the specificity was 86% (60-98, 95% Cl) whiles for swabs the sensitivity for detection of *M. ulcerans* was 57% (47-66, 95%Cl) and the specificity 94% (70-100, 95% Cl).

Table 8: Evaluation of two immediate slide smear microscopy against the gold standard PCR

	No. of patients with				
	PCR positive	**PCR negative**	**Total**	**Sensitivity (%) [95% CI)]**	**Specificity (%) [95% CI)]**
FNA microscopy positive	53	2	55	**55**	**87**
FNA microscopy negative	43	13	56	**45 - 65**	**60 - 98**
Total	96	15	111		
Swab microscopy positive	62	1	63	**57**	**94**
Swab microscopy negative	47	15	62	**47 - 66**	**70 - 100**
Total	109	16	125		

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4.2 The immune status and T cell cytokine expression pattern of BUD patients under therapy and healthy contacts

4.2.1 Immune response of patients with Buruli ulcer versus controls

To determine the IFN gamma response of patients with Buruli ulcer compared with controls, patients and age-matched controls were recruited in the Ahafo Ano North District, and Asante Akim North District.

Table 9 shows the demographic data of patients recruited. There were 102 with confirmed Buruli ulcer of which there were 28 (27.5%) nodules, 18 (17.6%) plaques, 8 (7.8%) oedema and 48 (47.1%) ulcers. Forty-four (43.1%) were category 1, 26 (25.5%) were category II and 32 (31.3%) were category III. Fifty-one age-matched healthy individuals from the same districts were recruited to serve as controls.

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Table 9: Demographic characteristics of patients and controls

	Buruli ulcer cases (n=102)	Controls (n=51)
Sex (male/female)	47/55	26/25
Mean age (yr)±SD	21±13	20 ±9
Presence of BCG scar		
Yes/no	58/44	31/20
Clinical form		
Nodule	28 (27.5 %)	
Plaque	18 (17.6 %)	
Oedema	8 (7.8%)	
Ulcer	48 (47.1%)	
Category of lesion		
I	44 (43.1%)	
II	26 (25.5%)	
III	32 (31.3 %)	

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Figure 2 shows that there were nonspecific IFN gamma responses between patients and controls to Mu *lysates, A85A tub, A85A para, Ag85A ulcerans, PPD tub* and *PPD sens* when PBMC were stimulated for 5 days.

After stimulation of whole blood of patients and controls for 18 hours with mycobacterial antigens (Figure 3). There was significant increase in median IFN gamma production to MU *lysate 1* [1295 (870-2786) vs 652.5 (570.4-947.7) pg/ml p = 0.02] and *Ag85A ulcerans* [335.8 (262.8-931.8) vs 135.3 (69.5-397.8) pg/ml p= 0.0176] in patients compared to controls but not to *Ag85A tuberculosis* (p=0.16).

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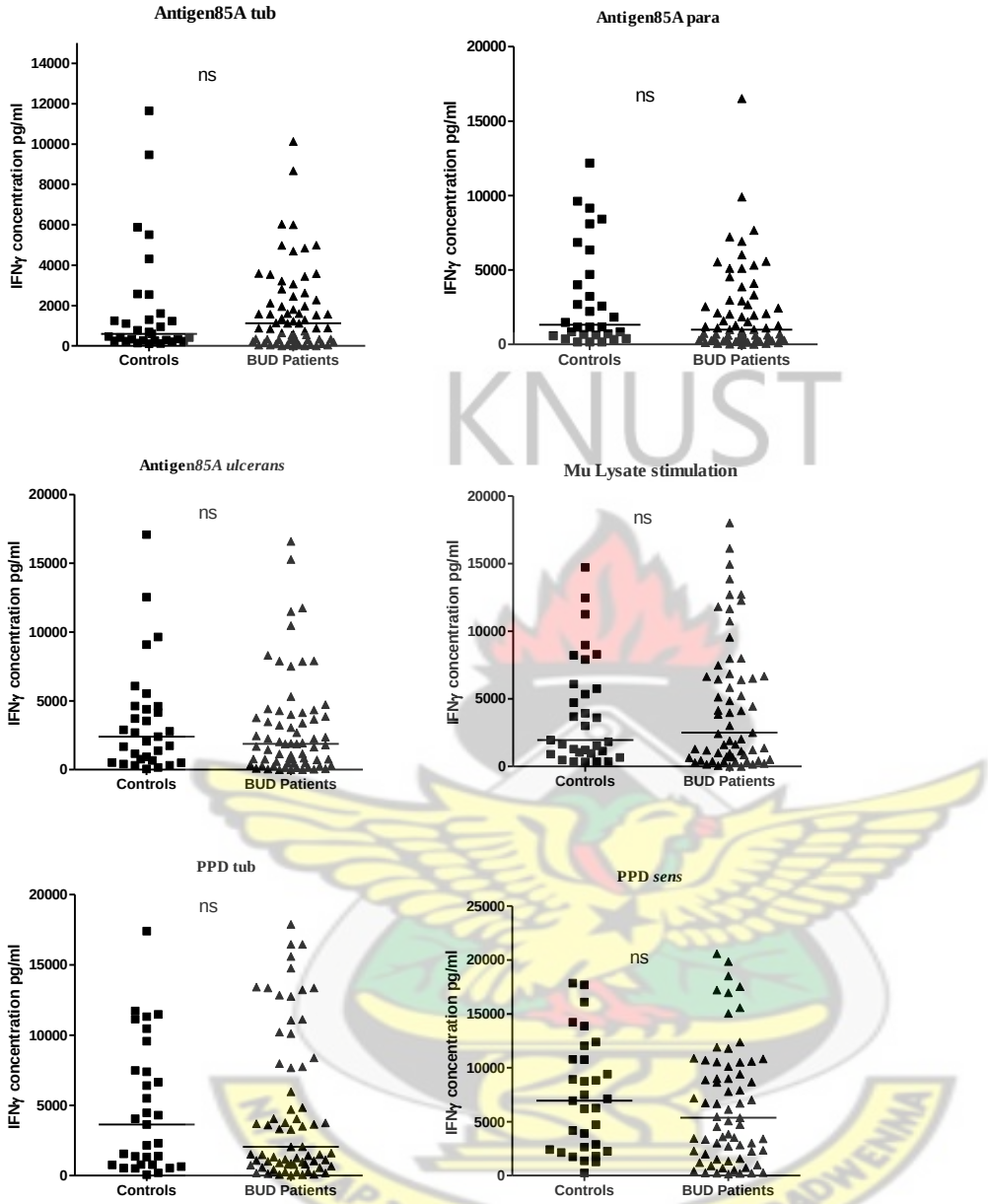

Figure 2: Non-specific IFN gamma responses between patients and controls when PBMC were stimulated for 5 days (ns – no statistically significant difference)

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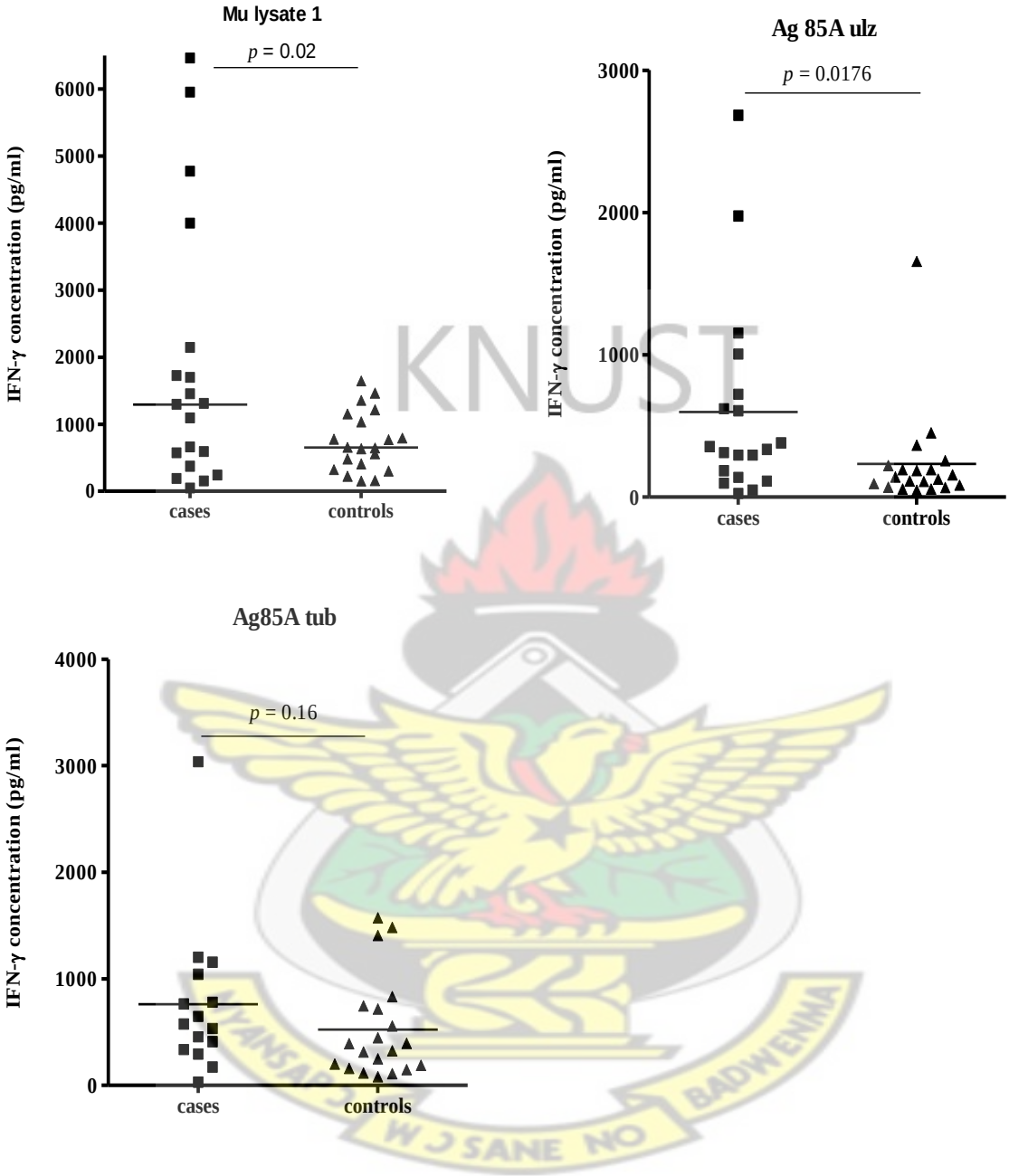

Figure 3: IFN gamma responses after stimulation with Mu lysate, Ag85A ulcerans and Ag85A tub of whole blood of patients and controls for 18 hrs.

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4.2.2 Immune response of patients with Buruli ulcer before, during and after treatment with antibiotics

Figure 4A shows that after 5days stimulation of patients' PBMC with mycobacterial antigens, a significantly higher IFN gamma secretion was produced to *Mu lysate 1* at 12 weeks after the start of treatment as compared to baseline (p=0.005) but not to *Ag85 ulcerans*, *PPDtub* or any of the other mycobacterial antigens.

Similarly after stimulation of whole blood with 5ug/ml of mycobacterial antigens for 18 hours significantly higher IFN gamma was produced after 6 weeks compared to baseline for Mu *lysate* 1 [1095 (589.2-1718) vs 4855 (3429-5749) pg/ml *p*=0.0001] and also at 12 weeks for Mu *lyaste* 1 but not for any of the other mycobacterial antigens (Figure 4B).

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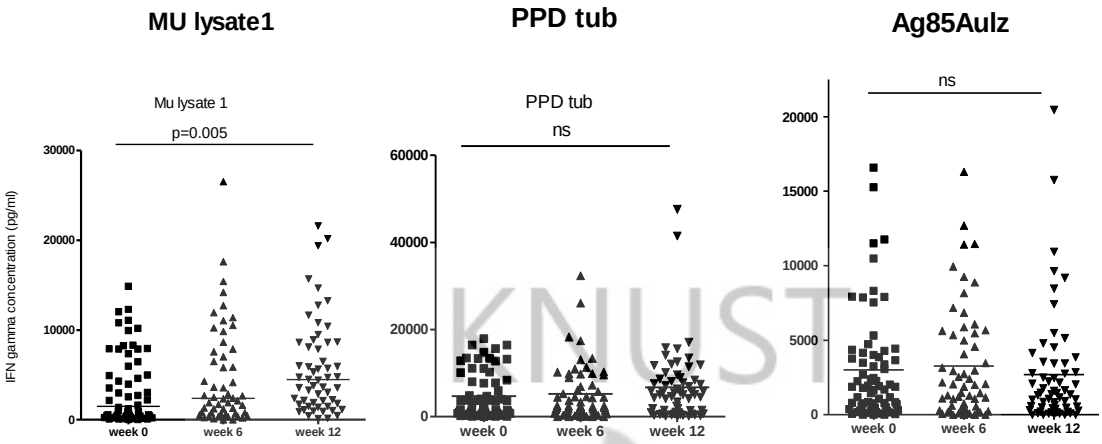
A. IFN-γ after 5 days stimulation of Patients PBMC with treatment



B. IFN-γ after 18 hrs stimulation of Patients whole blood with treatment

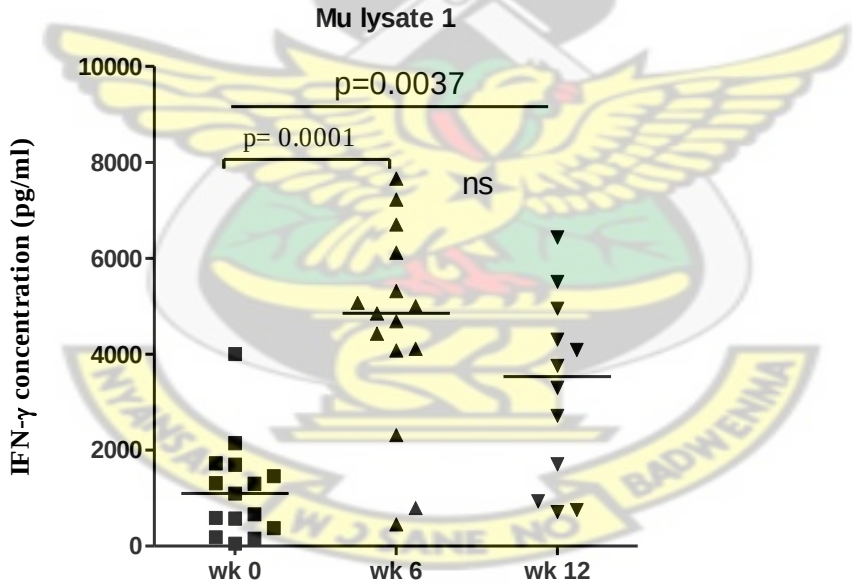


Figure 4: IFN gamma responses before, during and after treatment

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4.2.3 Immune response of patients with Buruli ulcer of different severity

To determine the IFN gamma production with different categories of Buruli ulcer disease, patients were classified into categories and their IFN gamma responses compared.

Definitions:

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Category I: a single lesion <5cm in diameter;

Category II: a single lesion between 5cm and 15cm in diameter;

Category III: a single lesion >15cm in diameter, multiple lesions, lesions at critical sites (e.g. eye, breast, genitalia) and osteomyelitis)

Figure 5 shows that when PBMC were stimulated for 5 days there was no difference in IFN gamma response across the different categories. However in the whole blood assay there was statistically significant increase in IFN gamma [834.1 (306.2-3842) vs 5276 (3140-7425) pg/ml p=0.01] response in the category I lesions compared with category II lesions respectively. These results suggest that the different categories of Buruli ulcer disease could not be discriminated with their IFN gamma response in the 5 day PBMC stimulation but category II lesions appeared to respond with higher IFN gamma levels in the short term whole blood assay but the numbers were small.

In addition patients with ulcerated forms of disease produced significantly higher IFN gamma responses to *Mu lysate*1 compared to non ulcerated forms (p = 0.008) in the 5 day stimulation and not in the short term assay.

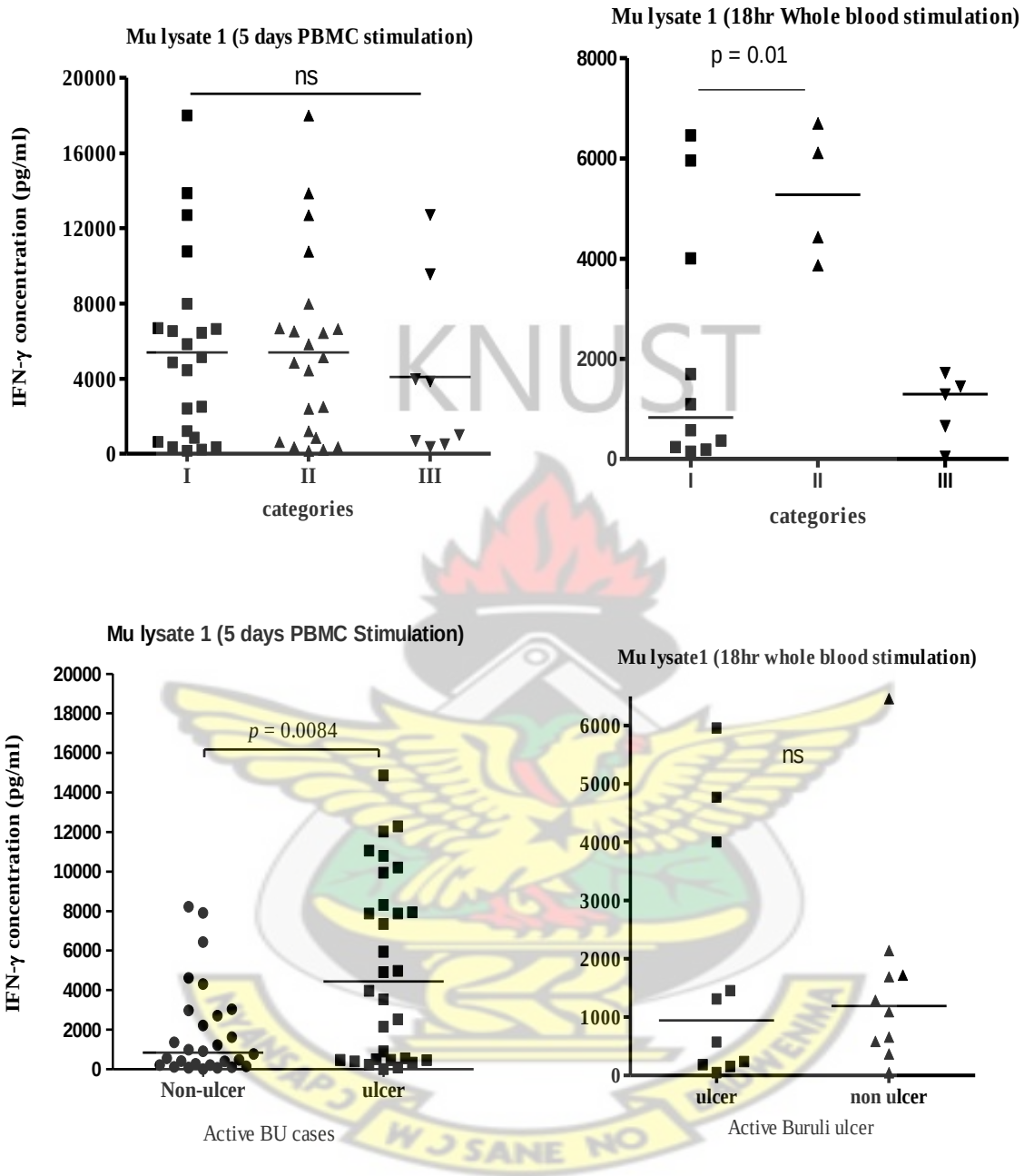

Figure 5: IFN gamma response of patients with Buruli ulcer of different severity

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4.2.4 Correlation of clinical response with IFN gamma response in BU patients

To determine if time to healing in these patients correlates with IFN gamma levels at baseline. Prior to therapy, 50 patients had small lesions (25 nodules and 25 ulcers) with a median surface area of 9.1 cm^2 (range of 0.28 to 78.5 cm^2). Forty-seven of the 50 patients were included in the analysis because 3 samples could not be stimulated due to hemolysis. After 6 weeks of antibiotic treatment, there was a significant difference in median (p = 0.0014) lesion size (7 patients had lesions healed completely) and a further difference (p <0.0001) 6 weeks later (additional 14 patients healed) with one paradoxical enlargement after 12 weeks.

Figure 6 shows that there was a gradual reduction in surface area of all lesions with median measurement of 9.1 cm^2 at baseline, 2.54 cm^2 at 6 weeks and 0.50 cm^2 at 12 weeks after treatment. After treatment for 6 weeks, the median IFN-γ secretion increased in both pre-ulcerative and ulcerative lesions (2934 pg/ml and 5097 pg/ml respectively), but not statistically significant ($p > 0.05$). After 12 weeks (i.e. 4 weeks after the mandatory 8 weeks treatment), the difference from the baseline was significant in patients with pre-ulcerative lesions (median 6093 pg/ml [range of 93.31 to 17722 pg/ml]; p = 0.0005). However, increase in IFN-γ secretion for ulcerative lesions as compared to baseline level (median 4626 pg/ml [range of 72.95 to 27046 pg/ml]) was not statistically significant as shown in Figure 7.

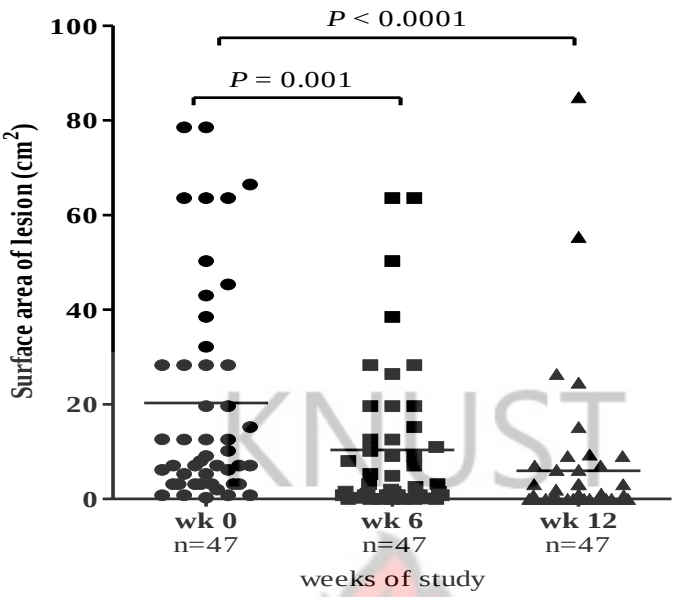

Figure 6: Changes in surface area of small Buruli lesions before and after RIF-STR treatment for 8 weeks.

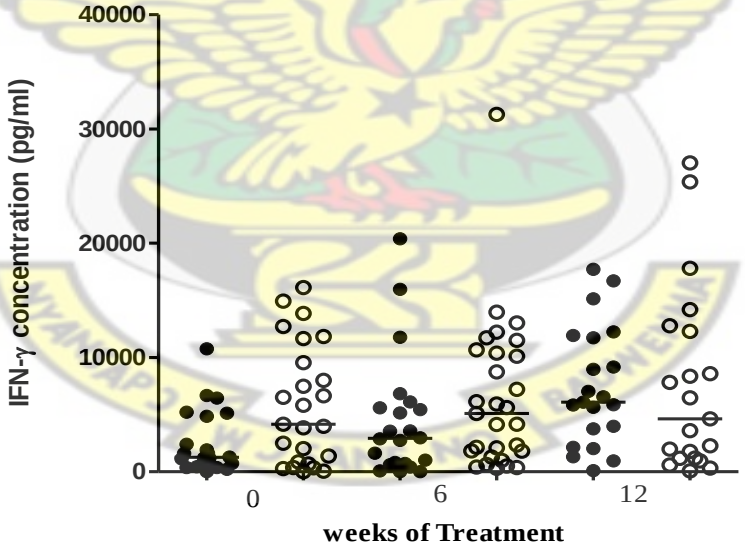

Figure 7: IFN-γ production after stimulation with MU lysate 1 of PBMC from patients with pre-ulcerative Buruli lesions compared with that from patients with ulcerative Buruli lesions before, during and after treatment for 8 weeks.

4.2.5 Effect of BCG vaccination on IFN-γ response

Following PBMC stimulation with *M. ulcerans* lysate antigen, subjects with active *M. ulcerans* disease were divided into those with BCG vaccination and those without vaccination. Determination of BCG vaccination was solely based on the presence of BCG scar on the shoulders of patients, since almost all the patients could not provide any record of vaccination. As shown in Figure 8 at baseline (pre-treatment) patients with BCG vaccination, IFN-γ response was very heterogeneous (median 1633 pg/ml [range of 0.0 to 18015 pg/ml]). The response was similar to those without BCG vaccination (median 3441 pg/ml [range of 65.85 to 12721 pg/ml]: $p = 0.4737$), no significant difference (ns) between patient groups.

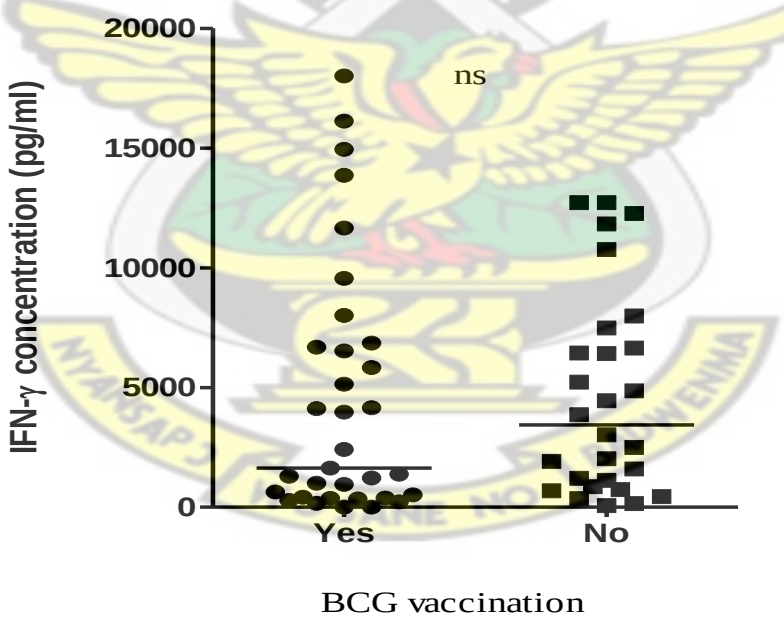

Figure 8: IFN-γ production after stimulation with MU lysate 1 of PBMC from subjects with active disease. The horizontal lines represent the median, ns – not significant, referring to comparison with patients with BCG scar and those without BCG scar.

4.3 Evaluation of immunological reactivity of subunit proteins with lymphocytes of BU patients

4.3.1 IFN gamma response to MUL4978 and MUL3720 in BU patients and Controls

Individual IFN-γ responses to MUL4978 (Figure. 9, left) and MUL3720 (Figure.9, right) were heterogeneous and widely overlapping within donors. There was no statistically significant difference between BUD patient and contact whole blood IFN-γ secretion. It was therefore, concluded that MUL4978 and MUL3720 do not discriminate between BUD patients and controls.

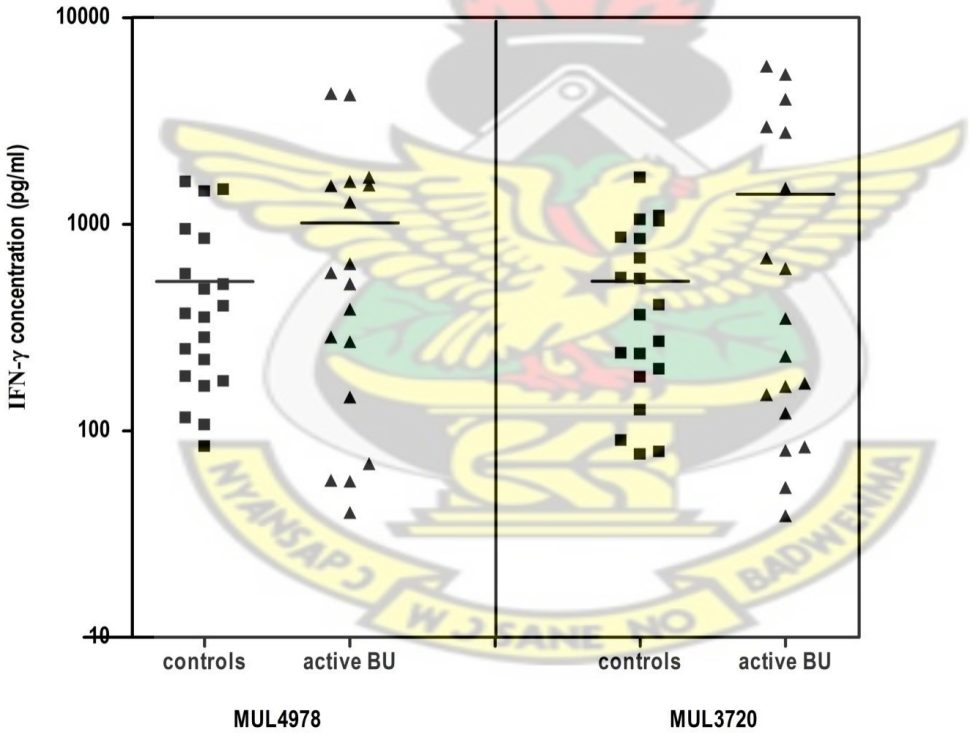

Figure 9: Stimulation of BUD contact (squares) or BUD patient (triangles: before treatment) whole blood by vaccine candidate MUL4978 (left) or MUL3720 (right). After 18 h supernatant was taken and IFN-gamma concentrations were measured by ELISA.

4.3.2 Immune response associated with severity in patients with Buruli ulcer

BUD patients were further grouped into three categories according to lesion size:

Figure 10 shows that the median IFN-γ response to MUL4978 was higher in category I compared to II and III but the analyses did not reveal significant differences between the subgroups (which were small to exclude possible differences. There was also no significant difference in the categories in IFN gamma response to MUL 3720.

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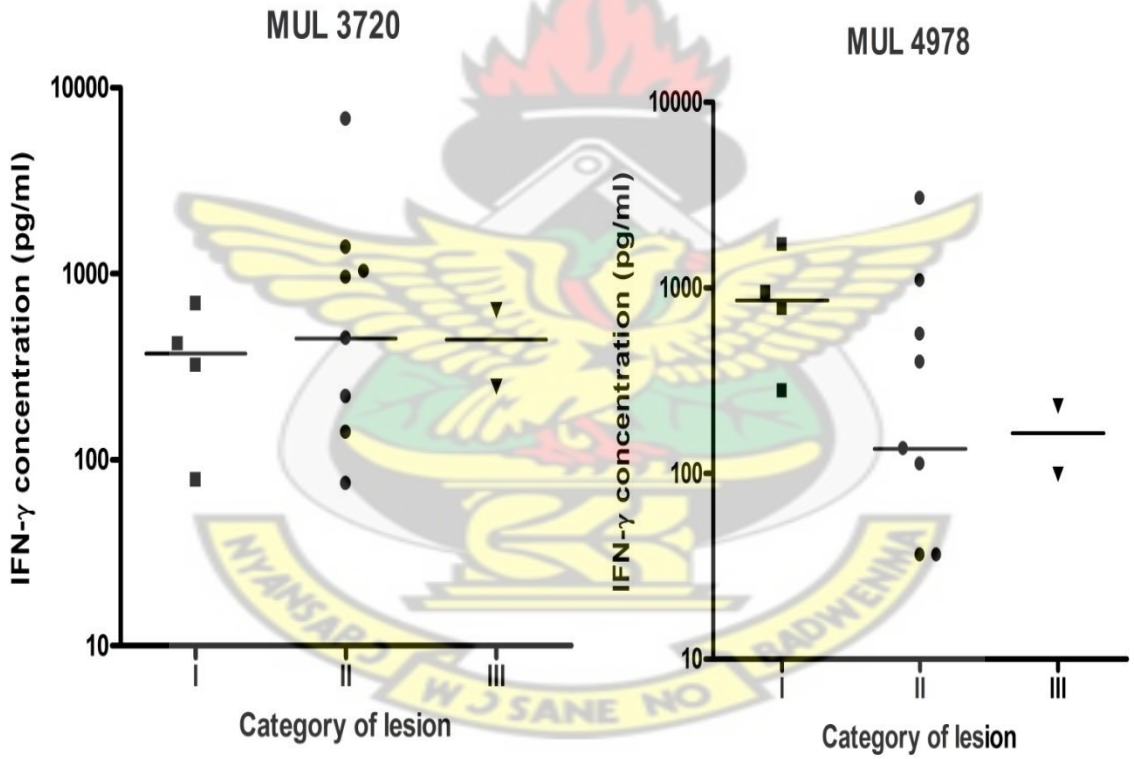

Figure 10: IFN gamma response to MUL 3720 and MUL 4978 associated with categories of BUD

4.3.3 IFN gamma response to Subunit *M. ulcerans* proteins with treatment

Figure 11 shows analysis of IFN-γ expression of BUD patients assays from supernates obtained after the whole blood assay prior to treatment, 6 and 12 weeks after treatment onset) against MUL3720 (Fig. 10A) and MUL4978 (Fig. 10B). There was a significant increase of IFN gamma response (*p*=0.03; *p*=0.005) to both candidates after six weeks of therapy. At week 12 similar levels were detected as compared to week 6.

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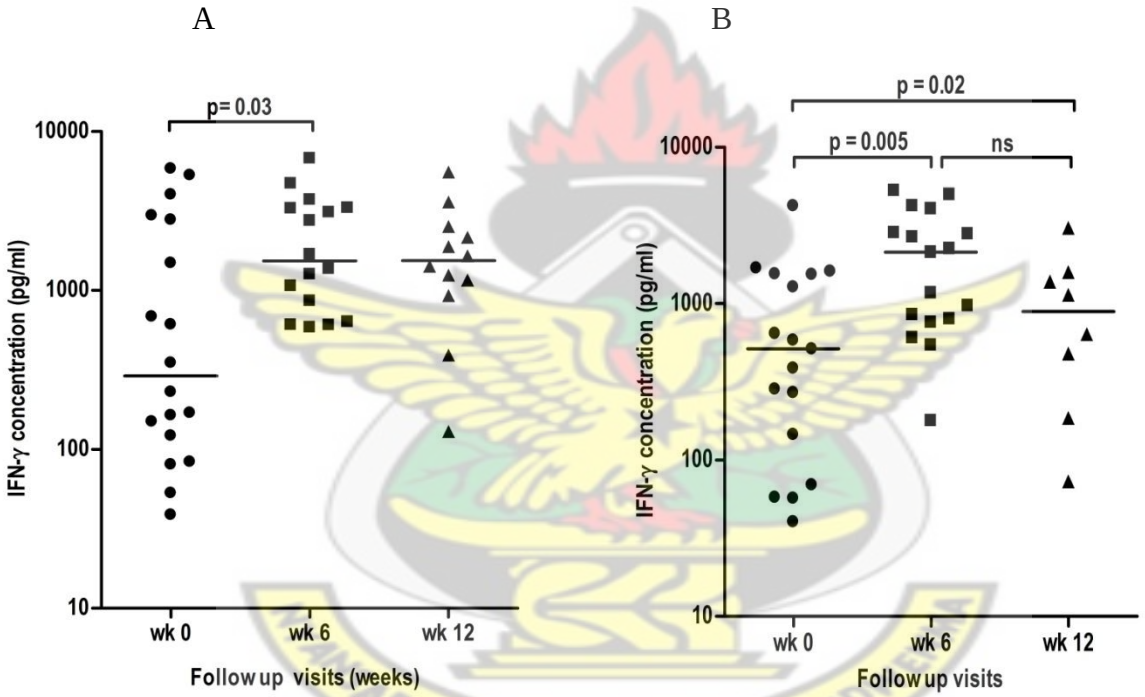

Figure 11: **Analysis of the IFN-γ secretion by BUD patient whole blood against vaccine candidate MUL3720 (A) and MUL4978 (B) under treatment. Blood was taken at week 0 (circles), 6 (squares) and 12 (triangle). After 18 h supernatant was taken and IFN-γ concentrations were measured by ELISA.**

By contrast PBMC *in vitro* stimulation assays partly verified results from whole blood (Figure. 12). Responses at all time points analysed were widely spread. There was a significantly (p=0.03) increased IFN-γ response to MUL4978 (Figure. 12B) after 12 weeks of chemotherapy. There was no significant difference in IFN-γ response before and after treatment when stimulated with MUL 3720 (Figure. 12A).

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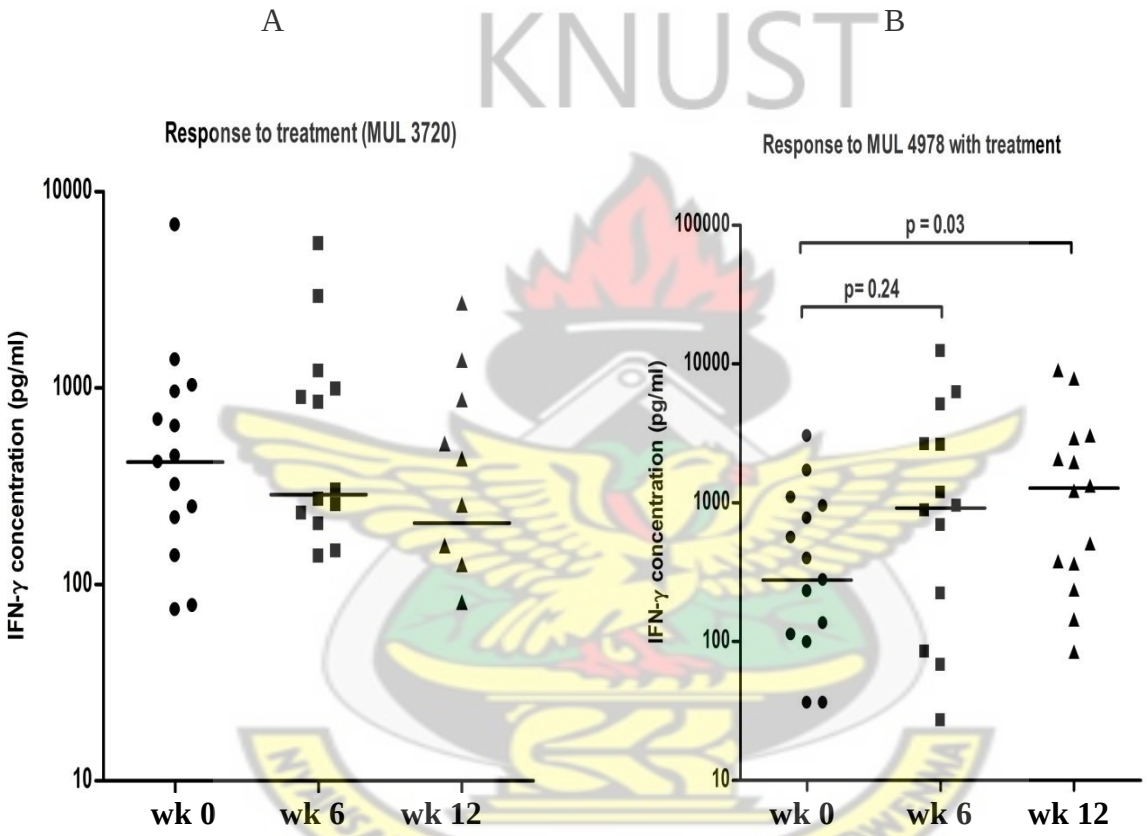

Figure 12: Analysis of the IFN-γ response by BUD patient PBMCs against vaccine candidate MUL3720 (A) and MUL4978 (B) under treatment. Blood was taken at week 0 (circles), 6 (squares) and 12 (triangle). On day 5 supernatant was taken and IFN-γ concentrations were measured by ELISA.

4.4 Alteration in Cell Populations in the peripheral blood of patients with *Mycobacterium ulcerans* infection

To determine if the down-modulation of lymphocyte-derived cytokines in BUD patients could be due to infection-induced cell death, we assessed the total white cell count together with $CD4^+$, $CD8^+$ and $CD19^+$ peripheral blood lymphocytes by means of complete blood count and flow cytometry analysis in order to determine if there are any differences in blood cell counts between patients and controls, if there were changes in the lymphocyte subpopulations in patients with BUD and if there were any changes in the lymphocyte subpopulations with treatment.

4.4.1 Characteristics of BUD Patients and Healthy controls recruited

Table 10 shows the characteristics of 58 patients and controls recruited as part of this aspect of the study. The median age (range) of BUD patients was 15 (5-47) years; 20 were males and 17 were females. Twenty-one healthy subjects from the same BU endemic communities with median (range) age of 16 (8-35) years (9 males and 12 females) served as controls. The age range of all the study participants was 5 – 47 years. There were no statistically significant differences in age or gender among the subject groups. Of the 37 patients, 8 presented with nodules, 9 plaques with surrounding edema and 20 with established ulcers. Based on the WHO categorization of BUD, of the lesions presented 14 were category I, 20 category II and 3 category III lesions.

Table 10: Characteristics of Study Participants

Parameters	BUD n =37	Controls n =21	Total n =58	*P* value
Sex (%)				
Male	20 (54)	9 (43)	29 (50)	0.585
Female	17 (46)	12 (57)	29 (50)	
Age				
Median (Range)	15 (5-47)	16 (8-35)	15 (5-47)	0.789
Lesion type (%)				
Nodule	8(22)			
Plaque and edema	9 (24)			
Ulcer	20 (54)			
Cat. of lesion				
I	14(38)			
II	20 (54)			
III	3 (8)			

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4.4.2 Blood cell counts and flow cytometry analysis

Absolute counts of neutrophils, monocytes, lymphocytes and eosinophils in fresh peripheral blood were determined with a hematology analyzer to find out if there were differences between patients with Buruli ulcer and healthy controls. While one would expect variations in the numbers of white blood cells in infected individuals, absolute counts of neutrophils, monocytes and eosinophils were comparable in BUD patients and controls (Figure 13). The total number of lymphocytes was slightly reduced in patients, but remained within normal values (1000-3700 cells per µl).

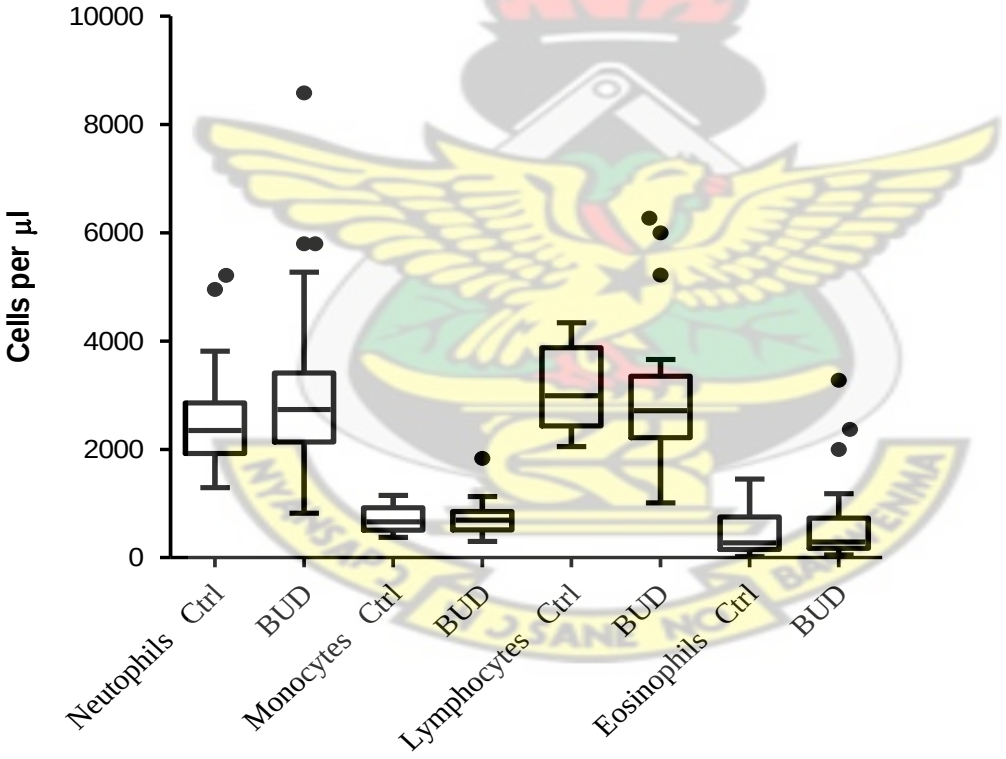

Figure 13: Leukocyte counts, presented as Box and Whiskers, in the blood of Buruli ulcer (BUD) patients and Healthy controls (Ctrl), as determined by complete blood count.

Using flow cytometry analysis as demonstrated in Plate 5 to 8, there was no difference in the lymphocyte subsets between the BUD patients and healthy controls (Figure 14A). But when the data was stratified based on the category of the disease presented, there was a statistically significant difference between the BUD patients with severe disease (Category II and III) and the healthy controls (*P*= 0.0395) as shown in (Figure 14B).

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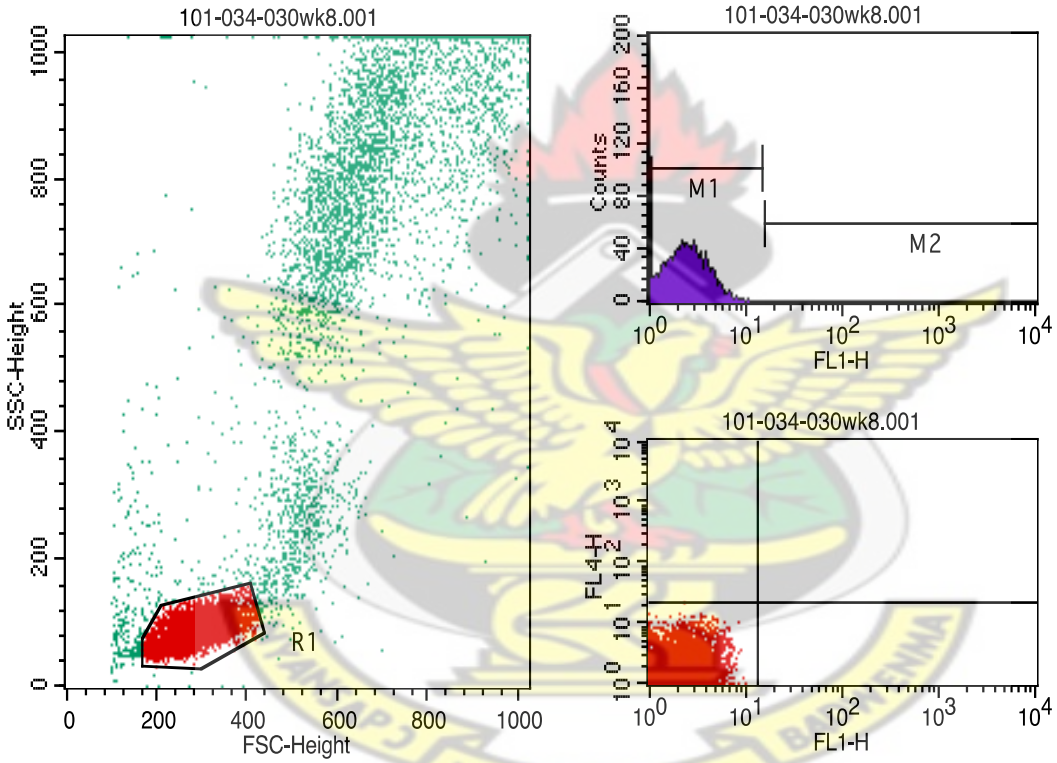

Plate 5: BUD Patient peripheral blood lymphocytes analysis by flow cytometry, unstained sample to serve a background control. Sample acquisition and analysis by BD CellQuest software.

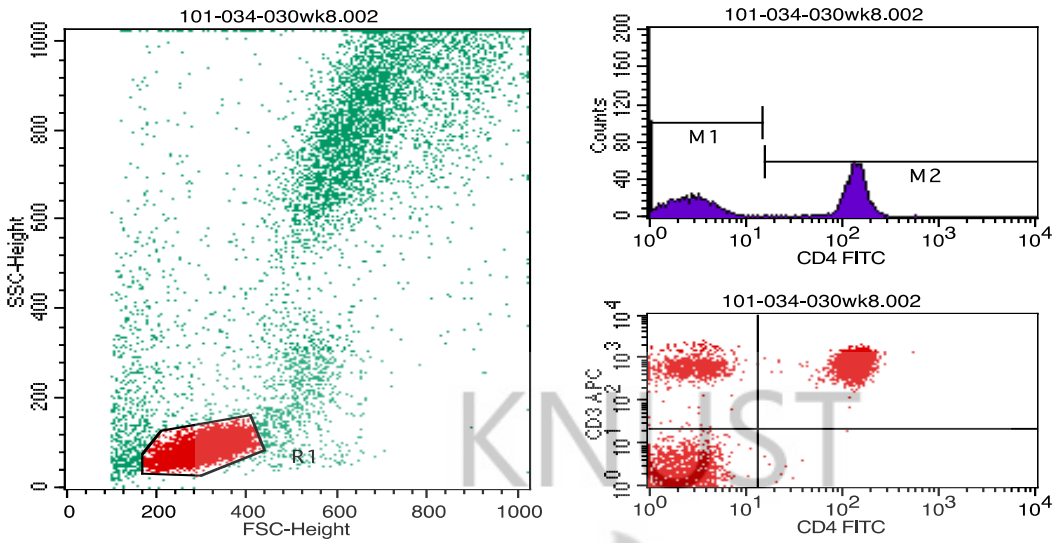

Plate 6: BUD Patient peripheral blood lymphocytes analysis by flow cytometry, sample was stained with FITC-conjugated mouse Anti-Human CD4 monoclonal Antibody and APC-conjugated Anti-Human CD3.

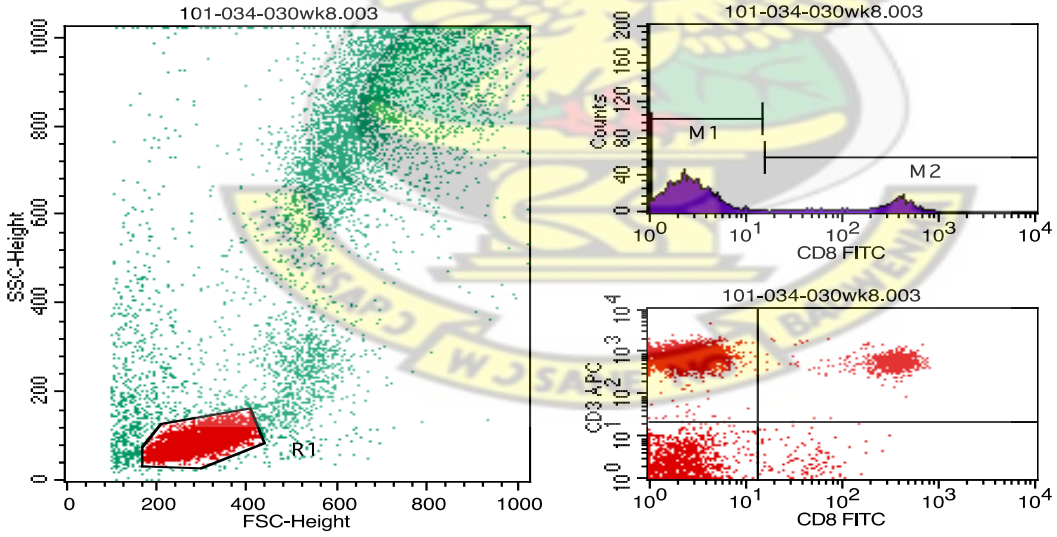

Plate 7: BUD Patient peripheral blood lymphocytes analysis by flow cytometry, sample was stained with FITC-conjugated mouse Anti-Human CD8 monoclonal Antibody and APC-conjugated Anti-Human CD3.

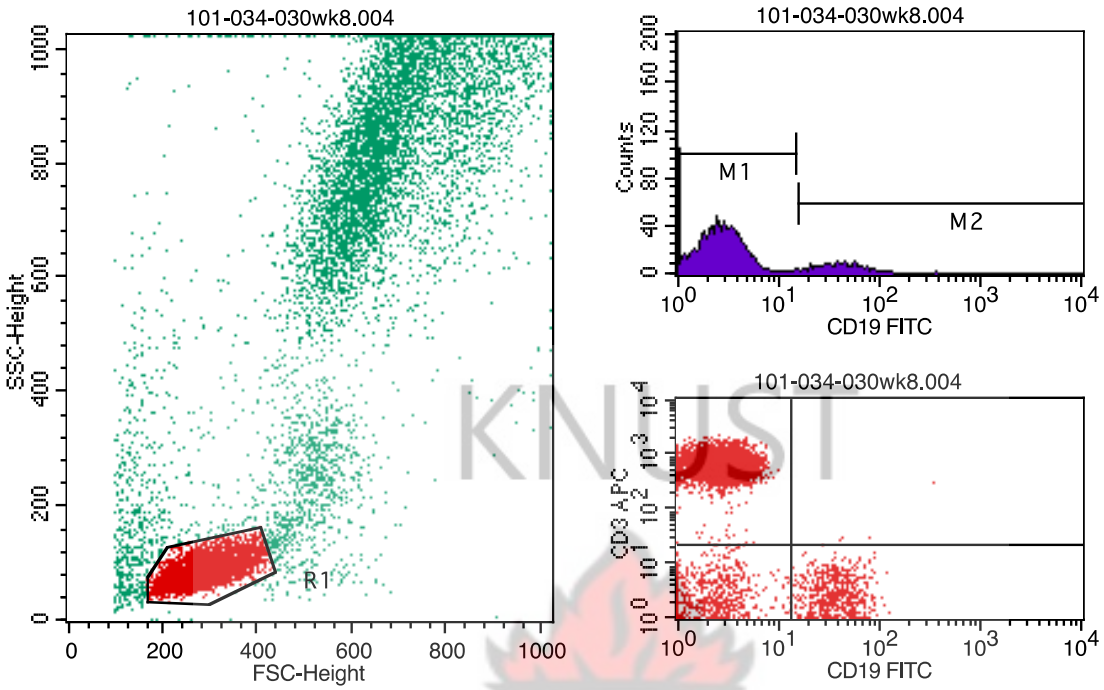

Plate 8: BUD Patient peripheral blood lymphocytes analysis by flow cytometry, sample was stained with FITC-conjugated mouse Anti-Human CD19 monoclonal Antibody and APC-conjugated Anti-Human CD3.

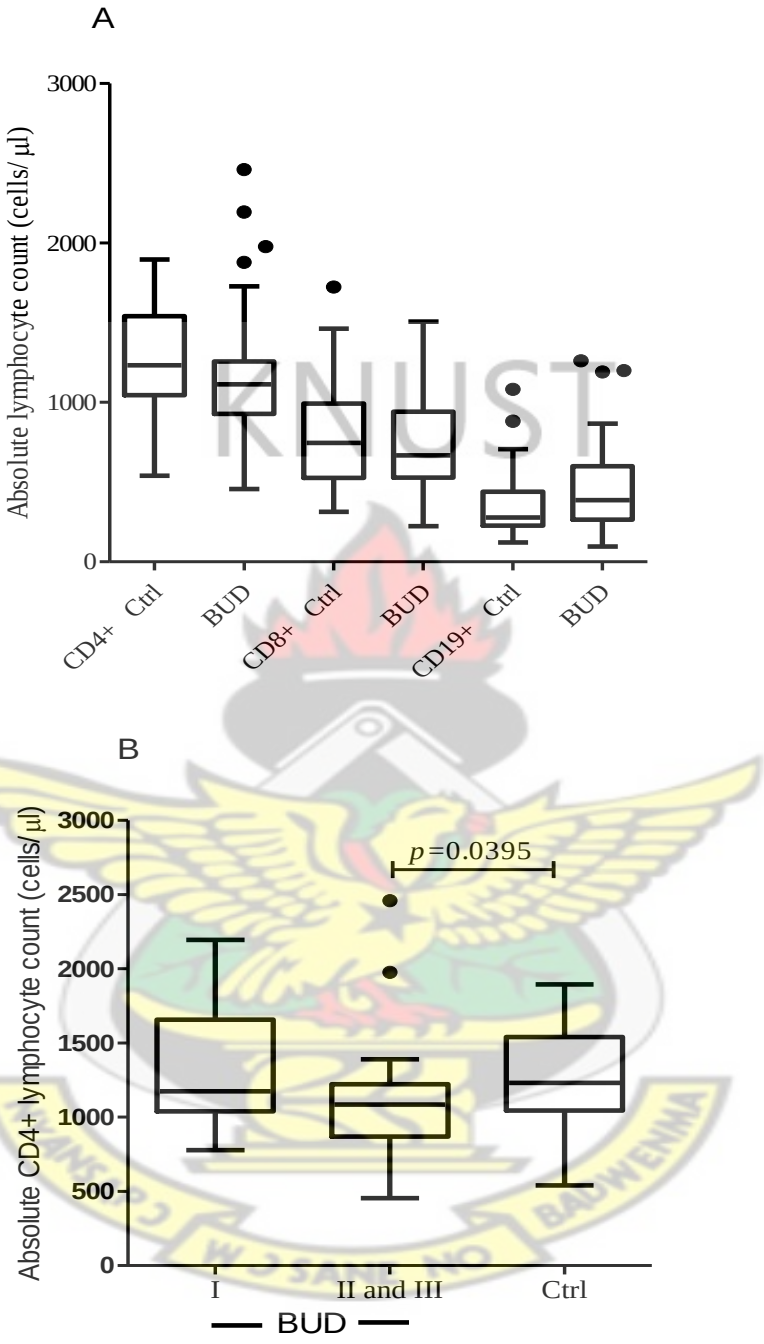

Figure 14: Comparison of the lymphocyte subpopulations between BUD patients and healthy controls, presented as Box and Whiskers plots, outlier cut-off determined by Tukey's test.

4.4.3 Lymphocyte subpopulations in Buruli ulcer patients with standard chemotherapy treatment

To find out if a change in lymphocyte sub-populations in Buruli ulcer patients contributed to this, patients were administered standard therapy by streptomycin 15mg/kg and rifampicin 10mg/kg by the attending physician. Whole blood from BUD patients were obtained at baseline and at week 8 after standard SR8 therapies and analyzed by flow cytometry.

Table 11 shows the lymphocyte cell populations of 25 patients before and after treatment. Six patients failed to comply with treatment. The mean CD4+ cell count was 1180 $\pm$ 471.6 before and 1196 $\pm$ 488.3 after treatment; mean CD8+ cell count was 740.7 $\pm$ 315.6 before, and 793.1 $\pm$ 486.5 after treatment; mean B cell 477.3 $\pm$ 318.7 before, and 452.2 $\pm$ 273.8 after treatment. There was no significant difference in lymphocyte sub-populations before and after treatment.

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Table 11: Comparison of the mean count of T-lymphocyte subsets and B-lymphocytes in BUD patients before and after standard therapy

Lymphocyte Subsets	Cell Count (Mean ± SD)		*P* value
	Before treatment	After treatment	
CD4+	1180 ± 471.6	1196 ± 488.3	0.5615
CD8+	740.7 ± 315.6	793.1 ± 486.5	0.4788
B-cells	477.3 ± 318.7	452.2 ± 273.8	0.5493

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4.5 Co-infection with *Mycobacterium ulcerans* and *Mansonella perstans* among Buruli ulcer patients in Ghana and the effect on the immune and treatment response

During an investigation into the immunopathogenesis of Buruli ulcer the filarial nematode *Mansonella perstans* was observed in preparations of peripheral blood mononuclear cells (PBMCs) from 15 patients. This led us to consider whether this organism was involved in transmission or pathogenesis of *M. ulcerans* infection which causes Buruli ulcer or if it was just an incidental finding.

4.5.1 Report of Patients with M. ulcerans and *M. perstans* co-infection

The first patient with Buruli ulcer and *M. perstans* infection was a 40 year old man who presented with a 5-week history of fever and an ulcerative lesion of the hand measuring 170mm by 135mm in diameter. Swabs obtained from undermined edges of the ulcer were confirmed positive for *Mu* by IS2404 PCR. Microscopy of PBMC from 10ml of heparinised blood incubated in culture wells for an overnight assay showed motile filarial worms identified as *Mansonella perstans* by their small size, and absence of sheath (Plate 9). Eosinophilia was not investigated in this patient. Two weeks later, similar observations were made in a second patient who was found to have 15.6% eosinophilia on a full blood count. Subsequently all Buruli ulcer cases and household contacts attending clinic between August 2010 and December 2011 were assessed for *M. perstans* co-infection and 66 clinically suspected cases of *Mu* disease were identified. Thirty of their household contacts were traced for investigation as controls. Nine communities in Agogo sub-district of Asante Akim North District which had *M. perstans* infections included Agogo-Zongo, Senkyeaso, Nshyieso, Ananekrom, Sempon, Kwame Addo, Bebuso, Afrisere, and Dukusen.

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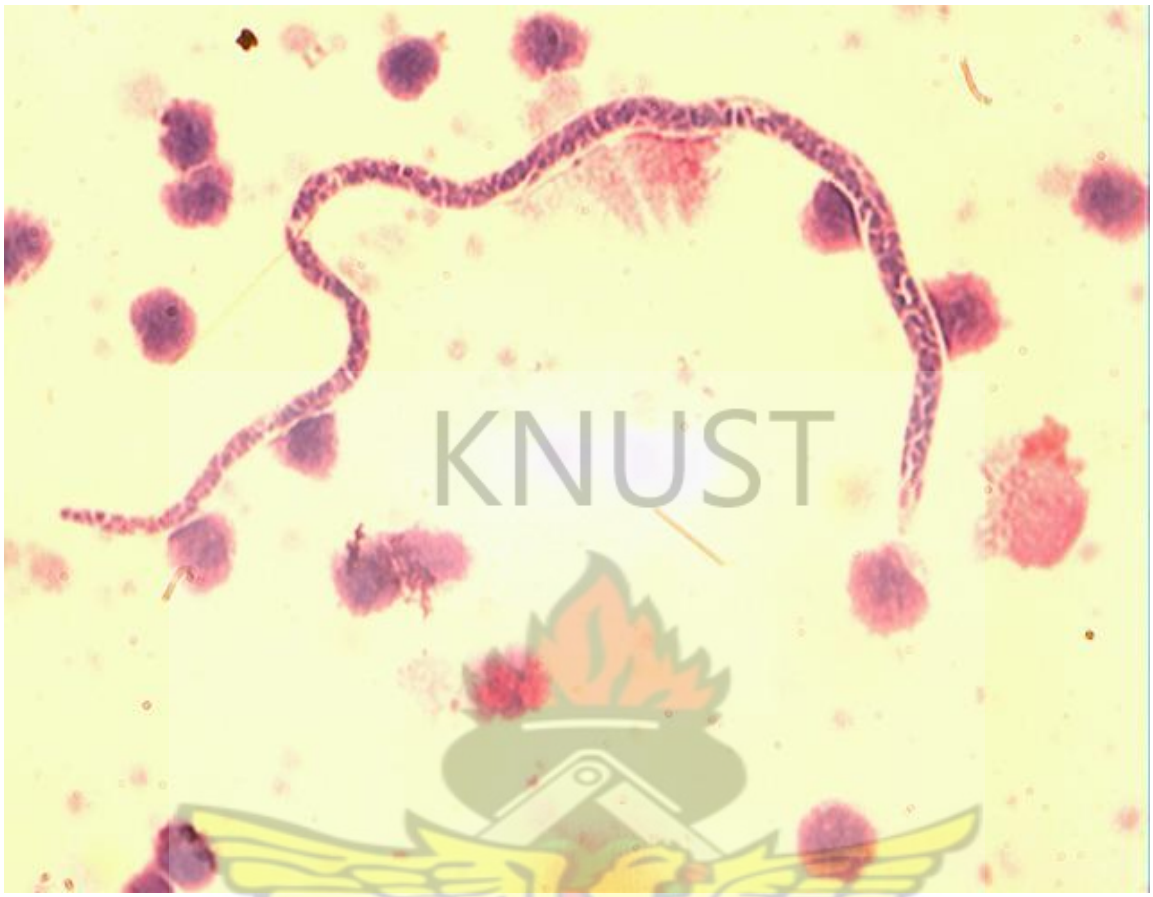

Plate 9: *Mansonella perstans* nematode in peripheral blood mononuclear cells from Buruli ulcer patients. (Magnification X1000). *M. perstans* can be distinguished from those of *Loa loa* and *Wuchereria bancrofti* by their relative small size, detection in blood taken during the day.

4.5.2 Demographics of patients co-infected and mono-infected cases

Table 12 shows a comparison of demographic and clinical features between co-infected patients and those with *Mu* disease. There was male gender preponderance in the co-infected group. There were no significant differences in the forms and categories of *Mu* disease between the two

groups. Three patients in the co-infection group complained of pruritus compared with none in the Buruli ulcer mono-infected group.

Table 12 shows that the median age (range) of *Mu* disease cases at 21 (5-53) years was similar to that of household controls at 19 (7-62). There were 30 males and 36 females with *Mu* disease while their household contacts were 14 males and 16 females ($p>0.05$). All forms of *Mu* disease were seen, including 22 (33%) nodules, 19 (28%) plaques with surrounding oedema and 25 (38%) ulcers. All 66 clinically suspected cases of *Mu* disease were confirmed positive by PCR for IS*2404*. Fifteen out of 66 (22.7%) patients with *Mu* disease were co-infected with *M. perstans* while 4 out of 30 (13%) controls had infection with *M. perstans* with no significant difference.

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Table 12: Characteristics of patients with active *M. ulcerans* infection, (monoinfected and or co-infected with *M. perstans*) and of household contacts.

	***Mu* co-infected with *Mp*†**	***Mu* monoinfected**	***Combined Mu** monoinfected and Mu co-infected with Mp**	**Household contacts**
	n = 15	n = 51	n = 66	n = 30
Age years Median (range)	25(10-42)	20 (5 – 53)	21 (5 – 53)	19 (7 – 62)
Sex M: F	9:6	19:32	30:36	14:16
Forms of *Mu* infection N (%)				
Nodule	8 (53)	14(27)	22 (33)	-
Plaque/oedema	2(12)	17(33)	19(28)	-
Ulcer	5(35)	20(40)	25(38)	-
Category of *Mu* infection N (%)				
I	9(59)	32(63)	41(62)	-
II	4(29)	11(22)	15(23)	-
III	2(12)	8(16)	10(15)	-
Diagnostic confirmation by PCR +/-	15/0	51/0	66/0	-
***M. perstans* co-infection +/- (%) 95% CI**	-	-	15/51 (22.7) 14.1-31.3	4/26 (13.3) 6.3-20.3

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4.5.3 Effect of *M. perstans* co-infection on Buruli ulcer disease treatment response

Mu infection was treated for 8 weeks with daily rifampicin and streptomycin (RS8) in all patients. Doxycycline 200mg and ivermectin 150 µg per kilogram of body weight were administered to patients with confirmed *Mansonella perstans* co-infection for 6 weeks starting from the second to the eighth week after the start of RS8 for *M. ulcerans* disease. All patients completed treatment but 9 were lost to follow-up during the 12-month follow up as they had to migrate due to tribal conflicts. All Buruli lesions reduced in size during treatment and none enlarged. Fourteen lesions in patients with co-infection completely healed by 58 weeks (median 20 weeks, (95%CI 14.6 - 30.2) and 41 of those with monoinfection by 50 weeks (median 21 weeks (95%CI 16.7 - 25.5) without surgery. Figure 15 shows that there was no significant difference in the median time to healing between the two groups. Time to healing was not available for one patient in the *Mu and Mp* co-infection group and 9 in the *M. ulcerans* monoinfected group due to loss to follow-up. After treatment for microfilariasis, pruritus subsided but viable microfilariae were still seen in subsequent PBMC cultures from all patients.

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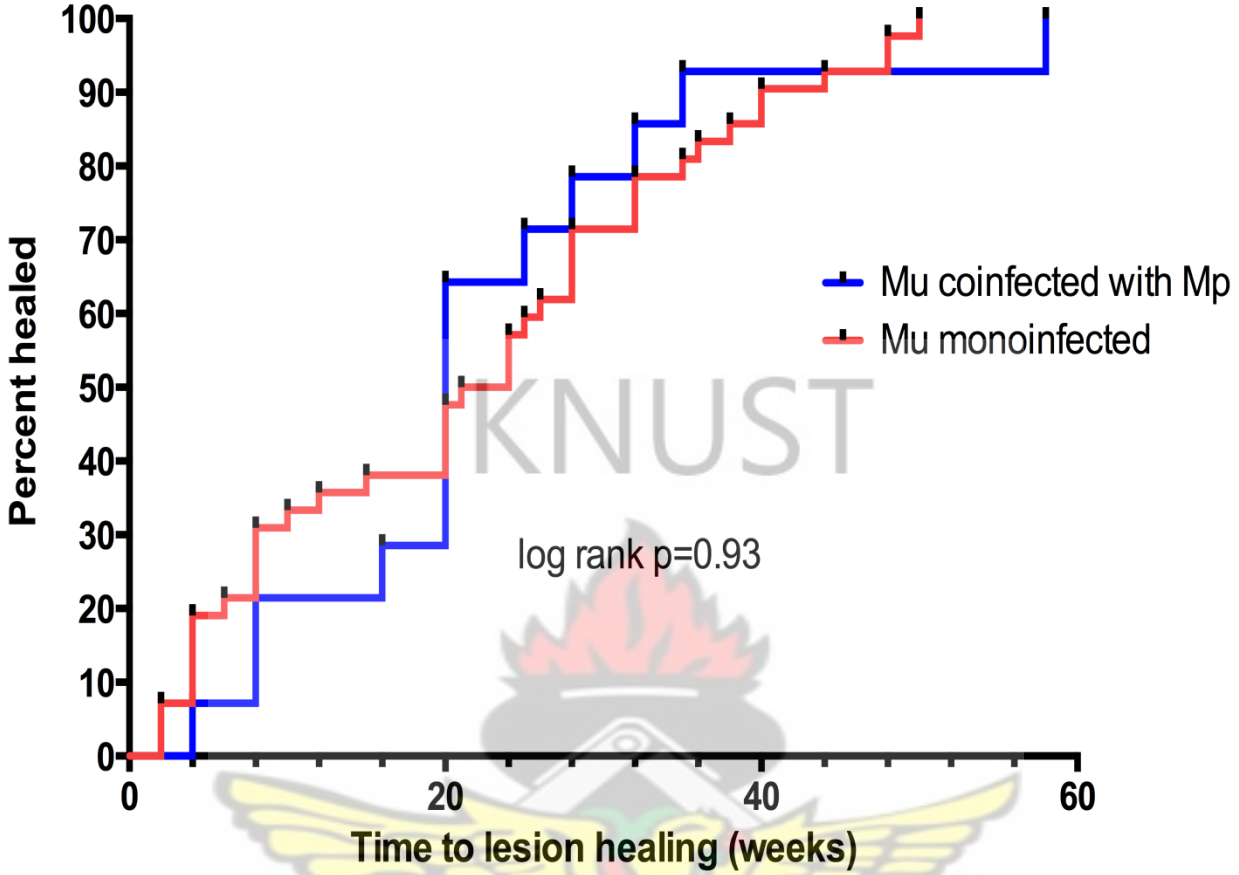

Figure 15: Survival analysis curve of cumulative healing for patients with *Mycobacterium ulcerans* infection who were co-infected with *Mansonella perstans* nematodes compared with those who had *M. ulcerans* monoinfection. No difference in cumulative healing was found between the 2 groups.

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4.5.4 Effect of *M. perstans* coinfection on TH-1 type immune response in BUD patients

The finding of *M. perstans* in the blood of patients with Buruli ulcer has not been reported before and it raises questions regarding the impact of human filariasis on the immunological polarization. We set out to first establish if coinfection made it less likely to develop a TH-1 type immune response measured by IFN gamma response. Supernatants from patients PBMC cultures stimulated with *M. ulcerans* lysate were collected from 34 monoinfected patients and 11 coinfected patients after 5 days incubation in 5% CO_2 at 37 ^{0}C and IFN- gamma response measured by ELISA. The median IFN-γ response of co-infected patients (3381pg/ml, 95% CI 915-8818) was not significantly different from those of monoinfected patients (1739 pg/ml, 95% CI 1774-4463) as shown in Figure 16.

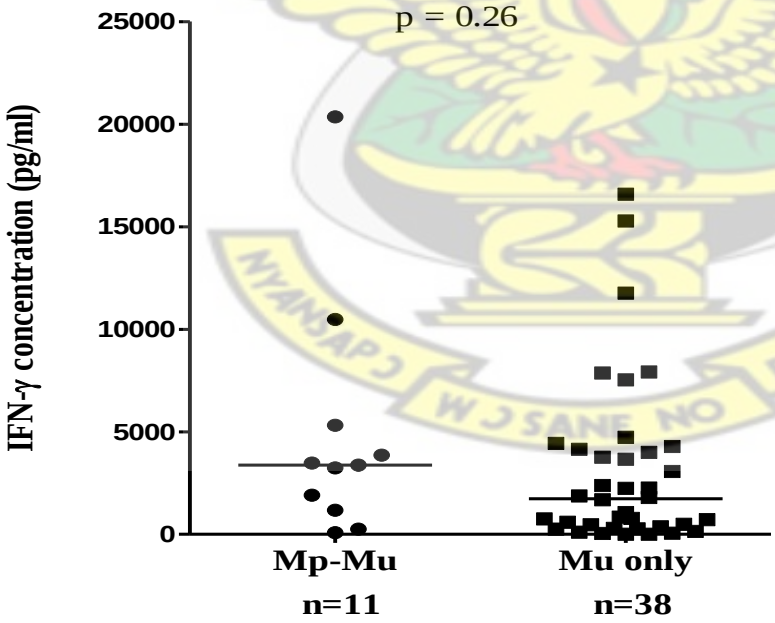

Figure 16: IFN-γ response to MU lysate 1 of patients with M ulcerans infection with co-infection with *M. perstans* (Mu+Mp) and *M. ulcerans* disease only (Mu).

CHAPTER 5

DISCUSSION

5.1 Laboratory confirmation of clinically suspected cases of *Mycobacterium ulcerans* disease (Buruli ulcer)

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Laboratory confirmation of clinically suspected cases has become an important step in management of the disease with the introduction of the WHO recommended antibiotic therapy (World Health Organization., 2012) as the first line of treatment. Accepted laboratory techniques available now for the confirmation of BUD are *M. ulcerans* isolation by culture, histopathology (Herbinger et al., 2009), smear microscopy for acid-fast bacilli (AFB) and polymerase chain reaction (PCR) for detection of the *M. ulcerans* specific insertion sequence IS*2404* (Phillips et al., 2009, Ross et al., 1997, Stinear et al., 1999). Due to the technical difficulties associated with culture for *M. ulcerans* and the low availability of histopathology, smear microscopy and IS*2404* PCR are the investigations most commonly used for case confirmation (Beissner et al., 2010). However smear microscopy and IS*2404* PCR present their own challenges. IS*2404* PCR is regarded as the gold standard due to its high sensitivity and specificity but it is expensive and needs a sophisticated laboratory setup and technical expertise that is not always available in resource-poor endemic communities. The need to transport diagnostic samples to specialized laboratories for case confirmation delays treatment in sites lacking experienced clinicians and treatment only initiated following laboratory confirmation and increases the cost of case management. Until a point of care diagnostic test that can be used in primary and secondary

health facilities is developed, the most suitable option is to improve the sensitivity of AFB detection by ZN microscopy as a first-line laboratory-confirmation technique for BU as it is for TB. Our study has demonstrated that obtaining two samples and immediately preparing smears on slides at the treatment centres for later microscopy in the laboratory increased the sensitivity from 50% to 55% for FNA samples and from 50% to 57% for swab samples. These sensitivities are comparable to when sample concentration techniques such as pooling or 3mm bead-beating with vortexing were used to release bacteria from swabs (Yeboah-Manu et al., 2011). The advantage of this technique over the concentration method is that it does not require expensive equipment and the processing time was significantly reduced. An independent person for quality assurance purposes could easily review stained slides kept securely in their slide cases. This is a larger study of the use of microscopy and employs a simpler technique than the concentration method in a retrospective study from Ghana and Togo, when similar sensitivity of microscopy of 58% for 36 FNA and 46% for 69 swabs was reported (Herbinger et al., 2010).

The sensitivity of PCR (86-87%) was significantly higher than that of ZN microscopic examination (55-57%) ($P < 0.01$) which is comparable to an earlier study (Yeboah-Manu et al., 2011). Further stratification by lesion type made the results more interesting since the sensitivity at 63% for oedemas remained the same whether one slide was or two were examined. These results show that it may not be necessary to take multiple samples from an edematous lesion, and that one sample is as good as processing two. Plaques and nodules however, show significant improvement in terms of sensitivity when two samples were processed. Notably, the sensitivity of FNA from plaques increased from 56% to 62% after examining two slides instead of one. A similar trend was observed for nodules where the sensitivity increased from 43% to 48%. These

results support the recommendation for two samples to be processed per case thereby increasing the sensitivity of the ZN microscopy for Buruli ulcer confirmation.

5.2 The immune status and T cell cytokine expression pattern of BU patients under therapy and healthy contacts

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Interferon gamma is a major cytokine of TH-1 cells. It promotes macrophage activation, nitric oxide production and cytotoxic-T lymphocyte proliferation. The pivotal role of IFN-γ in orchestrating a TH-1 immune response to mycobacterial infections has been shown extensively in animal models of tuberculosis (Cooper et al., 1993, Flynn et al., 1993) and in BU disease patients (Phillips et al., 2006, Sarfo et al., 2009). In this study, IFN-γ was used as a marker to describe the immune status of BU patients before, during and after standard treatment compared with healthy contacts. Patients produced statistically significant increase in IFN-γ to *M. ulcerans* antigens (*MU lysate and Ag85A ulcerans*) and not to the *M. tuberculosis* antigen *Ag85A tub* or PPD suggesting a level of specificity. The response was however non-specific with the 5 days PBMC stimulation. Long-term stimulation assays (5 days stimulation) induces central memory response, and may be the reason for the non-specificity. Since the controls are living in the same endemic communities and are contacts to the cases, they may have memory of the infectious agent as has been previously demonstrated (Diaz et al., 2006). But the results from the short time whole blood stimulation assay shows that, contrary to what has been shown in active tuberculosis patients, where most have depressed IFN gamma secretion in response to specific antigen stimulation of PBMC (Jo et al., 2003). Buruli ulcer patients are capable of mounting high

IFN-γ secretion in response to specific *Mu* antigens. This clearly indicates that Buruli ulcer patients are capable of mounting an appropriate immune response with the right stimulus.

In this study, patients with active BU were divided into those with established ulcers and those with pre-ulcerative/early lesions (nodules, plaques and edemas). They were also categorized (I, II, or III) based on the severity of their disease presentation. The result showed that, patients with established ulcers produce significantly higher IFN-γ response than those with pre-ulcerative lesions after peripheral blood mononuclear cells were stimulated with *M. ulcerans lysate* antigen and cultured for 5 days. This result is similar to that of the study carried out by Phillips and colleagues when they stimulated whole blood with the same antigen and cultured for only 24 hours (Phillips et al., 2006). In contrast to two earlier studies carried out in Australia and French Guyana by Gooding and colleagues, and Prevot et al., 2004 respectively in which IFN-γ secretion in response to antigen stimulation was lower in ulcerative lesions than in pre-ulcerative forms. Even though, assay technique in the current study was similar to these two studies (Gooding et al., 2002, Prevot et al., 2004). The long incubation assays may provide an insight into effector and regulatory T-cell responses; the 24h assay probably focuses on the prevailing memory responses to *M. ulcerans* antigen. Prevot et al. found that nodular forms (n = 4) of BU patients produced significantly higher levels of IFN-γ than ulcerative lesions (n = 9). Additionally, PBMC from patients when stimulated for 5 days, showed no difference in IFN-γ response across the different categories. However, in the whole blood assay, there was statistically significant increase in IFN-γ response in the category II lesions compared with category I lesions. From our results, we realized that IFN-γ response development in different individuals is highly heterogeneous and it is therefore essential to include adequate numbers to

obtain a balanced view as was suggested by Phillips et al. (Phillips et al., 2006). And clearly, the 102 subjects in this present study were higher than in Prevot's work, which could account for the difference.

The finding that IFN- γ secretion is depressed in early lesions compared to late established ulcers in this study supports the suggestion that in early stage of the disease, there is strong suppression of the protective cellular immune response facilitated by rapid proliferation of bacilli (Kiszewski et al., 2006). This is possible because, rapid proliferation of bacilli leads to increased mycolactone secretion, which is known to be cytotoxic to phagocytic cells by apoptosis and necrosis. Another possible explanation is that, because ulcerative lesions have been present for longer and more bacilli replication has taken place, coupled with the finding that longer-lasting lesions showed granulomas (Kiszewski et al., 2006). More quantities of antigens in the tissues of these lesions could stimulate a more rapid immune response compared to early lesions with fewer antigens.

The essential role of IFN-γ in orchestrating a TH-1 response to mycobacteria infection has been demonstrated in animal models infected with TB (Cooper et al., 1993, Flynn et al., 1993). Most patients with active tuberculosis have depressed IFN-γ secretion in response to specific antigens stimulation of peripheral blood mononuclear cells, which significantly improves after antituberculous therapy (Jo et al., 2003). In this study, the kinetics of IFN-γ production after stimulation of peripheral blood mononuclear cells with *M. ulcerans* specific antigens was investigated. After treatment with combination rifampicin and streptomycin, the results showed increase in secretion of IFN-γ after 6 weeks in both patients with ulcerative and pre-ulcerative

lesions (Figure 4) with the highest increases in those with pre-ulcerative lesions. This result is consistent with similar work done in Ghana, where it was shown that after 8 weeks combination rifampicin and streptomycin treatment, whole blood stimulated with *M. ulcerans* specific antigens produced significant IFN-γ response than at baseline (Sarfo et al., 2009). This could be attributed to antibiotic killing of *M. ulcerans* bacilli and hence decrease bacilli numbers leading to less mycolactone production which is known to be cytotoxic to macrophages and dendritic cells required for antigen presentations. These findings support the idea that the killings of M. ulcerans by antibiotics permit phagocytosis of organisms and provide macrophages and dendritic cells with more opportunities to present antigens resulting in increase secretion of IFN-γ.

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Patients with laboratory confirmed *M. ulcerans* disease (Buruli ulcer) responded well to treatment with rifampicin and streptomycin antibiotic combination therapy. All the lesions healed within 48 weeks of follow-up with no recurrence reported within the period. Larger lesions took longer time to completely re-epithelialize as expected, with some needing skin grafting. This result is similar to observations made in similar studies conducted in Ghana and Benin (Chauty et al., 2007, Sarfo et al., 2009) respectively, where the same antibiotics were used. Some lesions healed more rapidly than others despite being similar in size initially. Further analysis of their TH-1 response via IFN-γ gave an insight into why this happened. It was observed that patients with nodules that produced higher response of IFN-γ after stimulation of their PBMC with *M. ulcerans* specific antigens resolved or healed more rapidly than those with lower IFN-γ secretion. This observation could be the reason why about a third of pre-ulcerative lesions healed spontaneously without any treatment (Revill et al., 1973). It also supports the suggestion that in some early lesions, the production of mycolactone is low, allowing rapid

development of TH-1 response. The speculation that such early lesions that showed strong IFN-γ response could have healed without treatment (Sarfo et al., 2009) still remains a hypothesis yet to be tested and proven.

The BCG vaccination has been administered for over two decades (Einarsdottir and Huygen, 2011), and is routinely given to newborns in developing countries for protection against childhood tuberculosis (meningitis, miliary TB). This study investigated BCG-mediated protection against Buruli ulcer by comparing IFN-γ response pattern between patients with BCG vaccination and those without evidence of the presence of a BCG scar, which is an indication of an effective vaccination. We have shown that there is no difference in the IFN-γ response pattern between the two groups. This result is supported by the results of two large clinical trials in Uganda, involving approximately 2500 and 9000 people respectively, where the effect of BCG vaccination against Buruli ulcer was examined. The results of this trial showed that BCG confers transient protection against Buruli ulcer in 47% of vaccinated persons that can last for up to a year (Anonymous, 1969, Smith et al., 1976). So given the time (median 15 year ago) that these patients that were recruited for this study received their BCG vaccination, the results was not very surprising.

Another theory that could be used to explain this finding is that, the limited protection of BCG could be due to antigenic differences between BCG and the disease-causing *M. ulcerans* strains. This is supported by the findings that recombinant BCG expressing *M. tuberculosis* antigens confer greater protection than standard BCG upon challenge with *M. tuberculosis* (Pym et al., 2003). A similar strategy of developing a recombinant BCG vaccine expressing *M. ulcerans*

antigens to increase BCG-mediated protection against BU has been suggested (Einarsdottir and Huygen, 2011).

The results from this experiment have clearly demonstrated that an effective anti-mycobacterial therapy leads to recovery of IFN-γ secretion in response to *M. ulcerans* antigens stimulation of PBMC from patients with *M. ulcerans* disease. The results show that a successful antibiotic therapy is correlated with duration and severity of disease as seen in patients with early lesions (nodular forms), which initially produce low IFN-γ but over a period with treatment, there was a significant recovery of IFN-γ secretion. The above results collectively, seem to suggest that IFN-γ could be used as an immune correlate of protection against *M. ulcerans* infection. We further demonstrated that, there was no BCG-mediated protection against BU especially in patients with BCG vaccination over 6 years old.

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5.3 Evaluation of Subunit protein vaccine candidates against *M. ulcerans* infection

Buruli ulcer is a neglected tropical disease (Mwanatambwe et al., 2000) with little knowledge on the exact mode of transmission. Although significant progress has been made in the management of the disease in endemic countries during the last decade, the disease still remains a major economic and social burden for some developing countries, in West Africa (Grietens et al., 2008). A more effective *M. ulcerans* vaccine would help to control this debilitating disease that affects mostly children. The protective immune response against mycobacterial infections

appears to involve TH 1 response, including the production of IFN-γ by sensitized CD 4^+ and CD 8^+ T-cells to induce macrophage activation (Orme, 1993, Tascon et al., 1998). In order to provide a more accurate correlate of vaccine-induced protection, current studies have used in vitro assays, using IFN-γ production as an indicator of protection.

Antigen 85A is a major secreted component in the culture filtrate of many mycobacteria such as *M. bovis BCG*, *M. tuberculosis* and *M. avium* subsp. *paratuberculosis* (Rosseels et al., 2006). An earlier study with DNA-prime protein boost strategy using specific *M. ulcerans* antigen 85A showed protective efficacy in animal models. This reduced bacteria load by approximately hundred fold and offered protection comparable to that of BCG vaccine (Tanghe et al., 2008).

Majority of BU patients and their contacts responded to both subunit proteins (e.g. MUL3720 and MUL4978) an indication that both proteins are immunogenic. Differential T-cell responses against these antigens in Buruli ulcer patients and healthy contacts were not detected which does not suggest a specific activity of the vaccine candidates linked to protection. Under therapy, T-cell immunity against vaccine candidates is increased when compared to baseline. This argues for T-cell suppression in BU patients before onset of therapy.

5.4 Alteration in Cell Populations in the peripheral blood of patients with *Mycobacterium ulcerans* infection

T-lymphocytes subsets, especially CD4+, play an important role in immunity against mycobacterium infections (Orme et al., 1993, Wu et al., 2009, Yu et al., 1995). The CD4+ T-

cells secretes cytokines, such as IFN-γ, which activates macrophages to destroy the bacteria with which they are infected. Some CD4+ T-cells are also reported to act as cytotoxic effector cells that can destroy the infected cells (Raja, 2004, Rodrigues et al., 2002, Tsao et al., 2002). These essential roles of CD4+ T-cells are known to be supported by other T-cells subsets such as CD8+ in the case of *Mycobacterium tuberculosis* infection (Boom et al., 2003). CD8+ cytotoxic T-cells are an important source of IFN-γ and may have the ability to kill bacteria via cytotoxic mechanisms (Serbina and Flynn, 1999, Smith et al., 1999). The role of B cells during mycobacterium infections is not well defined.

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The major finding of this study was a decrease in CD4+ T-lymphocytes counts in BU patients compared to their controls. There were no significant differences in the absolute lymphocyte count, CD8+ T-lymphocytes subsets and B-lymphocytes (CD3- CD19+). This result cannot be compared to any study in BU immunology since this is the first investigation to study the changes in lymphocyte subsets in peripheral blood of BU patients, despite the pivotal role of CD4+ and CD8+ lymphocytes in antimicrobial immunity. The results in however similar to several studies conducted in tuberculosis patients. In an observational study conducted in newly diagnosed pulmonary tuberculosis patients, the investigators observed a significant decreased in percentage of CD3+ and CD3+ CD4+ cells in patients compared with healthy controls (Wu et al., 2009). A similar result was reported in an earlier study, where patients with acute pulmonary tuberculosis had significantly lower percentages of CD4+ T-cells in the peripheral blood than controls (Deveci et al., 2006). A supportive mechanism to explain this finding was shown in a study using mice models. Fraga and colleagues showed that early during mouse infection with either mycolactone positive or negative strains, pathogen-specific IFN-γ producing T-cells

developed in the draining lymph node. CD4+ cells migrated to the infection foci, but as the infection progresses with virulent *M.ulcerans*, there is local depletion of CD4+ T-cells and abrogation of IFN-γ expression (Fraga et al., 2011). This may account for the decrease in CD4+ T-cells in peripheral blood of patients with severe forms of BU.

Overall, the concentration and viability of monocytes, neutrophils, T and B lymphocyte populations were intact in the peripheral blood of BU patients, showing that *M. ulcerans* infections does not impair innate and acquired immune responses by depleting these immune cell subsets. This result supports the suggestion that *M. ulcerans* infection may not lead to systemic immunosuppression.

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5.5 Co-infection of *Mycobacterium ulcerans* and *Mansonella perstans* among Buruli ulcer patients

We have shown that *M. perstans* was co-infected with *M. ulcerans* in 23% of 66 Buruli ulcer disease patients in the Asante Akim North District and in 13% of 30 healthy household contacts. This difference was not significant but the number of subjects, particularly in the group of household contacts, was small. The prevalence of *M. perstans* infection in the Ghanaian population is not known but in other African countries the prevalence has been found to be high, ranging from 0.4 to 50% in Uganda for example (Onapa et al., 2005).

There are several reasons why it could be important if there is a higher incidence of *M. perstans* infection in patients with *M. ulcerans* disease. Helminthic nematodes have been shown to polarize host immunity towards humoral and T helper type 2 mediated immunity and they may impede development of protective immunity (Salgame et al., 2013). *M. perstans* infection is a reasonable candidate for interference with BCG vaccination which is normally given at birth in Ghana and transplacental transfer of this nematode has been reported (Adolph et al., 1962). BCG confers some protection against *M. ulcerans* disease, particularly in the first 6 months after immunization. (Metenou et al., 2009). Wammes *et al*. found that children with helminth infestations responded poorly to BCG or malaria vaccination and this was independent of the type of worm infection (Wammes et al., 2010). *M. perstans* filariasis is predominantly found in rural populations and infection begins in childhood with maximal infection rates in children aged ten to fourteen years (Asio et al., 2009). Children of this age group are at a concomitantly higher risk of getting infected by *M. ulcerans*.

If immunity to *M. ulcerans* was affected by co-infection with *M. perstans* it might be expected that the healing rate of Buruli lesions would be slower in co-infected patients and also the immune response via IFN-γ will be reduced compared to those with monoinfection, but this did not seem to be the case in the patients. There was no difference in the median time to healing and IFN-γ of *Mu-Mp* co-infected patients compared to *Mu* monoinfected patients and there have been no recurrences. It was expected that recurrence rate would be higher in the co-infected patients due to possible compromised immunity but it was not the case and further studies are warranted. It is possible that the worm load may have an influence on the IFN gamma response, but in this study the worm burden was not assessed.

It is also possible that the same transmission route applies to *M. perstans* and *M. ulcerans* infection. *M. perstans* is transmitted by the bites of midges belonging to the genus *Culicoides* but it is not known whether *M. perstans* infected midges can be co-infected with *M. ulcerans*. Skin penetration seems to be a requirement for establishment of *M. ulcerans* disease in a guinea pig model (Marsollier et al., 2007) and it has been postulated that mosquito bites cause *M. ulcerans* disease in Australia (Johnson et al., 2007) so midge bites would be another possibility. Alternatively, *M. perstans* infection could be more likely to occur in patients with Buruli ulcer as a result of the open wound. However more than half of the co-infected patients in this series had nodules or plaques without any ulceration.

In contrast to onchocerciasis caused by *Onchocerca volvulus* and lymphatic filariasis caused by *Wuchereria bancrofti, M. perstans* responds poorly to treatment with ivermectin alone (Bregani et al., 2007). Based on the recent discovery of *Wolbachia* endosymbionts in *M. perstans* from Uganda (Buttner et al., 2003) and Mali (Coulibaly et al., 2009) and a report suggesting that doxycycline is effective (Hoerauf, 2009), patients with co-infection in the present study were treated with a combination of ivermectin and doxycycline for *M. perstans*, starting during WHO recommended rifampicin and streptomycin treatment for *M. ulcerans* infection. Although itching resolved during this treatment, *M. perstans* elimination did not occur after 12 weeks of repeat blood sampling. However, the time of repeat blood sampling may have been too early to demonstrate elimination. Others demonstrated reduction in pretreatment levels of *M. perstans* 6 months after treatment and undetectable levels 12 months after treatment that was sustained at month 36 (Coulibaly et al., 2009). There was no difference in the median time to healing of *Mu-Mp* co-infected patients and *Mu* monoinfected patients suggesting that the presence of *Mp* co-

infection did not significantly modulate the clinical response of BU to conventional antibiotic therapy. One may argue that since *Mp* infection was treated at the same time as treating BU, we cannot be sure that untreated *Mp* does not influence healing rate but we can since the treatment did not succeed in eliminating *Mp.*

Overall our findings suggest that *Mp* is a common infection in Buruli endemic communities of Ashanti Akim North district but a more interactive relationship cannot be ruled out. A larger study would be needed to find out if there is a greater prevalence of *Mp* infection in patients with Buruli ulcer.

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CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

Laboratory confirmation of clinical diagnosis of Buruli ulcer disease is vital; first to prevent the situation of misdiagnosis and also to have epidemiological data on new infections. Currently, there is over-reliance on PCR as the first line laboratory technique to confirm clinical diagnosis of the disease. This makes laboratory confirmation in resource poor endemic countries limited and disease management expensive. The usefulness of preparing two direct smears for each case instead of one on-site for ZN microscopy was evaluated as a means of improving the sensitivity of the technique, since this technique can easily be implemented in district hospitals where Buruli ulcer is endemic. After evaluation of the PCR technique with ZN microscopy, it was found out that examining two slides for each case improves the overall sensitivity of the technique to over 50% comparable to other methods previously described (Yeboah-Manu et al., 2011). This result clearly shows that about half of clinically diagnosed cases of Buruli ulcer could be laboratory confirmed on site, reducing the cost of management and delays in early treatment.

The main goal of this work is to describe the host immune response associated with *M. ulcerans* infection. Basic knowledge of the host immune response to *M. ulcerans* infection is necessary for preventive and treatment strategies. Until recently, there was very little literature available on the

human immune response to *M. ulcerans* infection (Gooding et al., 2001, Gooding et al., 2002, Gooding et al., 2003, Jo et al., 2003, Prevot et al., 2004). IFN-γ response was used as a marker of protective immune response to describe BU patients immune status associated with disease. It has been demonstrated that BU patients respond well to *Mu* specific antigens compared to other mycobacteria antigens, indicating immune specificity. Results from this study showed that patients with established ulcers produce more IFN gamma than those with pre-ulcerative lesions as described previously (Phillips et al., 2006). Furthermore, it has been shown that BU patients with depressed IFN-γ response prior to treatment recover after standard antibiotic therapy.

Buruli ulcer patients and healthy controls respond well to *MUL 3720* and *MUL 4987* antigens thus indicating that both proteins are immunogenic. However, there was no differential T-cell response to these antigens in patients and controls. In order to fully explore the potential of using these two as vaccine candidates, more work needs to be done to improve their specificity.

The concentration and viability of monocytes, neutrophils, T and B-lymphocytes populations were intact in the peripheral blood of Buruli ulcer patients, indicating that *M. ulcerans* infection does not impair innate and acquired immune responses by depleting these immune cell subsets.

M. perstans may be a common infection in Buruli ulcer endemic communities in the Ashanti Akim North district. We cannot also rule out a more interactive relationship, which requires future studies in this area.

6.2 Recommendations

1. The use of ZN microscopy as first line diagnostic tool in confirming Buruli ulcer disease as done in tuberculosis diagnosis should be encouraged.

2. Further studies to investigate recombinant *Ag85A* (*MUL 4978*) and *MUL 3720* from *M. ulcerans* as a potential candidates for vaccine against *M. ulcerans* infection.

3. A future study to further explore changes of lymphocyte subsets in the peripheral blood of Buruli ulcer patients in a larger cohort is advised. Further investigations on the various effector cells involved in protective immune response to *M. ulcerans* infection.

4. It is recommended for future studies to determine the predominant cell types responsible for interferon gamma secretion in Buruli ulcer patients

5. A study to investigate the interactive relationship between *M. ulcerans* and *M. perstans* co-morbidity in a Buruli ulcer endemic area and epidemiological survey to ascertain the extent of *M. perstans* infection in the Asante Akim-North district.

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APPENDIX

I. Procedures

Collecting Clinical samples for Laboratory Confirmation

Procedure for Obtaining Fine-Needle Aspiration (FNA)

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Before samples were taken patients were assured that the samples are required to find out what is causing the lesion (Nodule, Plaque or Edema), so that correct treatment can be given. The procedure is briefly explained to the patient.

Materials for FNA

1. 10 ml syringe and 12 or 22 gauge needle
2. Labeled tubes with transport media (CLS for PCR)
3. Labeled Microscope slides
4. Cotton - wool pads and disinfectant (70% ethanol)
5. Dressing materials
6. Disposable gloves

Procedure for FNA

Lesion was carefully cleaned with cotton-wool pads soaked in 70% ethanol by wiping the skin several times. The skin was palpated to locate the affected site, the needle inserted around the estimated centre of the lesion. With the needle in the lesion, a full suction is applied to create pressure and slowly the needle is moved back and forth in at least 3 different directions while maintaining suction pressure. The suction was gently released and the needle slowly withdrawn. Dry gauze was applied to prevent possible bleeding. Samples were the transferred to appropriate tubes and glass slides for microscopy.

Procedure for obtaining Swab Samples

Materials for Swab samples

1. Sterile cotton-wool on an applicator (Swab)
2. Disinfectant (70% ethanol)
3. Disposable gloves
4. Labeled tubes with appropriate media
5. Cotton-wool pads

Procedure for swab sample

After disinfecting the site of lesion, a sterile swab was gently inserted underneath the edges of the ulcer. The swab was used to wipe the tissue beneath the edges of the ulcer in a clockwise manner. The swabs are then placed in well-labeled tubes containing appropriate media and another to make direct smear on glass slides.

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APPENDIX

II. Buffers and reagents

Wash Diluent Reagents (WDR)

1X Phosphate Buffered Saline without calcium or magnesium, ready-to-use.

Note: Store opened bottles at 2-8° C as recommended until manufacturer's expiration date. Discard if visible signs of contamination, such as a cloudy appearance, develop.

Heat Inactivated FBS (HI-FBS)

FBS was heat inactivated using the instructions below:

- FBS was removed from the -20°C freezer. Thawed in the refrigerator (2 to 8°C), preferred, or for several hours at room temperature. Gently swirl two or three times over the course of the thaw.
- Placed FBS in a 56°C (55 to 57°C) water bath. Carefully monitor the water bath temperature. Higher temperatures can degrade components of the FBS.

Note: The water level in the water bath should cover the level of the FBS in the bottle, but not touch the cap of the bottle. This will help ensure even heating of the FBS and avoid contamination.

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- Once the water bath has returned to 56°C (55 to 57°C), heat the FBS for 30 minutes, mixing every 5 to 10 minutes. Heating for longer periods of time can degrade components of the FBS.

Note: If the top of the bottle comes in contact with the water bath then swab the top of the bottle with 70% v/v ethanol before opening.

- Mix the FBS gently but thoroughly using aseptic technique. Aliquot into sterile, labeled 50mL conical tubes.

Note: Labels should identify these tubes as "HI-FBS" (heat inactivated FBS) and include the lot number, the aliquot date, the expiration date. FBS is stable for 1 month at 2 to 8°C, or until the original manufacturer's expiration date if stored at-20oC.

- Refrigerate (2 to 8°C) the number of aliquot tubes needed for the expected workload. Mix well before use.

Note: Repeated freeze/thaw cycles will have an adverse effect on the quality of the FBS. Do not refreeze aliquots that have been stored at refrigerated temperatures.

The remaining aliquot tubes can be returned to the -20°C freezer and are stable until the original manufacture's expiration date. When ready to use the frozen aliquots, thaw in the refrigerator overnight, preferred, or for several hours at room temperature. Change the expiration date to one month. Mix well before use.

Staining Solutions

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1) Stock alcoholic fuchsin

Fuchsin (basic)	3g
Ethanol (95%)	100ml

The basic fuchsin is dissolved in 100 ml ethanol.

2) 5% Phenol solution

Phenol melted	5ml
Distilled water	95ml

To liquefy pure phenol crystals, loosen the cap of the phenol reagent bottle; place it into a hand warm water bath. Measure it with a warm pipette to avoid re-crystallization. Add the melted phenol slowly to the distilled water while stirring.

3) Ziehl's solution (working carbol fuchsin solution)

Stock alcoholic fuchsin	10ml
5% Phenol solution	90ml

Mix the stock alcoholic fuchsin with 5% phenol while stirring.

Filter the solution before use to remove fuchsin crystals or particles.

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4) 20% Sulphuric acid solution

Sulphuric acid (conc H_2SO_4)	20ml
Distilled water	80ml

Add the sulphuric acid slowly to the distilled water using a safety pipette.

5) 0.3% methylene blue solution

Methylene blue	0.3g
Distilled water	100ml

Dissolve the methylene blue in the distilled water.

DRB-PCR

Lyophylization of primers

Reagents

Purified destilled water (Carl Roth, Karlsruhe, Germany)

Primer MU5 primer stock

agc gac ccc agt gga ttg gt

(TibMolbiol, Berlin, Germany)

Primer MU6 primer stock

cgg tga tca agc gtt cac ga

(TibMolbiol, Berlin, Germany)

DNA Extraction Reagents

Reagents contained in the Genomic DNA Purification Kit

Cell Lysis Solution (CLS)

DNA Hydration Solution (DNA Hyd)

Protein Precipitation Solution (PPS)

Reagents not contained in the Genomic DNA Purification Kit

Ethanol

Glycogen 20 mg/ml

Isopropanol

Lysozyme 10 mg/ml

Proteinase K 20mg/ml

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Agarose gel electrophoresis

10 X TBE Buffer

100 bp DNA ladder

Agarose

Ethidium bromide

Loading Dye

Preparation of 6X loading dye for agarose gel-electrophoresis

All reagents were mixed according to the suppliers´ protocol(s).

Invitrogen
http://www.resgen.com/products/GelBuf.php3
0.25% bromophenol blue
(0.25% xylene cyanol FF)
30% glycerol

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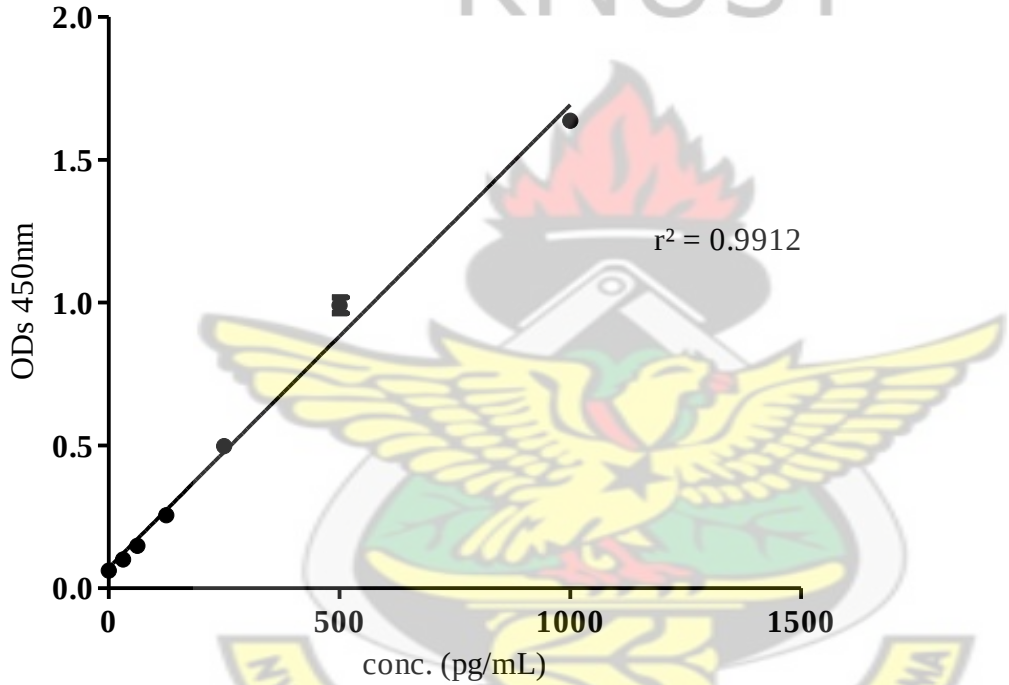
Materials and Reagents for Flowcytometry

Items	Catalogue Number	Company
5 ml Polystyrene Round-Bottom tubes	352054	BD Biosciences
FACS Lysis Solution	00-5333-57	eBioscience, USA
FACS Calibrite Beads	340486	BD Biosciences
FITC Mouse Anti-Human CD4	555346	BD Biosciences
FITC (SK1) CD8	345772	BD Biosciences
FITC (SJ25C1) CD19	345788	BD Biosciences
APC Mouse Anti-Human CD3	555335	BD Biosciences
Phosphate Buffer Saline 1X	14190	Gibco, Life Technologies

ELISA STANDARDIZATION

Example of IFN-γ standard Curve



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APPEDIX

III. Forms

Research data entry form, Patient

Laboratory Data Entry Form Country: Ghana Category: Patient

Date ……201……..

Use separate form for each "visit" and each lesion (if multiple lesions) per patient!

A: Patient ID

Hospital ……

Family name

…… First name ……

Village…… **Distric** ……

Age ……years **Sex** m ☐ f ☐ **BCG Scar** yes ☐ no ☐

B. **Classification** **New case** ☐ **Recurrence** ☐

C. **Clinical Presentation** **&** **Number of lesions**

☐ Nodule ☐ Papule ☐ Plaque Single lesion
☐ Ulcer ☐ Edema ☐ Osteomyelitis or Multiple lesions (……..)

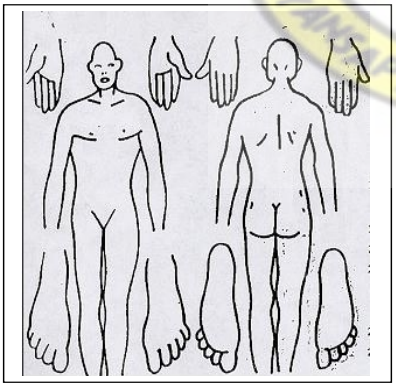
D.Visit (Time of sample collection)

Pre-treatment (V1) week 6 (V2) week 12 (V3) week 48 (V4) other: week……

E. **Duration of Disease** ..... day ..... weeks ..... months

F: Matched Control ID of control sample:……

G: Location of the Lesion



(Photographical) documentation: no☐ yes☐
(by……)

H: Treatment Data

Dosage of Rifampicin : ……mg/d

Dosage of Streptomycin : ……g/d
Dosage of other (name) : ……

Treatment Start Date : ……
Treatment End Date (if completed) :
……

I: Treatment outcome

☐ Antibiotic treatment not completed

☐

☐

Page 2

BURULIVAC Country: Ghana Category: Patient

J. Clinical samples WP6 ☐ WP1 ☐ WP5 ☐

Nodule, Papule, Plaque, Edema

ø **of nodule**...../ ..... mm Category .....

WP6 (*PCR **MIC+CUL)

FNA*	**O**	**Tissue punch ***	**O**	**Tissue surgery ***	**O**
FNA**	**O**	**Tissue punch ****	**O**	**Tissue surgery ****	**O**

WP 5 (individuals > 5 years only!)

Heparinized blood	**O**	**Serum**	**O**
> 10 years:	10 ml	>10 years:	3 ml
5-10 years:	6 ml	5-10 years:	2 ml

Ulcer

Ø **of ulcer**...../ ..... mm Category .....

WP6 (*PCR ** MIC+CUL)

Swab*	**O**	**FNA***	**O**	**Tissue punch***	**O**	**Tissue surgery***	**O**
Swab**	**O**	**FNA****	**O**	**Tissue punch****	**O**	**Tissue surgery****	**O**

WP 5 (individuals > 5 years only!)

Heparinized blood	**O**	**Serum**	**O**
> 10 years:	10 ml	>10 years:	3 ml
5-10 years:	6 ml	5-10 years:	2 ml

Other (Osteomyelitis)

WP6 (*PCR **MIC+CUL)

Swab*	**O**	**Tissue surgery***	**O**
Swab**	**O**	**Tissue surgery****	**O**

WP 5 (individuals > 5 years only!)

Heparinized blood	**O**	**Serum**	**O**
> 10 years:	10 ml	>10 years:	3 ml
5-10 years:	6 ml	5-10 years:	2 ml

K. **Remarks**

KNUST

Research data entry form, Control

Laboratory Data Entry Form **Country: Ghana** **Category: Control**

Date ……...201….

A: Control ID **Study Site**……..

Family name ……..…….. **First name** ……..……..

Village **District**

…….....……. ……..……..

Age ……years **Sex** m f **BCG Scar** yes no

B. Matched Control to ……..……(ID number patient)

Family member ☐ **Neighbour** ☐ **Other:** …….

Healthy Control without exposure

C.Visit (Time of sample collection)

week 0 (V1) week ..... (V2)

D. Clinical samples

WP5 (individuals > 5 years only!)

Heparinized blood	**O**	**Serum**	**O**
> 10 years:	10 ml	>10 years:	3 ml
5-10 years:	6 ml	5-10 years:	2 ml

E. **Remarks**

KNUST

Buruli ulcer clinical and treatment form – new case **BU 01.N**

Health facility: _____
Name of health worker treating patient : _____
Date of clinical diagnosis or admission (dd/mm/yy): ___/___/___
Date of complete healing (dd/mm/yy): ___/___/___

Name of patient: _____ ID#:_____
Address (village or town): _____ District: _____
Province/Region/State: _____ Country: _____
Age (yrs): _____ Sex: ☐ Male ☐ Female
Weight (kg): _____ Profession: _____

CLINICAL HISTORY AT DIAGNOSIS

Duration of illness before seeking care (weeks): _____
Use of traditional treatment: ☐ Yes ☐ No
Limitation of movement at any joint: ☐ Yes ☐ No
Previous treatment with streptomycin: ☐ Yes (duration in days: _____) ☐ No

REFERRED BY: ☐ Village health worker ☐ Self-referral ☐ Former patient ☐ Family member ☐ Schoolteacher ☐ Health worker ☐ Other (specify): _____

CLINICAL FORMS

☐ Nodule (N) ☐ Plaque (Q) ☐ Oedema (E) ☐ Ulcer (U) ☐ Osteomyelitis (O) ☐ Papule (P)

CATEGORIES ☐ **Category I:** A single lesion ≤ 5 cm in diameter ☐ **Category II**: A single lesion 5–15 cm in diameter ☐ **Category III**: A single lesion > 15 cm in diameter, multiple lesions, lesions at critical sites, osteomyelitis

LOCATION OF LESION(S) ☐ Upper limb (UL) ☐ Lower limb (LL) ☐ Abdomen (AB) ☐ Back (BK) ☐ Buttocks and perineum (BP) ☐ Thorax (TH) ☐ Head and neck (HN)

CRITICAL SITES ☐ Eye ☐ Breast ☐ Genitalia

LABORATORY CONFIRMATION

Specimen(s) collected: ☐ **Yes** ☐ **No** Date first specimen(s) taken: ___/___/___
Specimen(s) type(s): ☐ Swab ☐ Fine needle aspiration (FNA) ☐ Biopsy

Results				
☐ ZN :	☐ *Positive*	☐ *Negative*	☐ *Positive*	☐ *Negative*
☐ PCR :	☐ *Positive*	☐ *Negative*	☐ *Positive*	☐ *Negative*
☐ Histo :	☐ *Positive*	☐ *Negative*	☐ *Positive*	☐ *Negative*

TREATMENT TYPE (Tick all applicable) ☐ Dressings ☐ Antibiotics ☐ Surgery (date: ___/___/___) ☐ POD (prevention of disability)

DOSAGES **Rifampicin: _____ (mg)** **Streptomycin: _____ (g)** **Other (name): _____:_____ (mg)**

Cross out each day (X) after administering the antibiotics; if antibiotics are not taken, indicate with the symbol Ø

Day / Month	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	Total Doses

TREATMENT OUTCOME

☐ 1a: Antibiotic treatment completed ☐ 2a: Healed without surgery ☐ 3a: Healed without limitation of movement at any joint ☐ 4: Referred for further treatment
☐ 1b: Antibiotic treatment **not** completed ☐ 2b: Healed with surgery ☐ 3b: Healed with limitation of movement at any joint ☐ 5: Lost to follow up ☐ Died

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WJ SANE NO

Dosage Guide						
Weight of patient (kg)	Rifampicin (300 mg/tablet)		Streptomycin (1 g/2 ml)		Other:	
	Dose (mg)	Number of tablets	Dose (g)	Volume (ml)	Dose (mg)	Number of tablets
5–10	75	0.25	0.25	0.50		
11–20	150	0.50	0.33	0.70		
21–30	300	1.00	0.50	1.00		
31–39	300	1.00	0.50	1.00		
40–54	450	1.50	0.75	1.50		
>54	600	2.00	1.00	2.00		
If streptomycin is contraindicated (e.g. pregnancy, previous treatment with streptomycin), please contact the national programme manager or a designated referral treatment centre.						

FOLLOW-UP APPOINTMENTS AFTER TREATMENT	
DATE (dd/mm/yy)	COMMENTS
__/__/__	
__/__/__	
__/__/__	
__/__/__	
__/__/__	
__/__/__	
__/__/__	
__/__/__	

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