KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

COLLEGE OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

PROTEOMICS OF BURULI ULCER DISEASE HEALING

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BY:

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DECLARATION

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

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DEDICATION

I dedicate this work to my family and friends who supported and encouraged me throughout this study, especially Mr and Mrs. Thomas Adjei. I will forever be indebted to you.

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ABSTRACT

Background: Buruli ulcer (Bu) is a neglected tropical disease, caused by *Mycobacterium ulcerans*. The disease often presents itself as a painless subcutaneous nodule, plaque, or oedema that subsequently develops into an ulcer with undermined edges. The pathogenesis of Bu is linked to *M. ulcerans* secretion of a lipid toxin known as mycolactone. It serves as a biomarker for *M. ulcerans* and has been demonstrated to be present in all forms of Bu disease before or after antibiotic treatment. This toxin has been demonstrated to be responsible for immunosuppression and tissue necrosis which are characteristic of the Buruli ulcer. Bu disease is reported to down-regulate the circulating levels of a large array of proteins involved in immune response. These proteins contribute to acute phase reaction, lipid metabolism, coagulation and tissue remodeling; processes involved in healing of mycobacteria disease and hence may interfere with wound healing. In this study novel and unique proteins capable of predicting fast and slow healers of *M. ulcerans* disease were analysed and validated.

Methods: This study was a prospective hospital-based cohort study. Suspected Buruli ulcer cases were confirmed by dry reagent based standard Polymerase chain reaction (PCR). Protein profiling was done using mass spectrometry and bioinformatics. This part was an *in-silico* approach, where a high throughput data was generated by mass spectrometry for tissue samples obtained from fast and slow healing Buruli ulcer patients. Interferon Gama inducible Protein-30 (IFI30 / IP30), Cluster of Differentiation 74 (CD74), Proteasome activator subunit 3 (PSME3) and Classical component 4A (C4A) levels were investigated in sera of fast and slow healing Buruli ulcer patients, endemic controls and non-endemic controls using ELISA

Results: The results from this study show that Buruli ulcer cases, endemic controls and non-endemic controls generally express IFI30 / IP30, CD74, PSME3 and C4A proteins. Fast healing Bu patients expressed higher levels of IFI30 with median of 1.5 ng/mL and (0.8 – 2.2) ng/mL

interquartile range compared to slow healing Bu with median of 1.2 ng/mL (0.2 - 2.0) ng/mL. Although fast healers expressed slightly higher levels of the protein, compared to slow healers, it was not statistically significant ($p > 0.05$). The expression levels of CD74 in fast healers (0.4 (0.07 – 2.8) ng/mL) were lower, compared with those of slow healers (1.2 (0.2 - 4.1) ng/mL) but the difference was not statistically significant ($p > 0.05$). The expression level of PSME3 in fast healers (0.35(0.21 – 0.56) ng/mL) were lower when compared with those of slow healers (0.39 (0.20 – 0.63) ng/mL) but the difference was not significant statistically ($p > 0.05$). The expression level of C4A in fast healers (7.0 (0.31 – 35.29) ng/mL) was higher, compared with those of slow healers (4.0 (0.09 – 22.13) ng/mL) but the difference was not significant statistically ($p > 0.05$).

Conclusion: The study has shown that IFI30/IP30, CD74, PSME3 and C4A proteins were generally expressed by both Bu patients and control. Higher median level of IFI30 protein was associated with fast healing, in agreement with initial analyses of the three pathways of immune response. Though C4A was higher in fast healing, this was contrary to the initial immune pathway analysis. However, CD74 and PSME3 were of lower expression in fast healing. Interestingly, IFI30 showed opposing expression levels in plaques and nodules. In nodules, the higher the baseline concentration of IFI30, the longer the time to complete healing, but in plaques, the higher the baseline concentration of IFI30, the shorter the time to complete healing. This finding could be a useful guide for extending or shortening antibiotic therapy in nodules or plaques, based on their initial or baseline concentration of IFI30 if confirmed in larger studies.

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LIST OF ABBREVIATIONS

AFB	Acid-Fast Bacillus
APCs	Antigen-Presenting Cells
Bu	Buruli ulcer
$CD4^+$	Cluster of Differentiation 4
$CD8^+$	Cluster of Differentiation 8
CD74	Cluster of Differentiation 74
C4A	Complement Component 4A
DNA	Deoxyribonucleic Acid
DRB	Dry Reagent-Based
ELISA	Enzyme linked Immunosorbent Assay
EGF	Epidermal Growth Factor
ECM	Extracellular Matrix
FGF	Fibroblast Growth Factor
FNAs	Fine Needle Aspirates
fTLC	fluorescent Thin Layer Chromatography
IFI30/ IP30/GILT	Gamma-Interferon-Inducible Lysosomal Thiol Reductase
IS	Insertion Sequence
KATH	Komfo Anokye Teaching Hospital

KCCR	Kumasi Centre for Collaborative Research in Tropical Medicine
LJ	Lowenstein Jensen
MIF	Macrophage Inhibitory factor
MHC	Major Histocompatibility Complex
M. ulceran	*Mycobacterium ulcerans*
Mu	*Mycobacterium ulcerans*
NBUCP	National Buruli Ulcer Control Programme
PDGF	Platelet-Derived Growth Factor
PMNs	Polymorphonuclear Cells
PSME3	Proteasome Activator Complex Subunit 3
PCR	Polymerase Chain Reaction
T	Thymus
TEB	Tris EDTA Buffer
TGF	Transforming Growth Factor
WHO	World Health Organization
ZN	Ziehl Neelsen

CHAPTER ONE

INTRODUCTION

1.1 Background

Buruli ulcer (Bu) is a neglected tropical disease caused by *Mycobacterium ulcerans (M. ulcerans)* (Phillips *et al.*, 2014a). Initial identification and description of the disease was done in Uganda, but currently, it has been reported by many subtropical and tropical countries with majority of cases from West Africa. It is prevalent among children between the ages of 5-15 years who live in remote areas, where health service is inaccessible (Wansbrough-Jones and Phillips, 2006). In Australia it affects the older population (O'Brien *et al*., 2014).

The disease often presents itself as a painless subcutaneous nodule, oedema or plaque that subsequently develops into an ulcer with undermined edges (Etuaful *et al*., 2005). If left untreated, ulcers can lead to extensive destruction of soft tissues and skin which result in scars with contractures and sometimes disabilities when it is found at the joints (Huygen *et al*., 2009).

Mycobacterium ulcerans is a slow growing environmental pathogen, suggested to have evolved from *Mycobacterium marinum*, by acquiring foreign DNA from the environment (Stinear *et al*., 2004). It is an acid-fast bacillus (AFB) microscopically and can be isolated from human samples and cultured suitably on Lowenstein Jensen (LJ) media, supplemented with 0.75% glycerol. The organism is cultured at a temperature of 29 - 33^{o}C with 2.5% low concentration of oxygen (WHO, 2017).

The reservoir and specific mode of transmission of Bu is unknown, even though there is a relation between incidence rate of the disease with water bodies either flowing or stagnant (Bratschi *et al*., 2013). Moreover, the roles of aquatic insects (Marsollier *et al*., 2002) and mosquitoes (Johnson *et*

al., 2007) in *M.ulcerans* transmission has been elucidated. Other organisms such as fish, amphibians, snails, turtles and possums are said to be potential reservoirs or vectors of *M. ulcerans* (Eddyani *et al.*, 2004; Fyfe *et al.*, 2010; Röltgen and Pluschke, 2015). This gives a compelling evidence that the mode of transmission is environmental. Further research revealed that the frequency of the Bu disease varied from village to village, even in the same district (Johnson *et al*., 2005), supporting the idea of environmental exposure of the *M. ulcerans*.

Buruli ulcer is currently diagnosed by laboratory methods such as microscopy for acid fast bacilli, culture for viable mycobacteria and polymerase chain reaction (PCR), which targets specific *IS2404* repeat sequence of the *M. ulcerans* genome. The most sensitive among these methods is PCR and hence regarded as the gold standard for the confirmation of the disease. PCR has proven to be highly sensitive for deoxyribonucleic acid (DNA), extracted from punch biopsies and subsequently from fine needle aspirate of clinical samples (Phillips *et al.*, 2009a).

Prior to the advent of antibiotic therapy, Bu disease was largely managed by surgery (Beissner *et al*., 2013). Rifampicin and Streptomycin combination daily, for eight (8) weeks has been established as the standard treatment, combined with grafting of larger lesions (Etuaful *et al*., 2005). This combination has been proven to be effectual in healing all forms of the disease. Furthermore, this approach has led to reduction of the recurrence rate which was 6 – 47% after surgery to 0-2% after antibiotic treatment (Sarfo *et al.,* 2010). The combination of Rifampicin and Clarithromycin has also been recommended as an effective alternative of treating Bu. These alternative treatments are convenient, since community health workers can administer them and there will be no need of hospitalization, except cases involving skin grafting (Sarpong-Duah *et al*., 2017).

The pathogenesis of Bu is linked to *M. ulcerans* production of a lipid toxin known as mycolactone. Mycolactone is a macrocyclic polyketide toxin and it is encoded by several genes on a large plasmid (Stinear *et al*., 2007). It serves as a biomarker for *M. ulcerans* and has been demonstrated to be present in all forms of the disease before or after antibiotic treatment (Sarfo *et al.*, 2010). Studies using animal models reported that mycolactone causes tissue destruction and eventually leads to ulceration (George *et al*., 2000). Histology of Bu disease lesions show cluster of extracellular *M. ulcerans* surrounded by necrotic subcutaneous fat, suggesting that mycolactone diffusing from the organisms is responsible for tissue damage (Evans *et al*., 2003).

Skin wound healing is a systematic process which is usually explained by four overlapping classical phases. These phases include haemostasis, inflammation, proliferation and maturation (Simon *et al*., 2016). During haemostasis, blood platelets aid in releasing cytokines, chemokines and hormones to the site. During inflammatory phase, there is debridement of tissue by inflammatory cells. Angiogenesis, fibroplasia and epithelialization occur during the proliferative phase and finally there is collagen cross linking which involves the expression of certain protein molecules in the blood, to increase the scar's tensile strength of (Simon *et al*., 2016).

In bacterial diseases, bacterial antigens present in wounds activate the secretion of pro-inflammatory cytokines, growth factors and other immune cells to aid in healing. However, in Buruli ulcer disease, *M. ulcerans* suppresses the expression and actions of these cells necessary to elicit immune response for healing to take place. However, patients express numerous proteins in their serum from onset to the elimination of infection. Fast healing Buruli lesions are lesions that heal before week twelve (12) and slow healing Buruli lesions are lesions that heal after week twelve (12).

1.2. Rationale of the study

Buruli ulcer disease is treated for 8 weeks with a combination of streptomycin and rifampicin with marked inter-personal differences in time for healing of lesions. Studies have confirmed that some Bu lesions heal faster, whilst others heal slower, independent of their size (Sarfo *et al.,* 2010). Report in 2013 suggested that the time for healing of Bu ulcer lesions ranges from 4 to 36 weeks (Phillips *et al.*, 2014a). The reason for this differences in time for healing, irrespective of size is still unclear and hence there is the need to properly investigate the reasons for these differences.

A research conducted by Sarpong-Duah *et al.* noted that the healing of the disease depends on the cessation of mycolactone production by viable *M. ulcerans* during antibiotic treatment and that healing of *M. ulcerans* lesions is tied to either fast killing of the organisms in fast healing or slow killing of the organisms, resulting in ulcers with poor healing (Sarpong-Duah *et al.*, 2017).

Another study involving the use of guinea pig model confirmed the presence of mycolactone in surrounding tissues and viable organism. In this study *M. ulcerans 98-912* were injected into the back or ear of the pig and the lesion monitored without treatment. Macroscopic observation of the initial lesion correlated with the presence of an abundant bacteria load, necrosis and acute inflammatory infiltrate. However, by the end of infection, viable organisms had decreased and subcutaneous tissue organization was returning to its initial state after the healing processes had taken place. They then reported that Bu can heal spontaneously without treatment (Silva-Gomes *et al*., 2015), but as to when healing will take place is still unknown.

Prior to the above study, previous animal models study, had reported that mycolactone secreted at the point of infection has access to the peripheral blood of the affected animals and concentrates in mononuclear cell subsets (Hong *et al.*, 2008a).

The presence of mycolactone's in the blood brings about immunosuppression at the systemic level and reduced ability of lymphocytes to produce interleukin 2 (Hong *et al.*, 2008a).

Moreover, mycolactone has been shown to impair T cells capacity to produce cytokines when stimulated *in vitro*. The circulation dynamics of these chemokines and cytokines during treatment shows that these proteins were not positively regulated during treatment (Phillips *et al.*, 2009b).

Further studies have also noted that mycolactone's presence in the blood of patients, though responsible for immunosuppression, may have certain controls on proteins such as growth factors needed for wound healing and this could account for longer healing time (Coutanceau *et al.*, 2007; Sarfo *et al.*, 2009).

According to some findings of Sarfo and colleagues, the persistence of mycolactone delays healing by inhibiting the production of growth factors and killing keratinocytes which are required for wound healing (Sarfo *et al*., 2014). Subsequently, Phillips and his collegues, conducted a follow-up research on serum proteins in Bu patients and endemic controls. The study revealed that in the disease, the circulating levels of a large array of proteins involved in immune response were down-regulated, without impacting on leukocyte composition of the peripheral blood (Phillips *et al.*, 2014a). The study also identified a number of proteins contributing to acute phase reaction, lipid metabolism, coagulation and remodeling of tissues involved in healing of mycobacteria disease and hence interfering with wound healing. The down-regulation of these proteins persisted after the elimination of bacteria with antibiotic therapy. Slow healers interestingly had deeper coagulation and metabolic defects at the beginning of antibiotic therapy which gives an indication that levels of serum proteins at the onset of the disease can predict the slow or fast healing of the disease.

In collaboration with the University of Southampton (UK), tissues from patients with fast and slow healing Bu were analysed for novel proteome biomarkers. These preliminary analyses generated a list of proteins expressed by Bu patients. The levels of expression of some proteins in fast healers were higher, compared to that of slow healers. This indicated that some of these proteins can serve as a biomarker for fast healing Bu patients and hence reduce the time for therapy. The aim of this study was therefore to analyse and validate novel and unique proteins capable of predicting fast and slow healers of *M. ulcerans* disease. These biomarkers will help in predicting whether some patients require less than / more than eight (8) weeks combination therapy of Rifampicin and Streptomycin.

1.3. Main hypothesis

It is hypothesised that Buruli ulcers that heal fast or slowly are linked to differential expression of novel and unique proteins.

1.4. Research questions

This study aims at addressing the following research questions;

- Which serum proteins has the potential of predicting fast and slow healing of Buruli ulcer disease?
- What are the predictive capability of the novel proteins for fast healing in plasma/serum of Buruli ulcer patients?
- Which pathways are significantly enriched with these novel proteins associated with fast healing?

1.5. Aim of the study

To analyse and validate novel and unique proteins capable of predicting fast or slow healing of *Mycobacterium ulcerans* disease.

1.5.1. Specific objectives

- To analyse and select protein(s) with potential for predicting fast and slow healing of Buruli ulcer.
- To quantify and evaluate the predictive capability/potential of the proteins for either fast or slow healing in Buruli ulcer patients.
- To determine the pathways significantly enriched with these proteins associated with fast/slow healing.

CHAPTER TWO

LITERATURE REVIEW

2.1. History and Geographical distribution of Buruli ulcer disease (BUD)

Buruli ulcer disease is from a similar family as leprosy and tuberculosis. It is the third most common mycobacterial disease of humans. The disease is not selective of sex and age, but it usually affects children under 15 year (Sarpong-Duah *et al.*, 2017).

It is caused by *Mycobacterium ulcerans* and was first seen by a British Physician in 1987, at a hospital called Mengo, in Kampala, Uganda. The Physician, Sir Albert Cook, saw a skin ulcer and described it but this was not reported in medical literature (Ward, 1970). Earlier in the 1920's, Kleinschindt, also in the same country saw skin ulcers and observed that these ulcers had undermined edges with high numbers of acid fast bacilli (Meyers *et al*., 1974).

In 1948, MacCallum and colleagues in Australia, reported the disease in Australian patients who presented a single ulcer on the limbs (MacCallum *et al*., 1948). The ulcers these patients presented had undermined edges, and one of the patients had a positive result when tuberculin skin test was done, though there was no evidence of tuberculosis. Specimen of tissues for histological studies revealed *Mycobacterium* species that were acid-fast bacilli present in the subcutaneous lesions of the patients and when the *bacilli* were inoculated into animals there was a progressive ulceration seen.

They named the *Mycobacterium* causing the disease Bairnsdale bacillus, where Bairnsdale was the region in which five of the six patients presenting the disease lived. People who live in that region still call the disease Bairnsdale ulcer (Wansbrough-Jones and Phillips, 2006).

Ballion and Van Oye, in 1950 reported the first African case from Zaire, but in Meyer's research in that area, *M. ulcerans* infection had been observed in Zaire as far back in 1935 (Meyers *et al*.,

1974). The epidemiology of the disease began from the 1960's to 1970's in Uganda and new cases were observed in Rwanda refugees who had gathered in an area close to the Nile river. More cases were also reported in Buruli County, near lake Kyoga and the disease was named Buruli ulcer. Following the first international conference on *M. ulceran* disease control and research, which was held in Cote d'Ivoire in 1998, the disease was declared by the World Health Organization as an emerging skin disease of Public Health concern (World Health Organization (WHO) and Public Relation Office, 1997). This declaration raised concerns about the little knowledge about the disease and therefore, the international community was called upon to support research and control of the disease. The disease was reported at that time as the third most common Mycobacterium disease, after tuberculosis and leprosy. Buruli ulcer disease was reported in most tropical countries and has been reported in at least, 30 countries (Johnson *et al*., 2005).

Currently, cases have been reported in 33 countries; from temperate, sub-tropical and tropical climates in Western Pacific, South Americas, and Africa, respectively (Phillips *et al*., 2016). However, majority of these cases occur in tropical and sub-tropical regions, except in Australia, China and Japan. In Africa, most cases come from Central and Western Africa, including Cameroon, Cote D'Ivoire, Democratic Republic of Congo and Ghana (WHO, 2017).

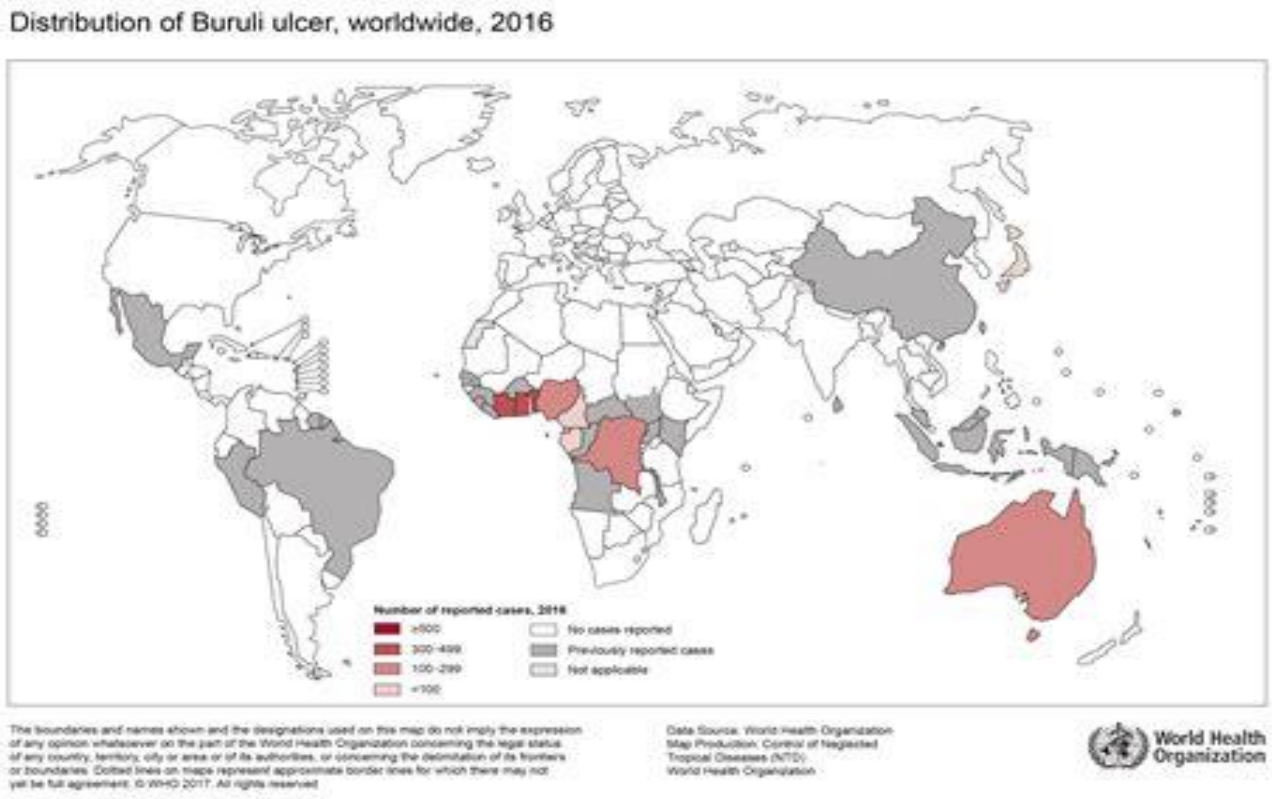

Figure 2.1: Countries reporting Buruli ulcer (WHO 2017)

2.1.1. Buruli Ulcer in Ghana

Buruli ulcer was first seen in Ghana in 1971 in the Greater Accra Region. The patient was seen at Korle Bu Teaching Hospital and was from Amasaman. Within that same year, a lot more cases were identified around the Densu river in the Greater Accra Region (Bayley, 1971). Subsequently, 96 cases were identified in 1986 at the Asante Akim North District, in the Ashanti Region. These cases were described by van der Werf and his team. This same group also reported several other cases in the Amansie West District, in the Ashanti Region and the region was noted as one of the endemic districts of Buruli ulcer in the country (Van der Werf *et al., 1999).* Several other cases were described and reported from the coastal belt and the middle of Ghana.

Due to the numerous cases being reported, the country commenced a surveillance system for Buruli ulcer case reports and this resulted in 1200 cases being reported within 5 years (Amofah *et al.,* 2002).

A National Buruli Ulcer Control Programme (NBUCP) was set up in 2000 to create awareness on Buruli ulcer in Ghana for early detection and treatment. The endemic districts over the years, increased from 6 districts (Asante Akim North, Amansie West, Ga West, Akuapim South, Upper Denkyira and Suhum Kraboa Coaltar) to 30 districts. A national survey in 1999 reported over 6000 cases and since 2005, 35 districts from 6 regions (Ashanti, Brong Ahafo, Central, Eastern, Greater Accra and Western) in Ghana have reported an average of about 1000 cases in a year (Amofah *et al.,* 2002).

2.2. Causative organism

The bacterium responsible for Bu is *Mycobacterium ulcerans (M. ulcerans)* (Wansbrough-Jones and Phillips, 2006). The bacterium is a slow growing environmental pathogen which falls into the same group with closely related Mycobacterium. Because of the slow growing nature, it enhances its growth as an endosymbiont and survives poor conditions (Stinear *et al*., 2000).

It is claimed that genetically, *M. ulcerans* evolved from *M. marinum,* by picking a foreign DNA from the environment (Stinear *et al*., 2007). *M. ulcerans* genetically consists of 5.8Mb genomic sequence. This sequence has a chromosome of 5632 kb, 2 circular replicons and a large virulence plasmid (pMuM) of 174b (Stinear *et al*., 2000). The bacterium has about 209 copies of insertion sequence (IS) 2404, accumulated in its genome. This insertion sequence has a nucleotide sequence length of 1,366 sequence length. It also has 91 copies of IS2606, two bacteriophages, 771 pseudogenes and multiple DNA deletions and rearrangement (Stinear *et al*., 2000).

IS2404 present in the *M. ulcerans* genome causes inactivation of several genes by disrupting coding and promoter sequence. This has led to the deletion of about 1Mb of the mycobacterium DNA and hence evidence of extensive gene function loss in *M. ulcerans* (Stinear *et al*., 2000).

M. ulcerans is an acid-fast bacillus microscopically. It can be isolated from human samples and cultured suitably on Lowenstein Jensen (LJ) media supplemented with 0.75%glycerol (WHO, 2017). The bacteria can however, thrive in other media such as Middlebrooks 7H10 and Middlebrooks 71. The organism is cultured at a temperature of 29 - 33ºC with 2.5% low concentration of oxygen (WHO, 2017).

The time for growth usually ranges between six to eight weeks and even beyond. However, the subcultures grow faster within three to four weeks. The growth isolates on LJ media are yellow in color and has a diameter of about 1-2mm (WHO, 2017).

2.3. Mode of transmission

The mode of transmission of Buruli ulcer is still unknown, but understanding of the mechanism of the disease has advanced from the time WHO Buruli Ulcer Initiative was established, which has improved diagnosis and management of the disease (Sarpong-Duah *et al.*, 2017). The disease is sometimes referred to as a "mysterious disease" because the exact mode of transmission is still not clear (Phillips *et al*., 2016). It has been widely associated with proximity to aquatic habitat, hence it is rare in the savanna regions of West Africa and drier areas of Australia (Merritt *et al*., 2010). The causative organism, *Mycobacterium ulcerans,* has been isolated from the environment, using environmental samples such as soil, water filtrates, frogs, snails and biofilms. The bacteria were isolated using the first developed probe PCR (Merritt *et al*., 2010).

A study was conducted where *M. ulceran* DNA was detected in *Hemiptera* which was obtained from one of the endemic areas in Africa. This suggested that *M. ulcerans* was present in aquatic bugs and may serve as a vector for *M. ulcerans* (Portaels *et al*., 2008). Another study which took place in Australia suggested mosquitoes, specifically *Aedes camptorhynchus* as a possible vector for *M. ulcerans* when the IS2404 insertion sequence was found in a group of mosquitoes trapped (Johnson *et al*., 2007).

A study led by Eddyani also indicated that some aquatic fishes (*Poeciliidae)* are passive reservoirs of Mu by eating insects which are known to be positive for the mycobacteria (Eddyani *et al.*, 2004). The mycobacteria were concentrated in the gill and intestines of the fish because they were found to be positive for IS2404 insertion sequence (Willson *et al*., 2013).

Currently, there has been a suggested transmission pathway of *Mu* to humans and this includes direct contact with contaminated vegetation or water, insect vector bites, aerosols from contaminated water, entrance of the mycobacterium through a pre-existing wounds after environmental exposure or mechanical trauma (Johnson *et al*., 2005, Portaels *et al*., 2008).

2.4. Risk factors

Even though the mode of transmission of Bu is still unclear, there are certain exposures that can increase an individual's likelihood of developing the disease. Quek and others in Australia reviewed that exposure to mosquitoes is a potential risk factor of the disease (Quek *et al*., 2007) while Jacobson and Padget also reported that living around water bodies, failure to wear protective clothing and poor wound care are risk factors associated with *Mu* infections (Jacobsen and Padgett, 2010).

2.5. Clinical forms of Buruli ulcer disease

There are two main stages by which Bu presents itself clinically; the pre-ulcerative stage and the ulcerative stage. The first stage of the disease is usually a painless nodule, which is firm, non-movable and extends from the skin to the subcutaneous tissue. This form is very common in Africa. In Australia, the early stage is known as papule (Boleira *et al*., 2010). Other pre-ulcerative forms of the disease include a painless intradermal plaque with large area of induration. It usually has a diameter greater than 3cm (WHO, 2017). The disease may also present as an oedematous lesion. This starts as a non-pitting, diffuse swelling which affects a part of the limb or all areas of the limb. It is known to be the severe form of the disease (Johnson *et al*., 2005). Another clinical manifestation of Buruli ulcer disease, although rare, is osteomyelitis (Portaels *et al.,* 2009). This affects the bones and can lead to amputation, if not managed properly.

The pre-ulcerative forms, over a period of days to weeks, enlarge gradually and erode through the surface of the skin, leaving an undermined edge ulcer with necrotic sloughing in the base (Portaels *et al.,* 2009). The undermined edges are irregular and often extends as far as 15cm and beyond with surrounding oedema in 10% of the cases (Sarpong-Duah *et al.*, 2017). These ulcers are also not painful, unless there is a secondary bacterial infection (Wansbrough-Jones and Phillips, 2006) and when left untreated can affect the bones and also cause scarring which may lead to deformities.

The lesions are most often seen on the limbs; 55% on the lower limbs, 35% on the upper limbs and 10% on other body parts (WHO, 2017). Other vital parts of the body such as the genitalia, breast and eye are severely affected and if not managed with care can cause damage to these vital organs.

Depending on the severity of disease, it can be classified into three categories: Category I, Category II and Category III (WHO, 2017). Category I are small single lesions; this is 32% of cases reported. Category II forms about 35% of cases reported and is usually non-ulcerative or ulcerative plaques and oedema. Category III which forms about 33% of cases reported are usually mixed and include disseminated forms like osteomyelitis and joint involvement (WHO, 2017).

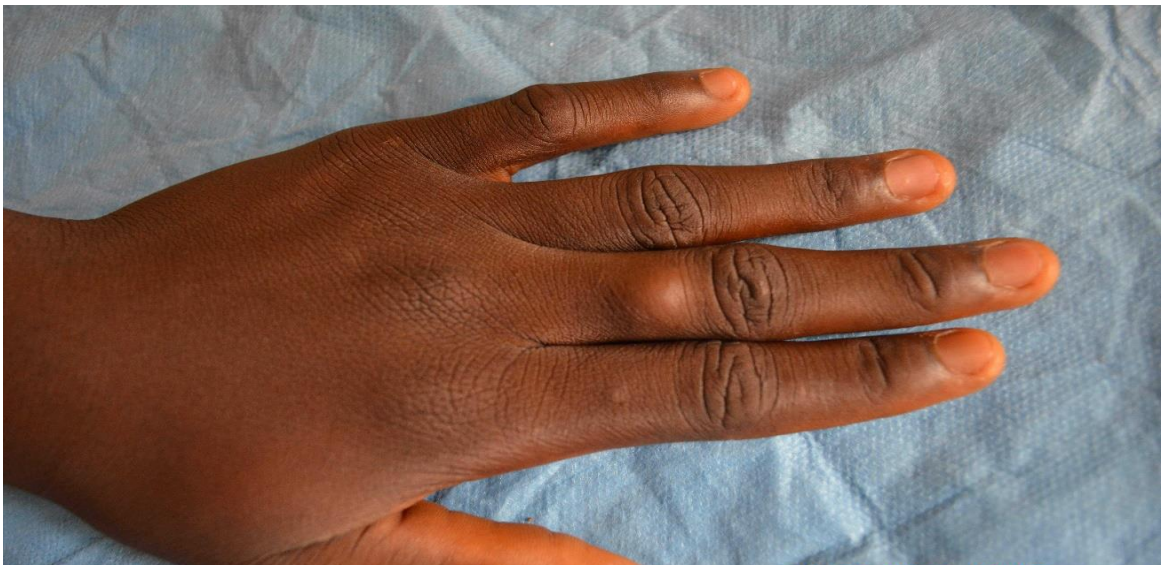

Figure 2.2: Nodular form of Buruli ulcer (Source: Buruli ulcer research team KCCR, Ghana)

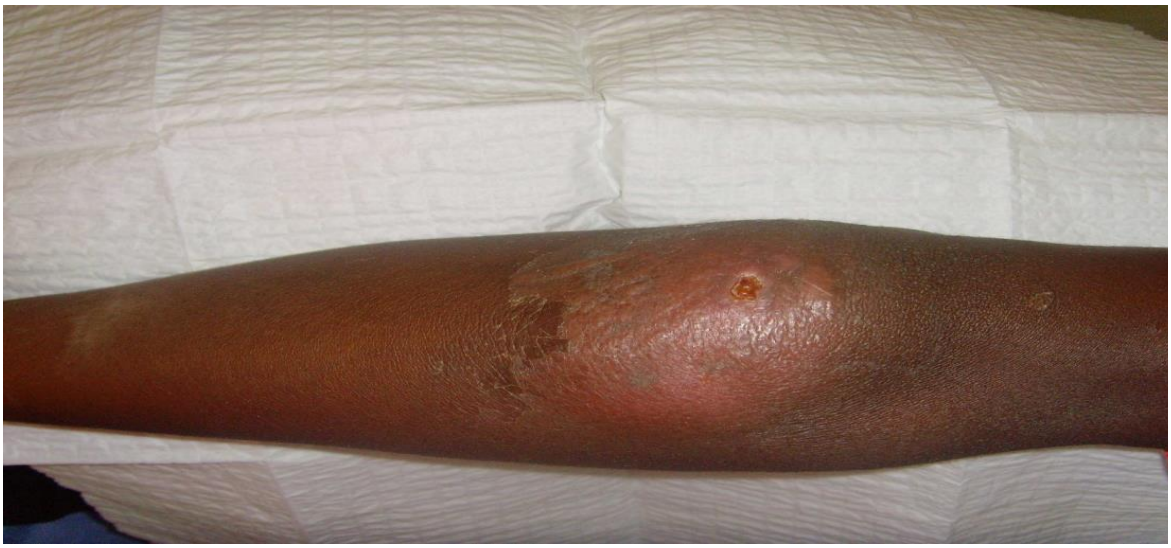

Figure 2.3: Plaque form of Buruli ulcer (Source: Buruli ulcer research team KCCR, Ghana)

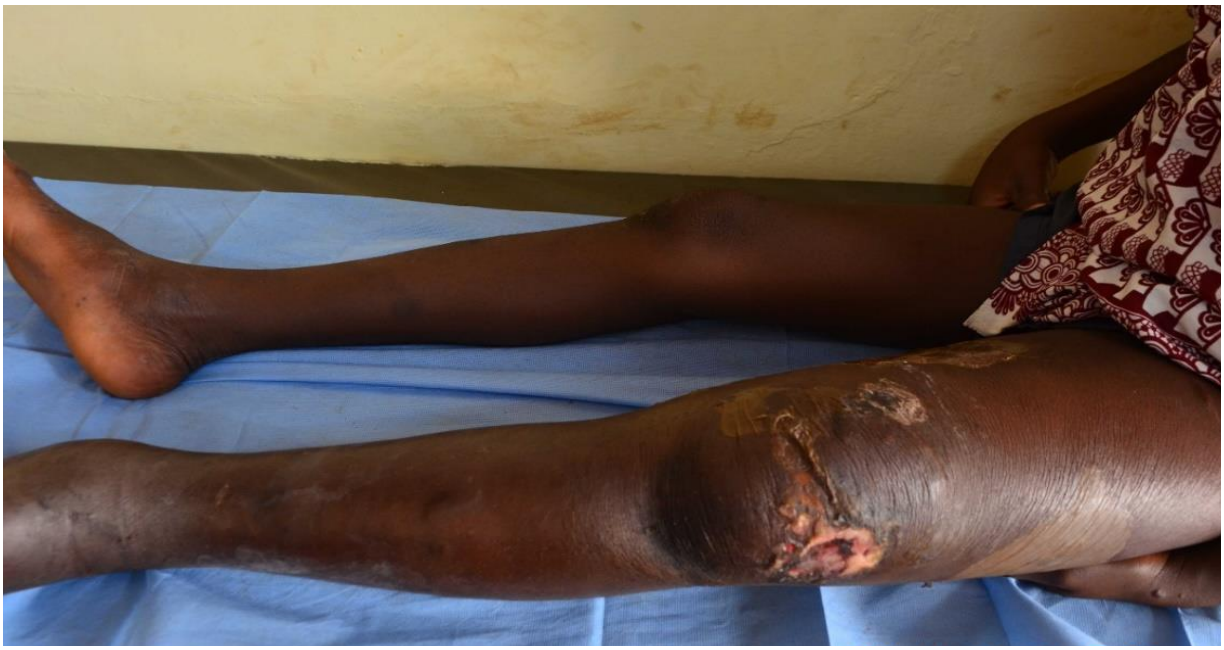

Figure 2.4: Oedematous form of Buruli ulcer (Source: Buruli ulcer research team, KCCR, Ghana)

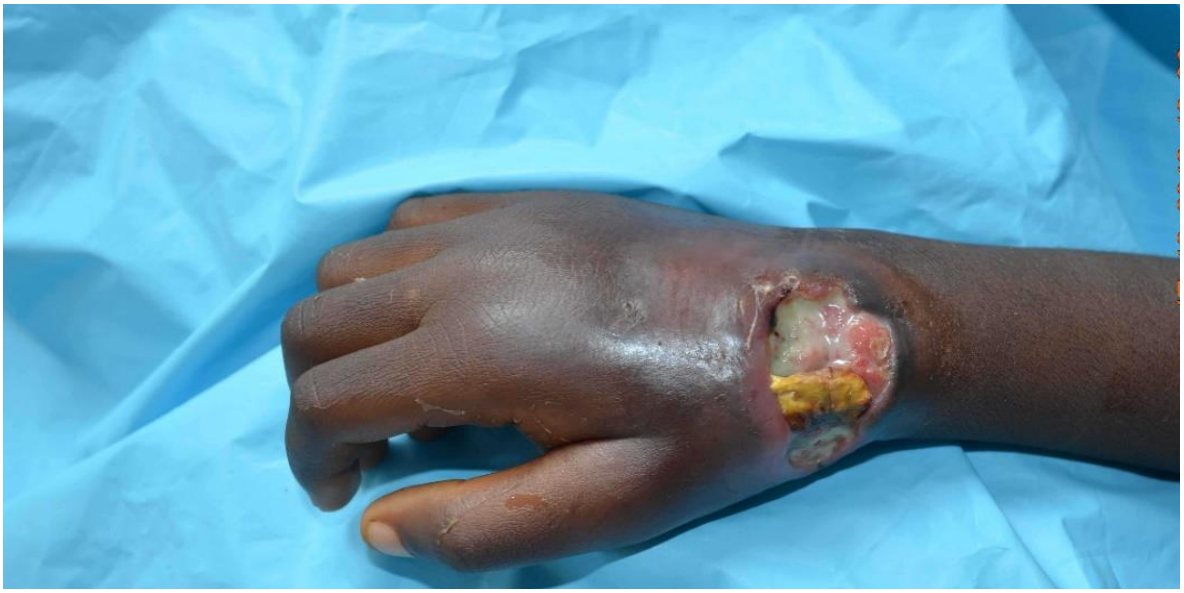

Figure 2.5: Ulcerative form of Buruli ulcer (Source: Buruli ulcer research team KCCR, Ghana)

2.6. Diagnoses of Buruli ulcer

Clinical manifestation of Bu makes it easier to be diagnosed in the endemic areas but in areas with less endemicity, there is a need for laboratory confirmation after the disease is clinically diagnosed. Laboratory confirmation is therefore very vital for the management of the disease and also helps in the identification and ruling out other diseases which are similar to Buruli ulcer disease, in terms of symptoms and presentation (Phillips *et al*., 2005). The type of sample taken for laboratory diagnosis is dependent on the form of lesion presented. Swab samples are taken from ulcerative lesions, whereas fine needle aspirates (FNAs) are obtained from pre-ulcerative lesions.

At the moment, there is no point of care diagnosis of Bu but there are four laboratory diagnostic methods (Beissner *et al.,* 2010). These are; microscopy for the detection of acid-fast bacilli (AFB), *in vitro* culture of viable organisms, histopathological examination of the lesions and polymerase

chain reaction (PCR) which targets IS2404 insertion sequence of *Mu* (Beissner *et al.,* 2010). The most sensitive among these four methods is PCR with a specificity and sensitivity of 98% to 100%, hence the gold standard for the diagnosis of the disease. Histopathology, with sensitivity of 82% follows; then culture 49% and lastly microscopy 42% (Phillips *et al.*, 2009a).

There are other diagnostic methods being developed and investigated and the most recent of them is fluorescent thin layer chromatography (fTLC) (Converse *et al*., 20014). This technique detects the lipid toxin produced by *M. ulcerans* known as mycolactone.

2.7. The pathogenesis and immunity to Buruli ulcer

Mycobacterium ulcerans which is the causative organism for the disease is suggested to have evolved from *Mycobacterium marimum*. Genetic analysis suggests over 98% nucleotide sequence identity between these two species (Stinear *et al*., 2000) but phenotypically and pathologically, these organisms are different. A research conducted by Stinear and colleagues suggested that, *M.marinum* evolved to *M.ulcerans* by acquiring a giant virulence plasmid(Pmum / 001) into its genetic make-up (Stinear *et al.,* 2004*).* This plasmid produces the toxic substance mycolactone which plays a key role in the pathogenesis of the disease.

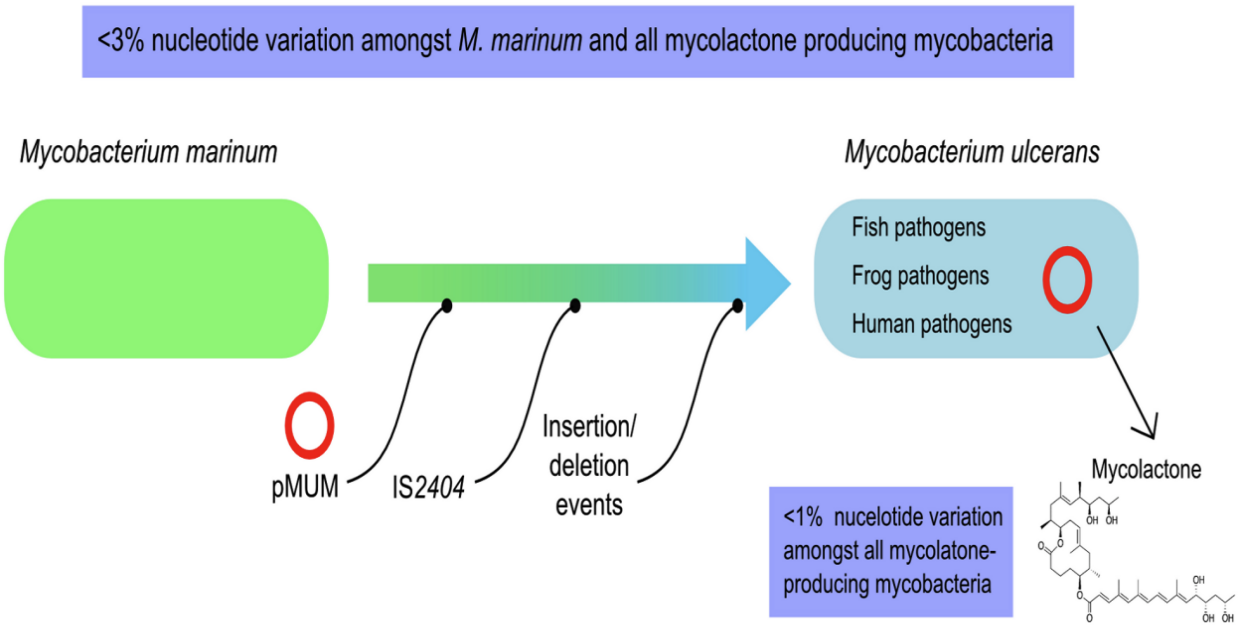

Figure 2.6: Overview of M. ulcerans evolution and principal species-defining features of organism (Pidot *et al.,* 2010).

Mycolactone is a macrocyclic polyketide responsible for the bacterial virulence (Hong *et al.*, 2008b). Histology of *M. ulcerans* disease lesions shows cluster of extracellular *M. ulcerans* surrounded by necrotic subcutaneous fat, suggesting that mycolactone diffusing from the organisms is responsible for tissue damage (Evans *et al*., 2003). Another study using animal models, had reported that mycolactone released at the point of infection gains access to the peripheral blood of the affected animals and concentrates in mononuclear cell subsets (Hong *et al.*, 2008a).

Buruli ulcer starts as a latent phase with inoculation deep into the subcutaneous tissue. This is characterized by proliferation of *M. ulcerans* and production of small amounts of mycolactone, leading to adipose tissue necrosis. During necrosis, cellular immune response is inhibited and this brings about immunosuppression (Walsh *et al.,* 2008).

Studies using human and animal models have confirmed that cell-mediated immunity plays an important role in the healing of the disease and that mycolactone has a huge immunosuppressive effect on patients' immune cells needed for both innate and adaptive immune response to *M. ulcerans* (Phillips *et al*., 2006). Mycolactone has been shown to impair the capacity of T cells to produce cytokines when stimulated *in vitro*. The circulation dynamics of these chemokines and cytokines during treatment shows that these proteins were not positively regulated during treatment (Phillips *et al.*, 2009). Another group reported mycolactone immunosuppressive property on professional antigen presenting cells, needed to elicit immune response against the disease. Tumour necrosis factor production by macrophages and monocytes were stopped, as a result of mycolactone and also dendritic cells needed to prime cellular responses and secretion of chemotactics, necessary for inflammatory response were suppressed (Demangel *et al.*, 2009).

Further studies have noted that the presence of this mycolactone in blood of patients, though responsible for immunosuppression, may have certain controls on proteins, such as growth factors, needed for wound healing and hence accounting for longer healing time (Coutanceau *et al*., 2007; Sarfo *et al*., 2009).

In 2014, Phillips and his collegues conducted a follow-up study on serum proteins in Bu patients and controls from an endemic area. The study revealed that Bu disease down-regulates the circulating levels of a large array of proteins involved in immune response, without impacting on leukocyte composition of the peripheral blood (Phillips *et al.*, 2014b). The study also noted several proteins that contribute to coagulation and tissue remodeling, lipid metabolism and acute phase reaction which are involved in healing and so may interfere with wound healing. The down-regulation of these proteins persisted after the elimination of the bacteria with antibiotic therapy. Interestingly, slow healers had higher coagulation and metabolic defects at the beginning of

antibiotic therapy, which gives an indication that serum proteins in Bu patients, whether down or up-regulated, can be used as a biomarker for anti-Bu disease therapy (Evans *et al.,* 2000; Gonzalez *et al.,* 2012; Heim *et al.,* 2012; Phillips *et al.,* 2014b).

2.8. Management of Buruli ulcer

Buruli ulcer is hardly deadly, but if it is not managed properly and early, can lead to destruction of skin, to the extent of affecting the bones, causing limitation to joints and disabilities. The disease, over the years was managed surgically with or without skin grafting. There has been evolution over the past years which has led to the replacement of surgery with antibiotic treatment (Phillips *et al*., 2016).

2.8.1. Surgery

Surgery has been the recommended therapy, used over the past decade (Sarpong-Duah *et al.,* 2017). In surgery, the affected tissues around the lesion are excised and closed. This sometimes leaves the patient with restriction of movement and deformities. The recurrence rate after surgery ranges from 6 to 28% (Huygen *et al*., 2009). Though antibiotics is the mainstay of current therapy for Buruli ulcer disease, surgical excision with skin grafting is still in place for advanced lesion (Phillips *et al.,* 2009).

2.8.2. Antimicrobial therapy

The management of the disease with currently introduced antibiotic therapy has been of great importance in the care of the patients. This can be administered by community health nurses or even the patient and hence no need for admission, except in cases where grafting is involved (Sarpong-Duah *et al.,* 2017). The recommended treatment is a combination of oral rifampicin at

10mg/kg body weight and intramuscular streptomycin at 15mg/kg body weight daily for 8 weeks or a combination of oral rifampicin at 10mg/kg body weight and oral clarithromycin at 15mg/kg body weight daily for 8 weeks and grafting for larger lesions (Johnson *et al*., 2005). This combination has decreased the recurrence rate of 6% - 47% experienced after surgery to 0% - 2% after antibiotic therapy. It is known to be effective in healing all forms of the disease (Sarfo *et al.*, 2010).

2.8.3. Wound care

Wound care is one of the major components in the disease management. It is very vital in the prevention of disabilities. This component involves the classification of the wound and preparation of the wound by cleaning the wound surface with appropriate solution and applying the appropriate dressing.

2.8.3.1. Wound cleansing

Proper Buruli ulcer wound wash is essential for the healing of the disease. Non-ulcerative forms of the disease are cleaned with normal saline till they ulcerate. Ulcerative forms of the disease are cleaned with normal saline to prepare it for dressing.

2.8.3.2 Wound dressing

Nodules and plaques are left undressed till they ulcerate. Oedemas, after washing with normal saline are compressed with short stretch compression bandages. Ulcers, after washing with normal saline (0.9% W/V), are kept moist with Vaseline gauze. Drawtex hydro conductive, a dressing material introduced by WHO is also applied to avoid longstanding complication of wounds, facilitate autolytic debridement of lesions and also serves as adsorbent. The wound is then bandaged or plastered (May, 2012).

2.9. Proteins associated with wound healing

2.9.1. Healing Process

Healing is a normal biological process that takes place in the human body. It is achieved typically through four highly programmed consecutive phases involving haemostasis, inflammation, proliferation and remodeling (Phillips *et al.*, 2014a). For successful healing, all these phases must occur in a proper sequence and time frame (Guo and DiPietro, 2010). Several factors can interfere with one or more stages of the healing process and can cause improper or impaired healing.

2.9.1.1. Haemostasis

This is the first phase of healing. During this phase, the affected tissue releases pro-inflammatory cytokines and growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF)-β. This leads to recruitment of inflammatory cells into the wound to promote inflammatory phase (Simon *et al*., 2016).

2.9.1.2. Inflammatory stage

This is characterised by infiltration of neutrophils and the presence of macrophages and lymphocytes at the site of the disease. Neutrophils play an essential role in the clearance of invading microbes and cellular debris in the wound area. Macrophages play several roles in wound healing. They initially release cytokines which promote inflammatory response by bringing on board additional leukocytes. Again, in conjunction with neutrophils, macrophages clear apoptotic cells and pave the way for the resolution of inflammation (Guo and DiPietro, 2010). As these cells clear dead cells, macrophages undergo phenotypic transition to reparative state which stimulates fibroblasts, keratinocytes and angiogenesis that promote tissue regeneration (Mosser and Edwards,

2008). Stimulation of these cells promotes the transition to the next stage which is proliferation stage.

2.9.1.3. Proliferation stage

T-lymphocytes, during this stage migrate to the inflammatory cells and macrophages and peak in the late stage of the disease. The specific role of T-cells is not completely understood but studies suggest that a decrease in levels of T-cell and delayed infiltration at the site of the disease causes impaired wound healing. Also, the skin gamma-delta T-cells regulate several aspects of wound healing which include maintaining tissue integrity, defending against pathogens and regulation of inflammation (Simon *et al*., 2016).

2.9.1.4. Remodeling stage

This is the last stage of wound healing and it is characterized by regression of severally formed capillaries in order for the vascular density of the wound to return to normal. One critical feature of this phase is extracellular matrix remodeling to a shape that approaches the normal tissue architecture (Guo and DiPietro, 2010).

Bacterial antigens present in wound activate the secretion of pro-inflammatory cytokines, growth factors and other immune cells to aid in healing of bacterial disease. However, as explained in the pathogenesis of Buruli ulcer disease, mycolactone production by *Mycobacterium ulcerans* suppresses the expression and actions of these cells, necessary to elicit immune response for healing to take place. The immune cells that are regulated by mycolactone contain several proteins contributing to acute phase reaction, lipid metabolism, coagulation and tissue remodeling (Phillips *et al.*, 2014a). The levels of these serum proteins persist from the onset of the infection to elimination of the bacteria with antibiotic therapy.

2.9.2. Some proteins found in the immune system expressed in serum of Bu patients

Buruli ulcer patients express numerous proteins from onset of the disease to the elimination of infection. A current study in collaboration with University of Southampton has revealed there are several proteins expressed in fast healing and slow healing Bu. Below are a few of them, their functions and where they are expressed;

2.9.2.1. Gamma-interferon-inducible lysosomal thiol reductase (IFI30/ IP-30/GILT)

IFI30/ IP30/ GILT, an enzyme which is encoded by IFI30 gene. It is located in the Major histocompatibility complex (MHC) class II and expressed constitutively in most antigen presenting cells (Haque *et al*., 2002). In other cells, it is induced by interferon γ, through signal transducer and activator of transcription. GILT reduces disulphide bonds in antigens and its action is maximal in an acidic pH (Hastings and Cresswell, 2011). Monocyte differentiation leads to secretion of disulphide-linked dimer of the enzymes, active precursor that may contribute to inflammation (Hastings and Cresswell, 2011). Studies show that GILT influences a variety of antigens by mediating in the reduction of their sulphide bonds. Among these antigens are melanocyte differentiation antigens. This was confirmed in tumour studies, where the absence of GILT altered antigen processing in melanomas. In these studies, it was stated that long-lasting tumor immunity needs functional mobilization of $CD8^+$ and $CD4^+$ T lymphocytes. $CD4^+$ T cell activation is strengthened by presentation of shed tumor antigens by professional antigen-presenting cells (APCs), together with display of similar antigenic epitopes by major histocompatibility complex class II on melanomas. Several self-antigens are processed and presented readily by APCs, but, T cell responses to these proteins were either absent or reduced in the context of class II melanomas. T cell recognition of select exogenous and endogenous epitopes was dependent on tumor cell expression of GILT (Haque *et al*., 2002). More so, cellular redox state is regulated by GILT such

that in cells with no GILT expression, glutathione state is shifted from reduced form to oxidized form, resulting in mitochondrial autophagy, decreased levels of superoxide dismutase 2 and elevated superoxide levels(Rausch and Hastings, 2015). This was confirmed by studies which reported that GILT expression increases the stability of superoxide dismutase 2 and decreases reactive oxygen species and hence decrease cellular proliferation. An example is its role in *Listeria monocytogenes* (West and Cresswell, 2013)

2.9.2.2. Cluster of Differentiation 74 (CD74)

CD74, also referred to as invariant chain (li). It is a transmembrane glycoprotein which is located in the MHC class II (Mun *et al*., 2013). Molecules present in MHC class II are synthesized and assembled through non-covalent bonds of MHC α and β chains to trimers of invariant chain in the endoplasmic reticulum. This protein is expressed in positive MHC class II types including monocytes, activated T-cells and fibroblasts (Mun *et al*., 2013). Studies have demonstrated that CD74 is a cell surface receptor protein for Macrophage Inhibitory factor (MIF) which plays a key role in healing and also leukocyte migration to sites of inflammation. This was confirmed in rodent studies, where there was increased levels of CD74 and MIF (Mun *et al*., 2013). During healing of skin wounds, MIF levels are strongly induced and widely expressed in the serum of the host (Mun *et al*., 2013).

2.9.2.3. Proteasome Activator Complex Subunit 3 (PSME3)

PSME3 plays a huge role in proteolysis that takes place in the nucleus and cytosols of eukaryotes. It performs vital roles such as haemostasis and cellular regulation (Rechsteiner and Hill, 2015)
PSME3 is a proteasome encoded by PSME3 gene. It is sometimes referred to as 26S and it is a multicatalytic proteinase with two highly ordered structural complexes; 20S and 19S. The protein

is located in the MHC class I and it is involved in the processing of MHC class I peptides in an ATP/ubiquitin-dependent process (Rechsteiner and Hill, 2015).

Studies have indicated that PSME3 regulates cell cycle progression and apoptosis. A study using mouse model, demonstrated spontaneous apoptosis, using embryonic PSME3 (Rechsteiner and Hill, 2015). Zhang and Zhang reported that PSME3 does this by degrading p53 which is a protein involved in the controlling of cell growth and differentiation. High levels of p53 induces apoptosis and cell cycle arrest and hence degradation of p53, by PSME3 results in the down regulation of p53 (Zhang and Zhang, 2008). Another study in breast cancer reported that PSME3 plays a key role in the disease. Report from this study suggested that PSME3 induces the epithelial-mesenchymal transition and contributes to the induction and expression of cancer stem cell markers and hence increases migration and invasion of the cells. *In vitro* analysis reported that PSME3 reduces $CD8^+$ T cells chemotaxis and kills T-cells *in vitro*. However, PSME3 gene knockdown or knock out upregulated $CD8^+$ T cells *in vivo* and reduced the subcutaneous tumor growth rate (Yi *et al*., 2017).

2.9.2.4. Complement Component 4A (C4A)

The term complements historically means a heat-labile serum component which has the capacity to lyse bacteria (Szilagyi *et al.*, 2006). However, complement is currently known to play a role in the host immune defence and it is the key constituent of innate immunity (Szilagyi *et al.*, 2006). Bacteria are opsonised by complement for enhanced phagocytosis for recruiting and activating various cells, such as macrophages and polymorphonuclear cells (PMNs). Complement can help in the clearance of immune complexes and apoptotic cells, though it can be detrimental to the host by contributing to inflammation and tissue damage (Mayer, 2017).

Complement has over 35 serum proteins which are released by several cells such as macrophages, hepatocytes, and gut epithelial cells (Beltrame *et al*., 2015). C4a, is one of the serum complement proteins and is essential in the activation cascades of the classical complement pathway (Szilagyi *et al*., 2006). It is encoded by complement factor 4 acidic form which is in the classical activation pathway. C4a is usually expressed as a single chain precursor but subsequently cleaved into alpha, beta, and gamma chains proteolytically (Beltrame *et al*., 2015). These chains provide an interaction surface for antigen-antibody complex and also for other antigen-antibody complexes. The alpha chain subunit may be cleaved to release C4 anaphylatoxin, which is a mediator of local inflammation (Beltrame *et al*., 2015). Neutrophils are activated by C4a which increases inflammation and free radicals (Beltrame *et al*., 2015). Studies have suggested that C4A appears to be a marker in diseases, such as psoriasis and systemic lupus erythematosus (Traustadottir *et al*., 2002).

2.10. Proteomics and Mass Spectrometry

Proteomics studies mainly consist of the identification of protein content of any given cell, their isoforms, splice variants, post-translational modifications, interacting partners and higher-order complexes, under different conditions (Kearney and Thibault, 2003). The study of living systems at the protein level is constantly providing essential insights into many biological processes across all kingdoms of life (Bantscheff *et al*., 2012).

Mass spectrometry is an extraordinary specific and sensitive analytical technique, able to give quantitative and qualitative analytical data in nanomolar to attomolar amounts of analytes. Mass spectrometry-based proteomics have basically revolutionized the way in which biological systems are investigated because of its capability to quantify thousands of proteins and post-translational modifications in parallel (Bantscheff *et al*., 2012). This is the technique of choice for a number of

studies obligated by regulatory authorities and most importantly, it is regarded the workhorse in the development of new methods (Bantscheff *et al*., 2012).

The successful application of this top-notched technology spans diverse fields of food and nutrition sciences. Proteomics studies are remarkably changing the way analytical problems are resolved. Mass spectrometry has an essential role in proteomics and has become an indispensable tool for molecular and cellular biology (Kearney and Thibault, 2003).

The main components of mass spectrometer consist of the ion source, mass analyzer and detection unit. Ion plume is produced and introduced into the mass spectrometer through the ion source; the ions are then separated in mass analyzer under an ultra-high vacuum, based on their mass-to-charge ratio and picked up by a detector (Pan *et al*., 2014).

Due to the advent of Proteomics technologies which are regularly improving and new technologies being introduced, a high throughput acquisition of proteome data is becoming increasingly important.

Bioinformatics which is currently at its cradle and a rapidly emerging domain, is playing a crucial role in proteomics by advancing new algorithms to handle enormous and diverse data sets and to enhance the knowledge discovery process (Blueggel *et al*., 2004). It is a newly categorised and swiftly emerging field of biomedical research, which has been recognised for about a decade. The relevance of bioinformatics in proteomics will gradually surge because of the advent of high-throughput methods, depending on powerful data analysis (Blueggel *et al*., 2004). Therefore, Bioinformatics tend to serve as a means or vehicle for deciphering the complexity associated with the enormous data generated in proteomics, using appropriate computer software.

CHAPTER 3

MATERIALS AND METHODS

3.1. Study sites

This study was carried out at four (4) different sites in Ghana. These sites were chosen because of the endemicity of the disease in those areas. They are; Agogo Presbyterian Hospital, Nkawie Toase Government Hospital, Tepa Government Hospital; all in the Ashanti Region, and Dunkwa Government Hospital in the Central Region. These sites have Bu clinics which serve as a centres for all the surrounding villages where Bu is endemic. Suspected Bu cases at these sites were enrolled into the study, after satisfying the inclusion and exclusion criteria.

Agogo Presbyterian Hospital is located in the Asante Akim North District. The district is about 80 km east of Kumasi in the Ashanti Region of Ghana and covers a surface area of about 1217. 7 sq km with a population of 170,882, with farming being their major occupations (Ghana District, 2017). It has neighbouring towns such as Hwidiem, Ananekrom, etc.

This hospital is one of the oldest mission hospitals in Ghana. It was founded in 1931 and is specialized in paediatrics, obstetrics, ophthalmology and surgery. Buruli ulcer disease is very common in the Asante Akim North District of Ghana which has Agogo as the capital. The hospital at Agogo has been receiving Bu cases since 1989, and case detection has been on the increase in and around the Agogo communities. The hospital has therefore been a centre for the management of Buruli ulcer and has been designated as a training centre for the disease by the National Buruli Ulcer Control Programme and WHO. The Bu team in Agogo engages in early case detection outreach with volunteers, who are based in the communities, in order to reduce progression to

larger lesions and hence reduce surgery (Abass *et al., 2015)*. The Bu clinic in the hospital operates every Wednesday and can boast of 50 trained Bu volunteers in the surrounding communities.

Nkawie-Toase Government Hospital is located in the Atwima Nwabiagya District in the Ashanti Region of Ghana. The district is situated in the Western part of the Ashanti Region and shares boundaries with Atwima Mponua District and Ahafo-Ano-South District. It has about 294.84 sq km surface area with an estimated population of 179,753. The occupation of the inhabitants is small scale farming and fishing, due to the presence of several streams such as Owabi, Tano and Offin. Nkawie Toase Hospital serves two main towns; Nkawie Panin and Toase. It is the only district hospital in the area. The hospital, in 2007, recorded 112 Buruli ulcer cases, making the district one of the highly endemic districts of the disease (Ghana District, 2017). The Buruli ulcer clinic day is Friday.

Tepa Government Hospital is located in the Ahafo Ano North District in the Ashanti Region of Ghana. The district has become one of the endemic districts of Buruli ulcer in Ghana and even reported the highest cases of Buruli ulcer in 2005. The district is bounded to the north by the Tano South District, south by the Atwima District, east by the Ahafo Ano South and west by Asutifi District (Ghana, District, 2017). The occupation of the populace is farming.

Tepa Government Hospital which serves as the centre for reporting Buruli ulcer cases in the neighboring villages endemic with Buruli ulcer is the only hospital for the district, though there are other small health service facilities. In view of this Ghana Health Service has trained people to help manage the disease. These people include school health teachers, community-based surveillance volunteers and traditional birth attendants. Early stage of the disease is identified by these people and reported to the Buruli ulcer clinic at the Tepa Government Hospital. The Buruli ulcer clinic operates on Thursdays.

Dunkwa Government Hospital is located in the Upper Denkyira East Municipal Assembly in the Central Region of Ghana. The district is known to be one of the highly endemic districts for Bu with a prevalence rate of 114.7 per 100,000 (Amofah *et al*., 2002). It is located in the southern part of Ghana and covers a projected area of 1020 sq km with a settlement population of 33,379 people. Dunkwa town is surrounded by numerous rivers and streams with river Offin being one of the principal rivers. The town has small scale mining and farming as their sources of livelihood (Ghana Districts, 2017).

Dunkwa Government Hospital is the only district hospital in the area and serves as a site for the management and treatment of Buruli ulcer disease in the district. In 2007, a theatre was built to help manage the disease effectively. The Buruli ulcer clinic operates on Fridays.

3.2. Study design

This study was a prospective hospital-based cohort study. Suspected Buruli ulcer cases were referred or brought to the four Bu management and treatment sites with the help of village volunteers and health workers in the community. The cases were carefully examined by professionals in the field and clinically suspected cases were recruited in the study from June 2013 to December 2016.

3.2.1. Sample size calculation

The sample size "n" was computed using the formula,

n =$z2pq\delta 2$

Where:

n=the desired sample

z=The standard normal deviate, usually set at 1.96, which corresponded to the 95 percent confidence level.

p=The proportion in the target population estimated to have recurrence of Buruli ulcer

q=The proportion in the target population who do not have Buruli ulcer,1.0-p

δ= degree of accuracy desired, usually set at 0.05

Assuming protein levels was expected to be at 3% in the study population.

n=

n=45.6

n=46.42

3.2.2. Inclusion criteria

1. All clinically suspected Buruli ulcer patients who gave their consent to the study.

2. All patients whose ages were 5 years and above.

3. All cases confirmed to be Buruli ulcer and has never been on Buruli ulcer antibiotics.

3.2.3. Exclusion criteria

1. Patients who refused to give consent to be recruited.

2. Patients who were already on Buruli ulcer antibiotic treatment before recruitment.

3. Patients whose ages were below 5 years.

3.2.4. Control Subjects

These were people who were either based in the endemic communities or non-endemic communities. They were sex and age-matched to the selected patients.

3.2.5. Ethical Considerations

Ethical approval was granted by the Committee of Human Research, Publications and Ethics, School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. Permission was sought from Agogo Presbyterian Hospital, Nkawie-Toase Hospital, Tepa Government Hospital and Dunkwa Government Hospital, to conduct the study at these hospitals. The study and consent processes were explained to each study participant in a language he/she understood. All information given by participants were treated confidential. Written and verbal consent were obtained from each participant who was older than 18 years, whereas parents or guardians consent were sought for participants who were younger. Patients who agreed to be part of the study appended their signatures or thumb prints on the written informed consent form. This whole process was done in the presence of witnesses. Consent was also sought from household contacts of patients who had no Bu and other people who were not based in the endemic communities to be recruited as controls. Participants had the right to withdraw from the study at any time during the study without any reason. Withdrawal did not affect participant's treatment and management negatively.

3.3. Study procedure

3.3.1. Patients' assessment

Patients seen at the various Buruli ulcer clinics were referred by village volunteers or health workers in the communities or districts. The participants were seen on Tuesdays, Wednesdays, Thursdays and Fridays at Dunkwa Government Hospital, Agogo Presbyterian Hospital, Tepa Government Hospital and Nkawie-Toase Government Hospital, respectively. Patients reporting at the sites were clinically examined by the Buruli ulcer team at the site to look out for the clinical features of the disease. The team was made up of disease control officer at the site, experienced

nurses, biomedical scientist from KCCR and specialist doctors from KATH. A decision was taken based on the features seen. Patients' vital information were recorded and this included blood pressure, height, weight, and temperature.

The demographic and clinical data of patients which included address, age, sex, lesion form, category of lesion and duration of lesion were taken, using the standard Buruli ulcer treatment form (WHO BU01 form) and (WHO BU04) form. Participants were given anonymous codes to protect their identity. A photograph of the lesion of each patient was taken with a digital camera; while a paper labelled with the given anonymous codes written on it, was fixed to the part being photographed.

Aranz silhouette was used to capture and measure ulcer, nodular and plaque lesions. The parameters measured by the silhouettes were area, volume, perimeter, width and depth of the lesion. They were used to measure the rate of healing and time of healing. Oedematous lesions parameters were measured using a tape measure to record the circumference and length of the lesion. The patients' lesions were then grouped into categories, based on the diameters measured by the silhouette. Lesions with diameter less than 5cm were put under category I, lesions with diameter between 5-15 cm were considered category II and lesions with diameter greater than 15 cm as well as lesions located at critical sites such as the genitalia, breast and eye were put under category III, according to WHO recommendations. Samples were taken for diagnostic confirmation. Fine needle aspirates (FNA) were obtained from pre-ulcerative lesions and swabs were taken from ulcerative lesions. The ulcerative lesions were dressed with normal saline, Vaseline gauze and drawtex, according to WHO standards (WHO, 2017).

Participants venous blood samples were obtained before onset of antibiotic treatment for protein expression assays. Patients were administered a standard antibiotic treatment for Buruli ulcer which is a combination of 10 mg/kg rifampicin and 15 mg/kg streptomycin for 8 weeks. Participants were reviewed every two (2) weeks during antibiotic treatment and monthly after treatment. Thirty (30) household contacts of patients with no Buruli ulcers and thirty (30) other people who did not live in any of the endemic areas were recruited for comparison.

3.3.2. Sampling method

3.3.2.1. Swab taking procedure.

Swab samples were taken, using sterile swab sticks. Since *Mycobacterium ulcerans* are usually located in the undermined edges of the ulcer, the swab stick was used to circle the entire undermined edge of the ulcer. The swab was then placed in 700 μL cell lysis solution for *Mycobacterium ulcerans* DNA extraction and subsequent PCR confirmation.

3.3.2.2. FNA procedure

Fine Needle Aspirates were obtained, using a 5mL syringe and a 21gauge needle. The lesion site was inspected and palpated. The site was then disinfected with cotton soaked in 70% alcohol. The needle was inserted into the centre of a non-ulcerated lesion or into a visibly inflamed skin, immediately adjacent to ulcers and moved back and forth within the subcutaneous tissue in different direction without withdrawing from the lesion. Suction was applied during movement. The needle was finally withdrawn and the tip with the aspirate flushed into a 300 μL cell lysis solution for *Mycobacterium ulcerans* DNA extraction.

3.3.2.3. Blood samples

Venous blood samples were taken by venipuncture using the BD vacutainer safety-lock blood collection set (BD Franklin Lakes, NJ USA). A tourniquet was tied around the upper arm near the medial cubital vein at the elbow of the patient. The area was disinfected with cotton soaked in 70% alcohol. Blood was collected into serum separator tube (BD Vacutainer Systems, Belliver Industrial Estate, Plymouth, UK). Ten millilitre of whole blood was obtained from participants above 10 years and 6 mL from those below 10 years before treatment. Same volume of blood was obtained from controls. The tourniquet was unwrapped and needle withdrawn. Gauze was used to press the area for some minutes to halt bleeding. The blood samples were kept at room temperature for not more than 6 hours before analysis.

3.3.2.4. Sample Transport

Sample containers uniquely labelled with each patient's details and type of sample were placed in a small carrier flask and transported to Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR) laboratories for further processing of the samples.

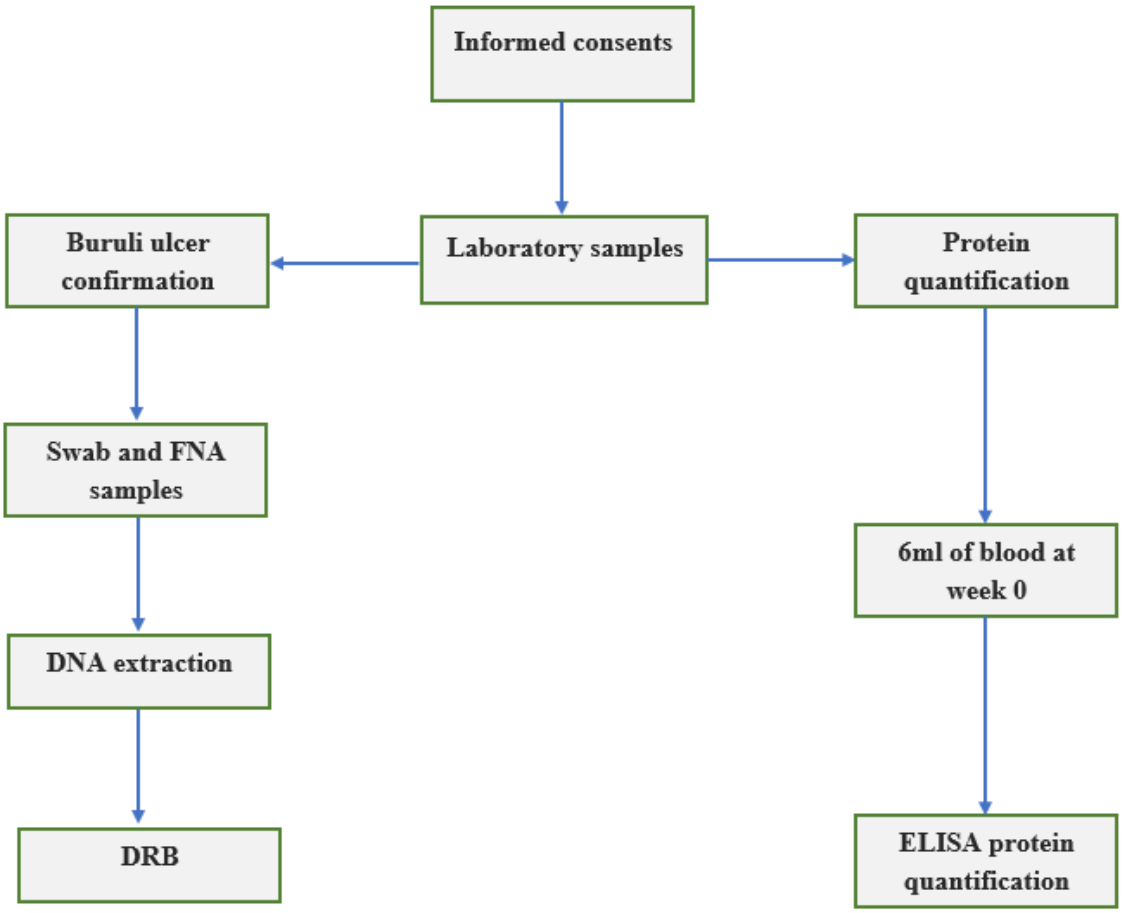

Figure 3.1: Study Procedure flow chart.

3.3.3. Laboratory Measurements

3.3.3.1 Diagnostic confirmation

Confirmation of clinically suspected Buruli ulcer lesions was performed at Kumasi Centre for Collaborative Research (KCCR) in Tropical Medicine. The samples taken from the field were placed in 700 µL of cell lysis solution and 300 µL of cell lysis solution for swab and FNA samples, respectively. The DNA of *Mycobacterium ulcerans* was extracted using a prepared protocol and dry reagent-based (DRB) PCR was performed.

3.3.3.1.1. DNA extraction

In the laboratory, upon arrival at KCCR from the clinical sites, the samples were inactivated at 95^{o}C in a thermomixer with a gentle mix for 15 min to stop the activity of the mycobacteria. The samples were then kept in a fridge at 4^{o}C.

The DNA of the mycobacteria were extracted using Puregene DNA isolation kit (Genomic DNA Purification kit, Gentra systems, Hilden, Germany). There were four main procedures involved in the extraction; these included cell lysis, protein precipitation, DNA precipitation and DNA hydration.

Cell lysis

For swab samples, 15 µL lysozyme (10 mg/mL) was added first and incubated at 37^{o}C for 1 hour in a thermomixer, while shaking gently to lyse the *Mycobacterium ulcerans* cells. Then10 µL Proteinase K (20 mg/mL) was added and incubated at 55^{o}C for 4 hours in a thermomixer, while shaking gently, followed by inactivation of Proteinase K at 80^{o}C for 20 min.

FNA samples, on the other hand, were treated firstly by addition of 10 µL Proteinase K (20 mg/mL) and incubated at 55^{o}C for 4 hours in a thermomixer, while shaking gently, followed by inactivation of Proteinase K, at 80^{o}C for 20 min. The samples were left to cool down to room temperature. 15 µL lysozyme (10 mg/mL) was then added and incubated at 37^{o}C for 1 hour in a thermomixer, while shaking gently.

Protein Precipitation

Protein precipitation was carried out in both swabs and FNA by incubating the samples on ice for 5 minutes in the first place. This was followed by the addition of 230 µL and 100 µL Protein

Precipitation Solution to the samples, and vortexed at a high speed vigorously for 20 seconds. The samples were then incubated on ice again for 5 minutes, followed by 5 minutes' centrifugation at 13,000 ×g. The supernatant after centrifugation was transferred into a labelled 2 mL Eppendorf tube, having 700 µL isopropanol (>99.7%) with 2 µL glycogen inside for swabs and 1.5 mL Eppendorf tube having 700 µL isopropanol (>99.7%) with 2 µL glycogen inside for FNAs.

DNA Precipitation

The supernatant and the content of the Eppendorf tube were mixed by gently inverting the tube about 50 times for swabs and 20 times for FNAs and then centrifuged for 5minutes at 13,000 ×g. The supernatant was discarded gently and 70% ethanol was added to the pellet and mixed gently by inverting 20 times. This mixture was then centrifuged for 5 minutes at 13,000 ×g and the supernatant poured off gently and carefully. The tube was inclined at 45^{o} angle on a clean absorbent paper for 1 hour to dry the DNA pellet.

DNA hydration

The dried DNA pellet was hydrated in 50 µL hydration solution for FNAs and 200 µL of hydration solution for swabs. This was achieved by pipetting up and down for about 20 times and then incubated for 1 hour in a thermomixer at a temperature of 65^{o}C for swabs and 30 minutes for FNAs.

3.3.3.1.2. DRB PCR

Dry reagent-based PCR was used to amplify the DNA extracted from each patient sample. This PCR targeted the IS2404 repeat sequence in the *Mycobacterium ulcerans* genome. Before the PCR was set, 1.25 µL each of the oligonucleotides MU5 forward primer (10 µM) (5‟ AGCGACCCCAGTGGATTGGT 3‟) and MU6 reverse primer (10 µM) (3‟

AGCACTTGCGAACTAGTGGC 5‟) (Frimpong *et al*., 2015), were lyophilized in 0.2 mL PCR reaction tubes, using a RVC 2-25 vacuum concentrator (Christ, Osterode, Germany). PuReTaq Ready-To-Go PCR beads (GE Healthcare Life Sciences, UK) were placed in each lyophilized primer reaction tube, and the beads were solubilized by the addition of 22.5 µL DNAse-free water. A volume of 2.5 µL of each extracted patient's DNA were then added to make up a volume of 25 µL. Four controls were set for quality control purposes and these were inhibition controls (patients DNA spiked with confirmed positive culture suspension, which is used to check false negative samples), negative PCR control (a tube containing water instead of patient DNA in the PCR process to check false positive and monitor the PCR process), negative extraction control (a tube containing cell lysis solution without clinical sample taken through the extraction process, used to monitor the extraction process) and positive control (confirmed *M.ulcerans* DNA from culture suspension).

Amplification was done in the Eppendorf Master cycler (Applied Biosystems, USA) and thermal cycling was done at 94^{o}C for 10 minutes and then the same temperature for 10 seconds for 40 cycles, followed by 58^{o}C for 10 seconds, 72^{o}C for 30 seconds, and finally 72^{o}C for 15 min. The amplicons were kept at 4^{o}C and later processed for electrophoresis and visualized under UV trans illumination and photographed for documentation (Siegmund *et al.*, 2007).

3.3.3.1.3. Electrophoresis

The amplicons were separated, by 1.5% agarose gel electrophoresis. Agarose gel was prepared by dissolving 1.8 mg agarose powder in 120 mL 0.5x Tris Boric EDTA (TBE) (Tris 53 g, boric acid 27.5 g, 0.5M EDTA 20 mL pH 8.0 in 1liter water) in a 500 mL conical flask. The flask was swirled to mix thoroughly. It was then heated in a microwave oven till it dissolved completely and allowed to cool down to a temperature of 50^{o}C. A volume of 10 µL of Gel red was added to the cooled

mixture, mixed gently and carefully, to avoid bubbles. It was poured gently into the electrophoresis tray, set with combs, well positioned in them. The liquid was then allowed to solidify at room temperature. It was placed in the gel electrophoresis chamber with 0.5× TBE. The buffer in the chamber was topped up to cover the gel. A volume of 2.5 µL of each patient's amplicon was mixed with 3 µL 6× loading dye and loaded into the gel wells, created by the combs. The controls were also loaded as well. A 6 µL volume of 100 bp DNA ladder was loaded in the first well.

The electrophoresis was run for 45 minutes, by applying 100 V current. The run gel with its DNA fragments was visualized under the UV trans illuminator linked to a computer. Infinity VX2, a 2-megapixel image software with an exposure time of 15 seconds was used to visualize the bands. The bands of each patient samples were visualized and compared to the positive control band which shows a band length of 492 bp length; the negative samples produced no band whilst the positive samples produced bands. The patients' samples bands were compared to their respective inhibition controls. The samples were recorded as positive, negative or inhibited and the picture documented.

3.3.3.2. Protein profiling using mass spectrometry and bioinformatics.

This part of the study was an *in-silico* approach. A high throughput data was generated by mass spectrometry for tissue samples obtained from fast and slow healing Buruli ulcer patients. This was done by Rules-Based Medicine Inc. (Austin, USA), which quantitatively measured patients' proteins, based on an iTrac heavy isotope labelling of samples and triple-phase chromatography allied with Orbitrap MS/MS. Data generated showed a list of proteins with their levels of expression for both fast and slow healing Buruli ulcer disease.

The data was analyzed with David.gov bioinformatics software, for differentially expressed proteins, in fast and slow healing Buruli ulcers. Bioinformatics showed a list of proteins which

included; Phosphoglycerate kinase 1, CD163, GTPase IMAP family member 4, Adenylate kinase, Insulin-like growth factor binding protein, Acid-labile subunit, Methyltransferase like 3, Annexin 8/ Annexin A8/like 2, Gluthathione peroxidase 3, Hexosaminidase B (Beta Polypeptide, Vacuola Protein Sorting 52 homolog (*S.Cerevisiae*). hematological and neurological expressed 1**,** Beta-2-microglobulin (B2M), Reticulin, Calnexin PSMB8, Antigen Peptide Transporter 1 and 2, Legumain, Cathepsin S and L, HLA-DRA, Interferon Gama inducible Protein-30(IP30), PSME3, Cluster of differentiation 74 (CD74), Complement component 4A (C4A).

To help throw light on processes underlying fast healing, the biochemical pathways that were enriched with novel proteins were identified. These pathways included the Major Histocompatibility Complex Class I and II, and Classical Complement cascade. Novel proteins that were highly expressed to enrich these pathways were identified and selected. Four of them; Interferon Gama inducible Protein-30 (IP30/ IFI30/ GILT), PSME3, Cluster of differentiation 74 (CD74) and Complement component 4A(C4A) were selected and these were determined in serum of fast and slow healing Buruli ulcer patients.

3.3.3.3. Protein quantification by enzyme linked immunosorbent assay (ELISA)

Prior to serum protein determination, blood samples taken from patients at the treatment sites were centrifuged at 100 ×g for 20 minutes to separate the serum from the cells.

3.3.3.3.1. Interferon Gama inducible Protein-30 (IP30)

The *in vitro* quantitative measurement of Interferon Gamma Inducible Protein 30 (IP30), also known as IFI30 was done, using Human Interferon Gamma Inducible Protein 30 (IFI30) BioAssay™ ELISA kit, from US Biological. The assay is a sandwich enzyme immunoassay.

Kit components: Microtiter plate1 x 96 wells pre-coated, standard 2 x1vial**,** standard diluent 1x20 mL**,** detection reagent A 1x120 µL**,** detection reagent B 1x120 µL**,** assay diluent A 1x12 mL**,** assay diluent B 1x12 mL, TMB substrate 1 x 9 mL, positive control 1.58 ng/mL 1 x 1 vial (lyophilized)**,** stop solution 1 x 6 mL**,** wash buffer 30× 1 x 20 mL, plate sealer 4pieces.

Assay Procedure

Kits components and patients' serum samples were brought to room temperature. Standards of IFI30 were prepared by reconstituting standard sample in 1mL standard diluent provided. The standard was then diluted in standard diluent serially, starting at a concentration of 10 ng/mL and ended at a concentration of 0 ng/mL which served as the blank.

A volume of 100 µL each of prepared standards and patients' serum samples were added in duplicate to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to IFI30. The plate was covered with a plate sealer provided and incubated at a temperature of 37^{o}C for 1 hour. The liquid was then removed from each well and 100 µL of Detection Reagent A working solution was added to each well. The plate was sealed with a plate sealer and incubated for 1 hour at 37^{o}C. After incubation, the solution was aspirated and each well was washed 3 times with 350 µL of 1× wash solution. A volume 100 µL of Detection Reagent B working solution was added to each well. The plate was sealed and incubated at 37^{o}C for 30 minutes. The wells were washed and aspirated 5 times with 350 µL of 1× wash solution after incubation. A volume of 90 µL TMB substrate was added to each well. There was a blue colour observed in the wells. The plate was then sealed and incubated for 10 minutes at 37^{o}C. This was protected from light. A volume of 50 µL of stop solution was added to each. There was a yellow colour observed after the addition of the stop solution. The liquid was mixed by gently hitting the side of the plate and drops

of water and fingerprints were removed with paper towel, to confirm the absence of bubbles on the surface of the liquid. The absorbance was read immediately at 450 nm.

3.3.3.3.2. Complement Component 4A

The *in vitro* quantitative measurement of Complement Component 4A(C4A) was carried out using Human Complement Component 4A(C4A) BioAssay™ ELISA kit from Biomatik. The assay is a sandwich enzyme immunoassay.

Kit components: Well strip plate 1x 96 wells pre-coated, standard 2 x 1 vial**,** standard diluent 1 x 20 mL**,** detection reagent A, 1 x 120 µL**,** detection reagent B, 1 x 120 µL**,** assay diluent A x 12 mL**,** assay diluent B 1 x 12 mL**,** TMB substrate 1 x 9 mL**,** stop solution 1 x 6 mL**,** wash buffer 30× 1 x 20 mL, plate sealer 4 pieces.

Assay Procedure

Kits components and patients' serum samples were brought to room temperature. Standards of C4A were prepared by reconstituting standard content in 1 mL standard diluent provided. The standard was then diluted in standard diluent serially starting at a concentration of 40 ng/mL and ended at a concentration of 0 ng/mL which served as the blank.

A volume of 100 µL each of prepared standards and 1:20 diluted patients' serum samples and 1x PBS were added in duplicate to the appropriate precoated microtiter plate well. The plate was covered with a plate sealer and incubated at a temperature of 37^{o}C for 1 hour. The liquid was then removed from each well and 100 µL of detection reagent A working solution was added to each well. The plate was sealed with a plate sealer and incubated for 1 hour at 37^{o}C. After incubation, the solution was aspirated and each well was washed 3 times with 350 µL of 1× wash solution. A volume of 100 µL of detection reagent B working solution was added to each well. The plate was

sealed and incubated at 37ºC for 30 minutes. The wells were washed and aspirated 5 times with 350 µL of 1X wash solution after incubation. A volume of 90 µL TMB substrate was added to each well. There was a blue colour observed in the wells. The plate was then sealed and incubated for 10 minutes at 37ºC. This was protected from light. A volume of 50 µL of stop solution was added to each. There was a yellow color observed after the addition of the stop solution. The liquid was mixed by gently hitting the side of the plate and drops of water and fingerprints were removed with paper towel, to confirm the absence of bubbles on the surface of the liquid. The absorbance was read immediately at 450 nm.

3.3.3.3.3. Cluster of Differentiation(CD74)

The *in vitro* quantitative measurement of Cluster of Differentiation(CD74) was achieved by using Human Cluster of differentiation(CD74) ELISA kit from Biomatik. The assay is a sandwich enzyme immunoassay.

Kit components: Microwell assay plate 1 x 96 wells pre-coated, standard 2 x 1vial**,** sample diluent 1 x 50 mL**,** Biotin antibody 1 x 120 µL**,** HRP antibody 1 x120 µL**,** Biotin antibody diluent 1 x 15 mL**,** HRP antibody diluent 1 x 15 mL**,** TMB substrate 1 x 10 mL**,** stop solution 1x 10 mL**,** wash buffer 25× 1 x 20 mL, adhesive strip 4.

Assay Procedure: Kits components and patients' serum samples were brought to room temperature. Standards of CD74 was prepared by reconstituting standard content in 1 mL standard diluent provided. The standard was then diluted in standard diluent serially starting at a concentration of 60 ng/mL and ended at a concentration of 0 ng/mL which served as the blank.

A volume of 100 µL each of prepared standards and patients' serum samples were added in duplicate to the appropriate microtiter plate wells. The plate was covered with an adhesive strip provided and incubated at a temperature of 37ºC for 2 hours. The liquid was then removed from

each well without washing and 100 µL of Biotin-antibody (1×) working solution was added to each well. The plate was sealed with an adhesive strip and incubated for 1 hour at 37°C. After incubation, the solution was aspirated and each well was washed 3 times with 200 µL of 1× wash solution. A volume 100 µL of HRP-avidin (1×) working solution was added to each well. The plate was sealed and incubated at 37°C for 1 hour. The wells were washed and aspirated 5 times with 200 µL of 1× wash solution after incubation. A volume of 90 µL TMB substrate was added to each well. There was a blue colour observed in wells. The plate was then sealed and incubated for 10 minutes at 37°C. This was protected from light. A volume of 50 µL of stop solution was added to each. There was a yellow colour observed after the addition of the stop solution. The liquid was mixed by gently hitting the side of the plate and drops of water and fingerprints were removed with paper towel, to confirm the absence of bubbles on the surface of the liquid. The absorbance was read immediately at 450 nm.

3.3.3.3.4. Proteasome activator subunit 3 (PSME3)

The *in vitro* quantitative measurement of proteasome activator subunit 3 (PSME3) was done using Human proteasome activator subunit 3 (PSME3) ELISA kit from Biomatik. The assay is a sandwich enzyme immunoassay.

Kit components: Microwell assay plate 1 x 96 wells pre-coated, standard 2 x 1 vial**,** sample diluent 1 x 50 mL**,** Biotin antibody 1 x 120 µL**,** HRP antibody 1x120 µL**,** Biotin antibody diluent 1 x 15 mL**,** HRP antibody diluent 1 x15 mL**,** TMB Substrate 1 x 9 mL**,** stop solution 1 x 10 mL**,** wash buffer 25× 1 x 20 mL, adhesive strip 4.

Assay Procedure: Kit components and patients' serum samples were brought to room temperature. Standards of PSME3 were prepared by reconstituting standard content in 1 mL standard diluent

provided. The standard was then diluted in standard diluent serially starting at a concentration of 1200 pg/mL and ended at a concentration of 0 pg/mL, which served as the blank.

A volume of 100 μL each of prepared standards and patients' serum samples were added in duplicate to the appropriate microtiter plate wells. The plate was covered with an adhesive strip provided and incubated at a temperature of 37^{o}C for 2 hours. The liquid was then removed from each well without washing and 100 μL of Biotin-antibody (1×) working solution was added to each well. The plate was sealed with an adhesive strip and incubated for 1 hour at 37^{o}C. After incubation, the solution was aspirated and each well was washed 3 times with 200 μL of 1× wash solution. A volume 100 μL of HRP-avidin (1×) working solution was added to each well. The plate was sealed and incubated at 37^{o}C for 1hour. The wells were washed and aspirated 5 times with 200 μL of 1× wash solution after incubation. A volume of 90 μL TMB substrate was added to each well. There was a blue colour observed in wells. The plate was then sealed and incubated for 10 minutes at 37^{o}C. This was protected from light. A volume of 50 μL of stop solution was added to each. There was a yellow colour observed after the addition of the stop solution. The liquid was mixed by gently hitting the side of the plate and drops of water and fingerprints were removed with paper towel, to confirm the absence of bubbles on the surface of the liquid. The absorbance was read immediately at 450 nm.

Figure 3.2: Removing samples from the ELISA plate after incubation.

3.3.3.3.5 Absorbance reading of ELISA proteins and calculations

Absorbance reading of the proteins was done using TECAN Sunrise ELISA plate reader (Tecan Group Ltd., Switzerland). Mean absorbance readings of the duplicated samples, standards and controls were calculated, using Excel and mean zero standard absorbance values were deducted. The results obtained were analysed with GraphPad Prism 6 software and standards best-fit curves were plotted.

Figure 3.3: Measuring protein absorbance using TECAN Sunrise.

3.3.4 Lesion measurement approach

Definition for rate of healing = mean diameter of ulcer (d) ÷ time for healing (t).

(d/t) = rate of healing. Patients whose lesions healed in less than 12 weeks, according to digital Planimetry Aranz silhouette, were considered as fast healers, whereas those that healed after 12 weeks were considered as slow healers.

3.3.5. Data management and statistical analysis

Statistical analysis was done, using Microsoft Excel 2016 and GraphPad Prism 6 software. The raw data obtained from the plate reader were entered in Excel, and GraphPad Prism 6 software was used in drawing standard curves for the various protein assayed. General descriptive information such as mean and standard deviation from the data were calculated. Fisher's exact test

was used to compare proportions, sex distribution and age distribution of study participants. One-way ANOVA (Kruskal-Wallis test) was used to compare group means. Results of proteins levels were expressed as medians and interquartile ranges. Protein concentrations in fast healers and slow healers were compared, using Mann-Whitney U test for continuous data; $p < 0.05$ was considered statistically significant.

CHAPTER FOUR

RESULTS

4.1.1. Patients and controls characteristics

Table 4.1, shows the demographics of the study participants. There were 55 Buruli ulcer cases confirmed by *M. ulcerans* IS2404 PCR, 30 endemic controls with known contact with Bu patients, and 30 controls who were residents in communities without the disease. The median (interquartile range) age for the Buruli ulcer cases was 16 years (12 - 33), 13 years (10 - 20) for endemic controls and 17 years (12 - 35) for non-endemic controls. Among the cases, there were 32 (58.2%) ulcers, 12 (21.8%) nodules and 11 (20%) plaques. The lesions were further categorized into categories I and II, and there were 33 (60%) category I and 22 (40%) category II lesions.

Table 4.1: Characteristics of cases and controls (endemic and non-endemic) enrolled in the study.

Parameters	**Cases = 55**	**Endemic n = 30**	**Non-endemic n = 30**	**Total**	**p value**
Age in yrs,					
Median (IQR)	16(12-33)	13(10-20)	17(11.75-34.50)	15 (11-28)	0.25
Sex, n (%)					
Male	19(46.4)	11(26.8)	11(26.8)	41(35.7)	
Female	36(48.6)	19(25.7)	19(25.7)	74(64.3)	0.97
Clinical form,					
n (%)					
Ulcer	32(58)	_	_		
Nodule	12(22)	_	_		
Plaque	11(20)	_	_		
Location of lesion, n (%)					
Upper limb	16(29)	_	_		
Lower limb	36(65)	_	_		
Other sites	3(6)	_	_		
Category of lesion, n (%)					
I	33(60)	_	_		
II	22(40)	_	_		

4.1.2. Characteristics of fast and slow healing Buruli ulcer patients.

Table 4.2, shows some demographic features of Buruli ulcer disease patients, grouped into fast and slow healers, according to time to complete healing. There were 55 cases, confirmed as *M. ulcerans* IS2404 PCR, of which 25 were fast and 30 were slow healers. The median (interquartile range) age for fast healers was 18 years (13-35) and 16 years (12-32) for slow healers. Among the fast healers, there were 18 ulcers and 7 nodules, which were further classified as 19 category I and 6 category II lesions. However, for the slow healers, there were 13 ulcers, 6 nodules and 11 plaques, classified as 14 category I and 16 category II lesions. Most lesions were located on the lower limbs for both fast 20 (55.6%) and slow healers 16 (44.4%), compared to the upper limb, 5 (31.3%) for fast healers and 11 (68.7%) for slow healers.

Table 4.2: Characteristics of cases who presented with Buruli ulcer, grouped into fast and slow healers.

Parameters	Fast healers n=25	Slow healers n=30	Total	p value
Age in yrs, median (IQR)	18 (13-35)	16 (12-32)	15 (12-33)	0.72
Sex, n (%)				
Male	7 (36.8)	12(63.2)	19(34.5)	
Female	18(50)	18(50)	36(65.5)	0.4
Clinical form, n (%)				
Ulcer	18(58.1)	13(41.9)	31(56.4)	
Nodule	7(53.8)	6(46.2)	13(23.6)	
Plaque	0(0)	11(100)	11(20)	0.0031
Location of lesion, n (%)				
Upper limb	5(31.3)	11(68.7)	16(29.0)	
Lower limb	20(55.6)	16(44.4)	36(65.5)	
Other sites	0(0)	3(100)	3(5.5)	0.07
Category of lesion, n (%)				
I	19(57.6)	14(42.4)	33(60)	
II	6(27.3)	16(72.7)	22(40)	0.03

4.2. Analyses carried out for the selection of novel protein(s) with potential for predicting fast and slow healing of Buruli ulcer.

This was determined using an *in-silico* approach. Mass spectrometer was used to measure patients' proteins expressed and their level of expression. This generated high throughput data, from tissue biopsies obtained from the patients. The data was subsequently analyzed with David.gov bioinformatics software, for the differentially expressed proteins in fast and slow healing Buruli ulcers. Bioinformatics showed a list of proteins and their functions. These are listed in Table 4.3.

Table 4.3: List of proteins, as obtained through bioinformatics.

Phosphoglycerate kinase 1
CD163
GTPase IMAP family member 4
Adenylate kinase
Insulin-like growth factor binding protein
Acid-labile subunit
Methyltransferase like 3
Annexin 8/ Annexin A8/like 2,
Gluthathione peroxidase 3
Hexosaminidase B (Beta Polypeptide)
Vacuola Protein Sorting 52 homolog (*S.Cerevisiae*)
Beta-2-microglobulin(B2M)
Reticulin,

Calnexin
PSMB8,
Antigen Peptide Transporter 1 and 2,
Legumain,
Cathepsin S and L,
HLA-DRA,
Interferon Gama inducible Protein-30(IP30),
PSME3,
Cluster of differentiation 74 (CD74),
Complement component 4A(C4A).

To help throw light on processes underlying the rate of healing, the expressed proteins for the biochemical pathways were identified. These pathways included, the Major Histocompatibility Complex Class I and II, and Classical Complement cascade, that play vital roles in healing of diseases. Hypotheses were generated to support the predictability of the identified proteins, for fast or slow healing in Buruli ulcer patients.

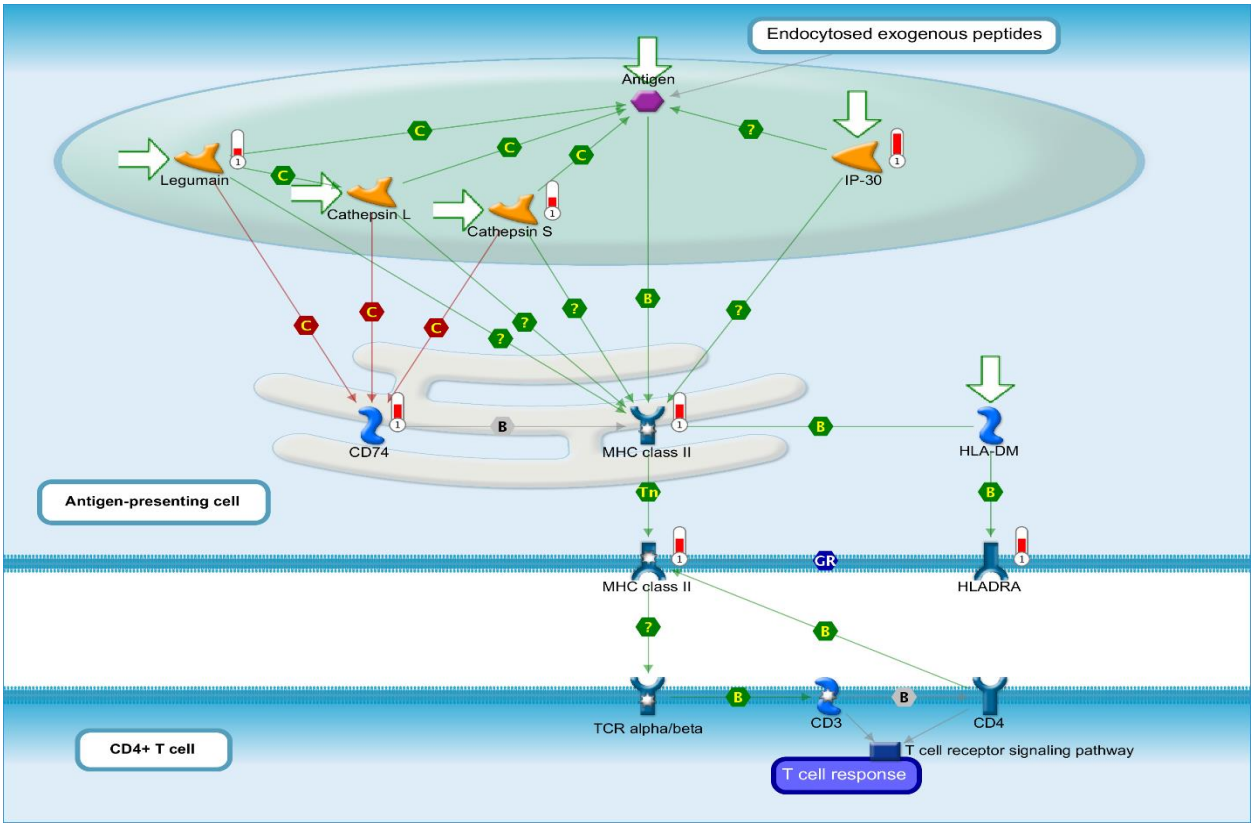

Figure 4.1: MHC class II pathway showing proteins expressed by fast healing Bu patients with their levels of expressions.

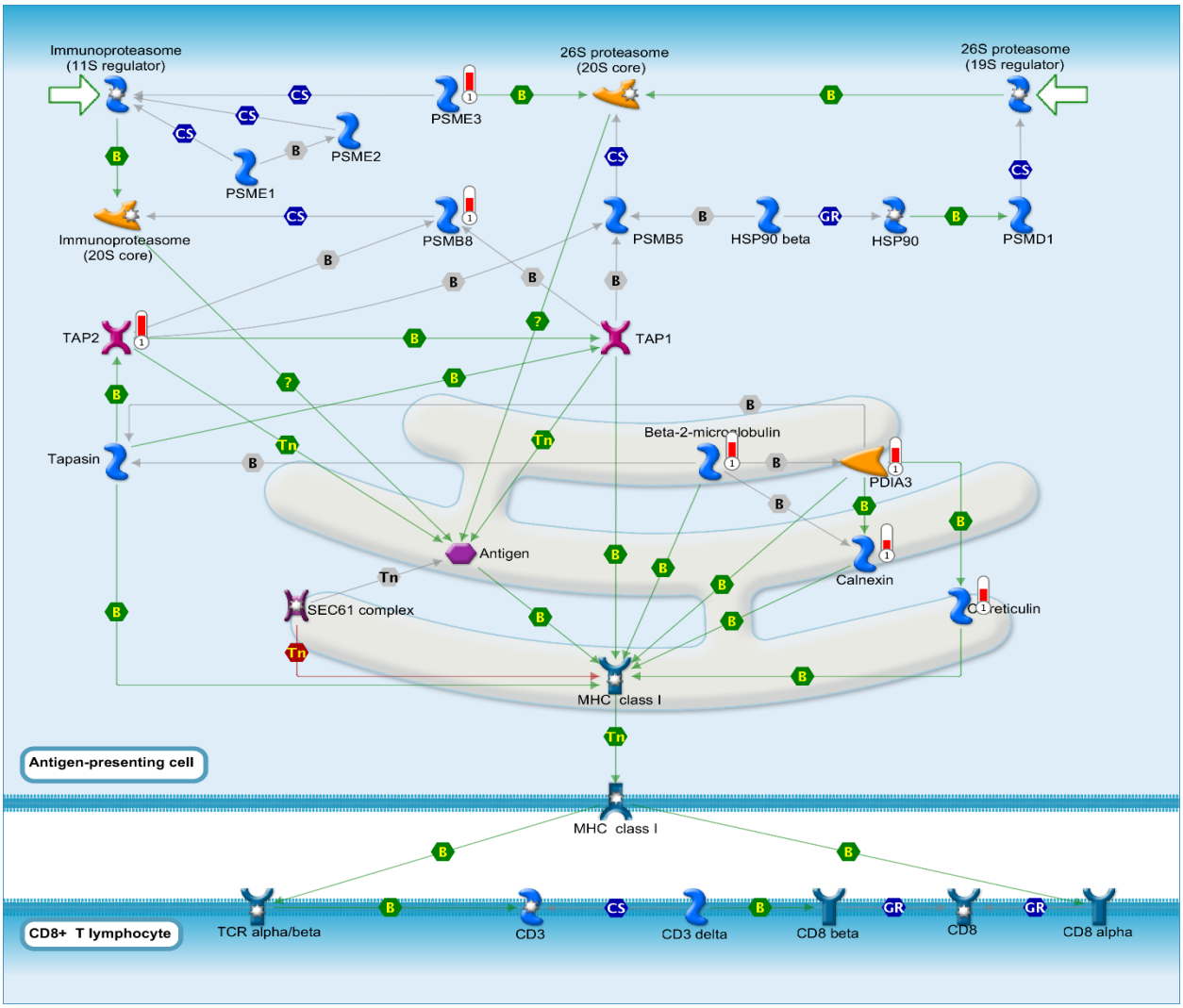

Figure 4.2: MHC class II pathway showing proteins expressed by fast healing Bu patients with their levels of expressions.

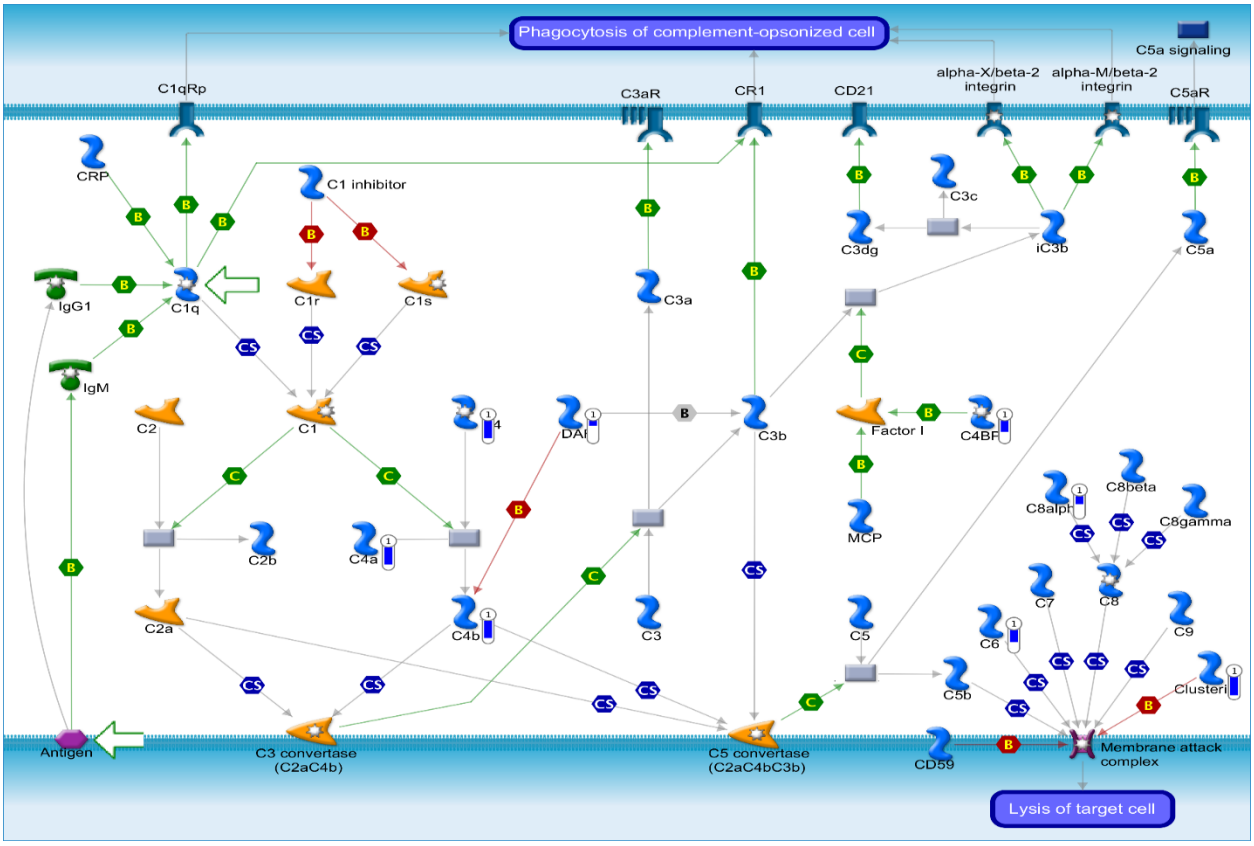

Figure 4.3: Classical complement pathway showing proteins expressed by fast healing Bu patients with their levels of expressions.

Among the nineteen (19) proteins identified from the above pathways, Interferon Gamma Inducible Protein - 30 (IP30/ IFI30/ GILT), Proteasome Activator Complex Subunit 3 (PSME3), Cluster of differentiation 74 (CD74) and Complement component 4A(C4A) were selected for further analysis due to their expression levels and assay availability/feasibility.

4.3. The predictive capability/potential of the IP30, CD74, PSME3 and C4A for fast/ slow healing in serum of Buruli ulcer patients.

To evaluate the predictive capability of selected proteins, serum samples of patients and controls were obtained at baseline. The serum proteins of patients and controls were determined using sandwich ELISA techniques, as described previously.

4.3.1. Interferon-γ inducible protein (IFI30) expression levels of patients; fast healers compared to slow healers.

Interferon-γ inducible protein (IFI30) expression by fast and slow healing patients with Bu was determined at baseline by Sandwich ELISA techniques, using the serum of patients with Bu. Fast healing Bu patients expressed higher levels interferon-γ inducible protein (IFI30) with median of 1.5 ng/mL (0.8 – 2.2) ng/mL, compared to slow healing Bu with median of 1.2 ng/mL (0.2 - 2.0) ng/mL. Although fast healers expressed slightly higher levels of the protein than slow healers, it was not statistically significant ($p > 0.05$) (Figure 4.4).

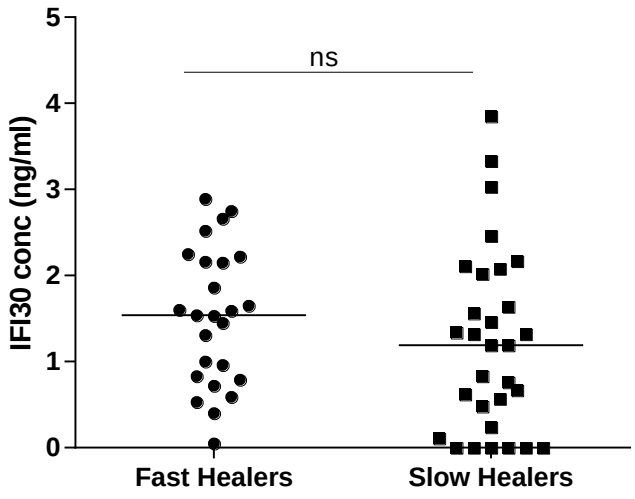

Figure 4.4: Comparison of differential expression of interferon-γ inducible protein (IF130) between fast and slow healers. Each dot represents protein concentration of each participant. The horizontal line represents the median for each group. (ns – no statistically significant difference)

4.3.2 Cluster of Differentiation 74 (CD74) expression levels of patients; fast healers compared to slow healers.

Cluster of Differentiation 74 (CD74) expression by patients with fast and slow healing Bu was determined at baseline by Sandwich ELISA techniques, were compared. The expression of CD74 in fast healers was (0.4 (0.07 – 2.8) ng/mL) and was lower when compared with those of slow healers, (1.2 (0.2 - 4.1) ng/mL), but the difference was not statistically significant ($p > 0.05$) (Figure 4.5).

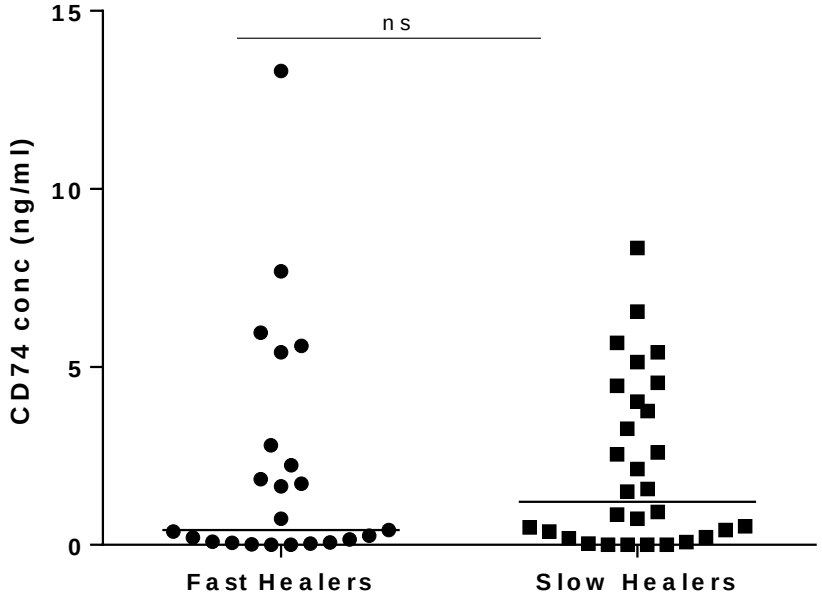

Figure 4.5: Comparison of differential expression of Cluster of Differentiation 74 (CD74) proteins between fast and slow healers. Each dot represents protein concentration of each participant. The horizontal line represents the median for each group. (ns – no statistically significant difference)

4.3.4. Proteasome Activator Complex Subunit 3 (PSME3) expression levels of patients; fast healers compared to slow healers.

Proteasome Activator Complex Subunit 3 (PSME3) expression by patients with Bu was determined at baseline by Sandwich ELISA techniques, in order to find the levels of expression between fast healing Bu and slow healing patients. The expression of PSME3 in fast healers was (0.35 (0.21 – 0.56) ng/mL): this was lower, compared with those of slow healers, (0.39 (0.20 – 0.63) ng/mL), but the difference was not statistically significant ($p > 0.05$) (Figure 4.6).

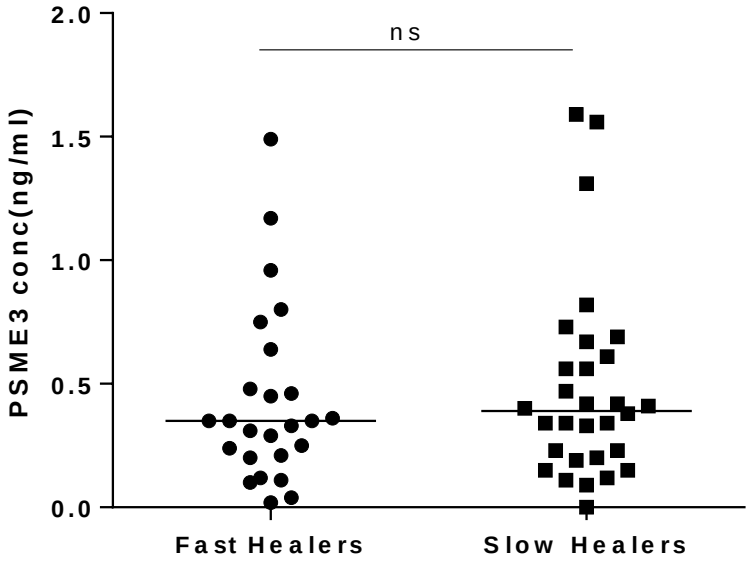

Figure 4.6: Comparison of differential expression of proteasome activator complex subunit 3 (PSME3) proteins between fast and slow healers. Each dot represents protein concentration of each participant. The horizontal line represents the median for each group. (ns – no statistically significant difference)

4.3.5. Complement Component 4A (C4A) expression levels of patients; fast healers compared to slow healers.

Complement Component 4A (C4A) expression by patients with Bu was determined at baseline by Sandwich ELISA techniques using serum of patients with Bu. C4A protein differential expression of patients with fast healing Bu and slow healing Bu were compared. The expression level of C4A in fast healers was (7.0 (0.31 – 35.29) ng/mL), was higher than those of slow healers, (4.0 (0.09 – 22.13) ng/mL) but the difference was not statistically significant ($p > 0.05$) (Figure 4.7).

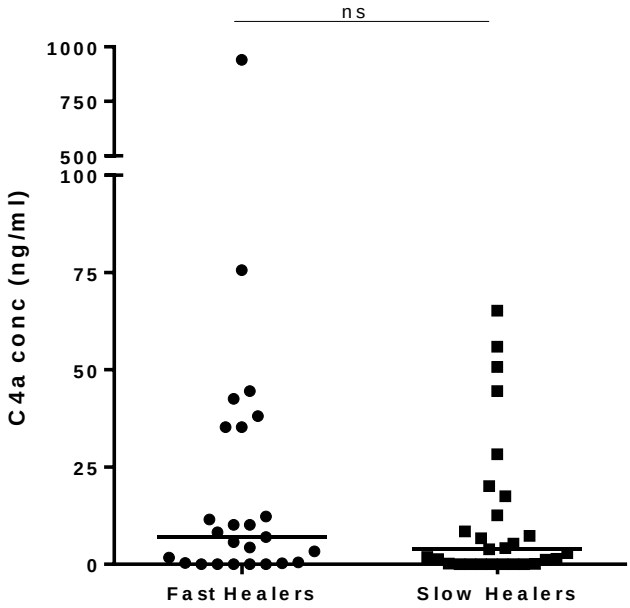

Figure 4.7: Comparison of differential expression of Complement component 4a (C4a) proteins between fast and slow healers. Each dot represents protein concentration of each participant. The horizontal line represents the median for each group. (ns – no statistically significant difference)

4.3.6. Correlation of selected proteins concentration to time to healing in the various forms of the disease.

The concentrations of the selected protein expressed in patients at baseline, were correlated with time to complete healing in the various lesion of the disease presented.

4.3.6.1. Correlation of Interferon-γ inducible protein (IFI30) concentration to healing completion time in the various forms of the disease.

Interferon-γ inducible protein (IFI30) differential expression by patients with Bu at baseline was determined in ulcers, nodules and plaques. These concentrations were correlated to their corresponding time to complete healing and a linear regression graph was drawn.

Figure 4.8 shows that the twelve **(**12) nodular cases showed a positive correlation of IFI30 with time to healing; that is, the higher the concentration of IFI30 protein at baseline, the longer the time to complete healing. For the 11 plaques presented, there was a negative correlation between IFI30 baseline concentration and the time to healing, meaning the higher the concentration of IFI30 the faster the healing in plaques. The 32 ulcerated forms showed different concentration at different time to healing and there was no significant correlation. The correlation was a negative one.

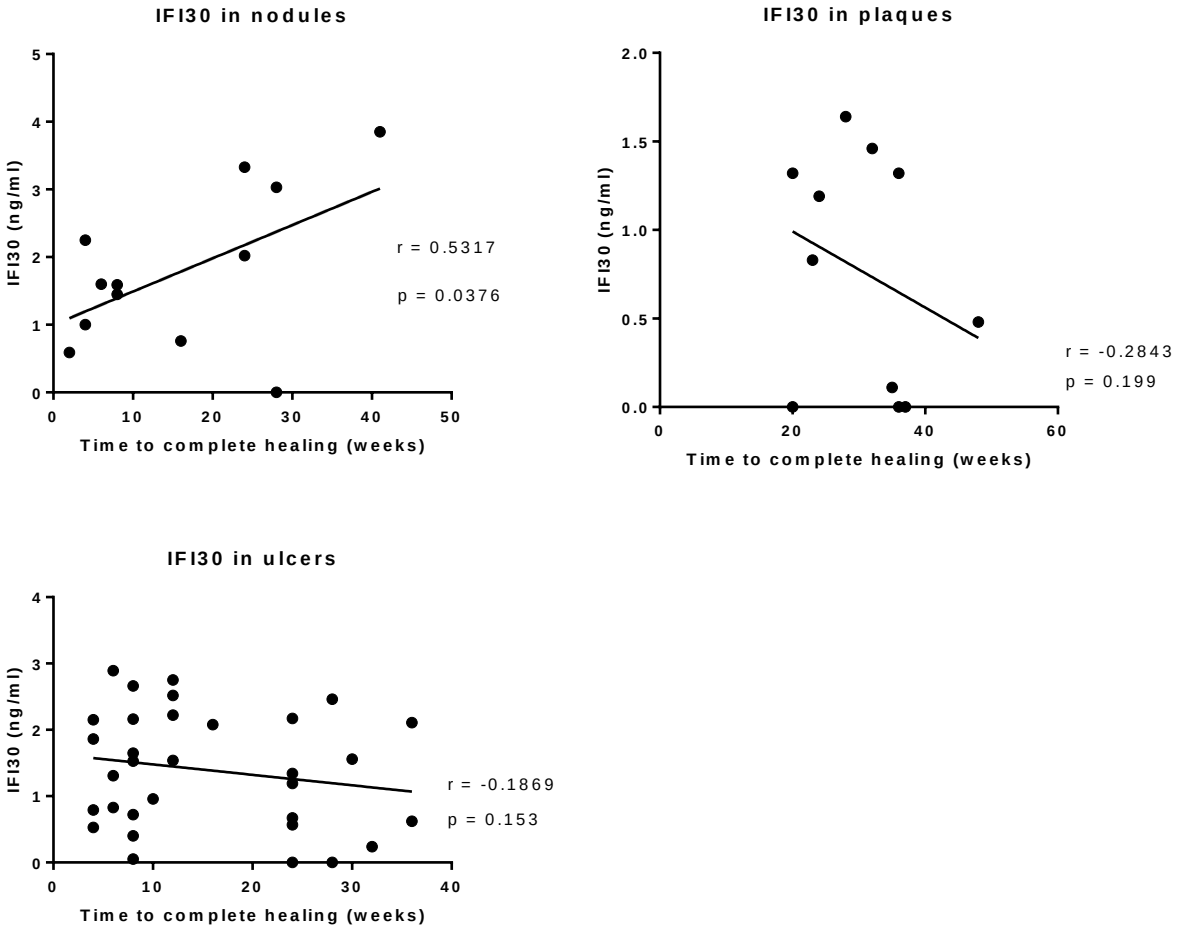

Figure 4.8: Correlation of IFI30 protein expression and time to healing in various lesions. Each dot represents protein concentration versus time to healing for a participant.

4.3.6.2. Correlation of Cluster of Differentiation 74 (CD74) concentration with time to healing in the various forms of the disease.

Cluster of Differentiation 74 (CD74) differential expression by patients with Bu at baseline was determined in ulcers, nodules and plaques. These concentrations were correlated to their corresponding time to complete healing and a linear regression graph drawn.

Figure 4.9 shows plots of CD74 baseline concentrations in the various lesion (ulcer, nodule and plaque) presented correlating to the time to complete healing**.** In all cases, there was a slightly negative correlation and there was no significant correlation of CD74 with complete time to healing.

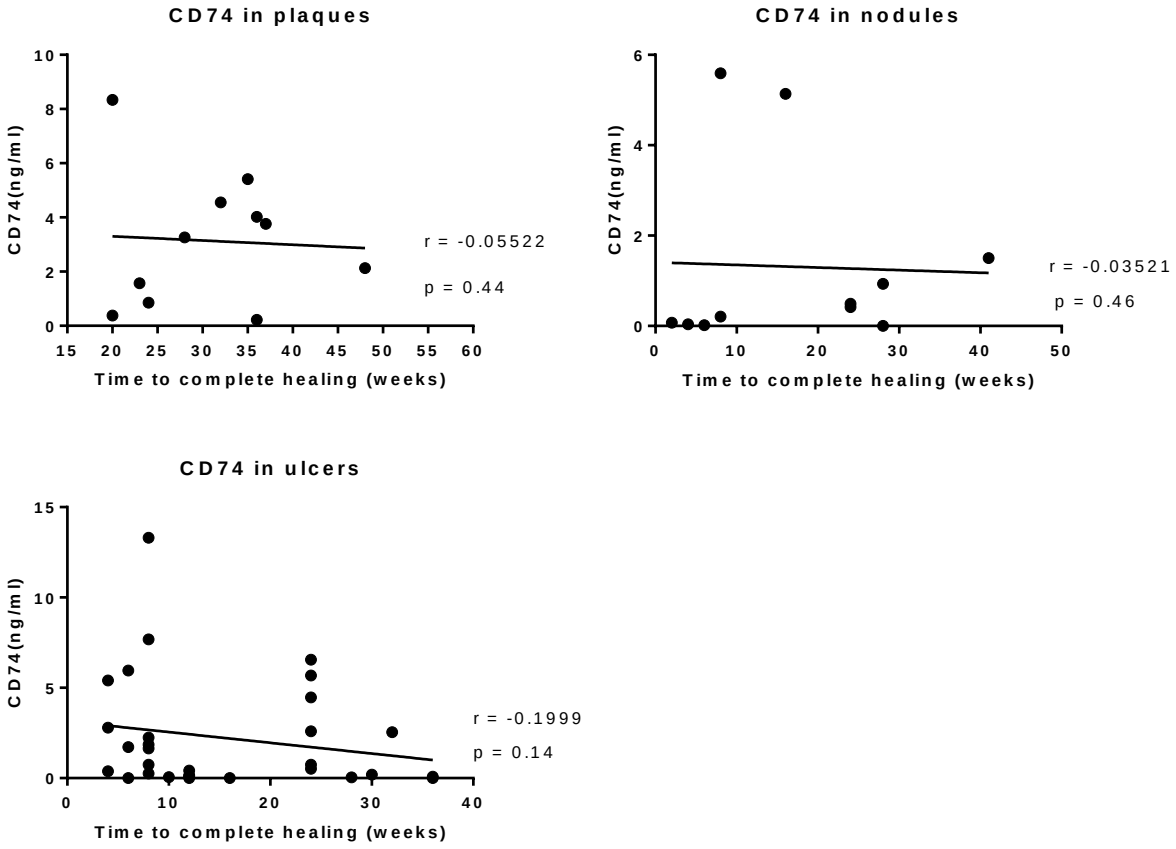

Figure 4.9: Correlation of CD74 protein expression and time to healing in various lesion. Each dot represents protein concentration versus time to healing for a participant. The line represents linear regression.

4.3.6.3. Correlation of Proteasome Activator Complex Subunit 3 (PSME3) concentration to time to healing in the various forms of the disease.

To determine this, Proteasome Activator Complex Subunit 3 (PSME3) differential expression by patients with Bu at baseline was analysed in ulcers, nodules and plaques. These concentrations were correlated to their corresponding time to complete healing and a linear regression graph was drawn.

Figure 4.10 shows the various lesions presented and the concentration of PSME3 at baseline plotted against the time to complete healing. There was no significant correlation of PSME3 concentration at baseline with complete time to healing

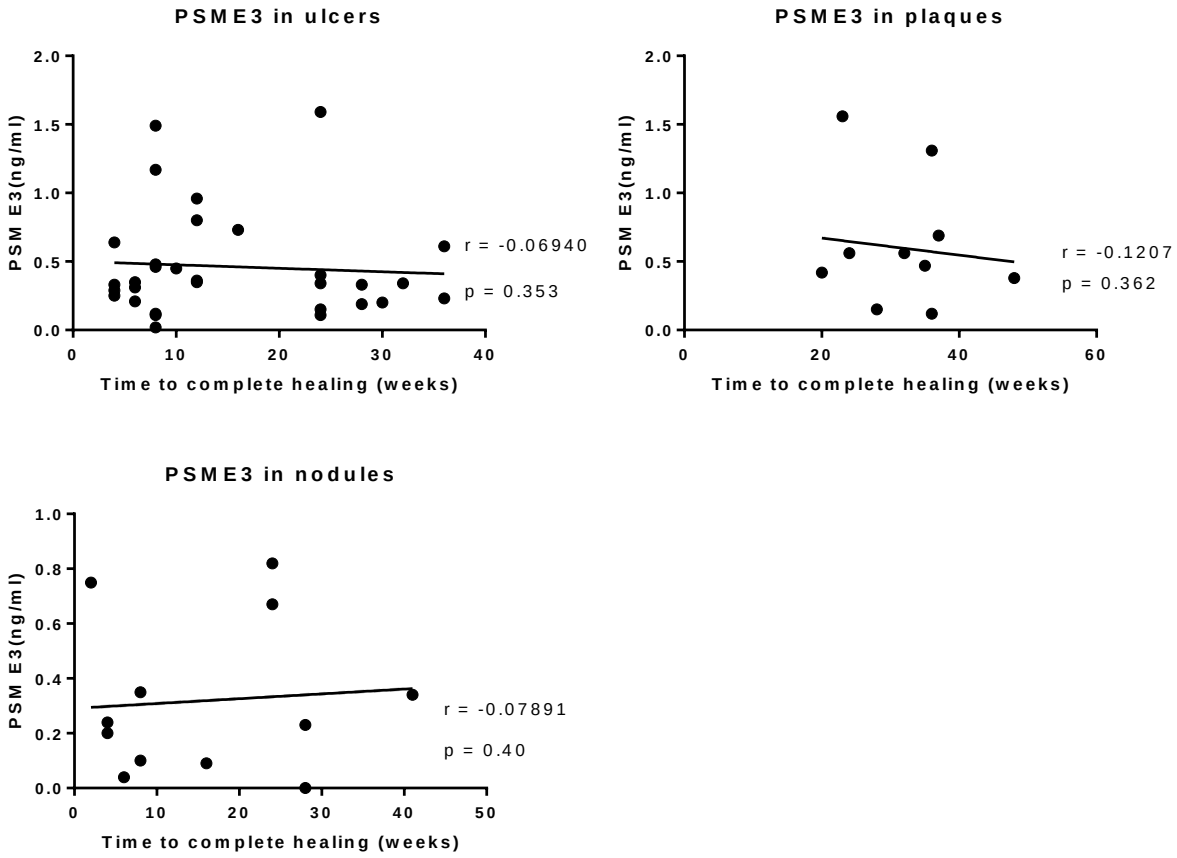

Figure 4.10: Correlation of PSME3 protein expression and time to healing in various lesion. Each dot represents protein concentration versus time to healing for a participant. The line represents linear regression.

4.3.6.4. Correlation of Complement Component 4A (C4A) concentration with time to complete healing in the various forms of the disease.

Complement Component 4A (C4A) differential expression by patients with Bu at baseline was determined in ulcers, nodules and plaques. These concentrations were correlated to their corresponding time to complete healing and a linear regression graph was drawn.

Figure 4.11 shows the various forms of Bu presented and the baseline concentration of C4A at the time to complete healing. There was no significant correlation of C4A with time to complete healing.

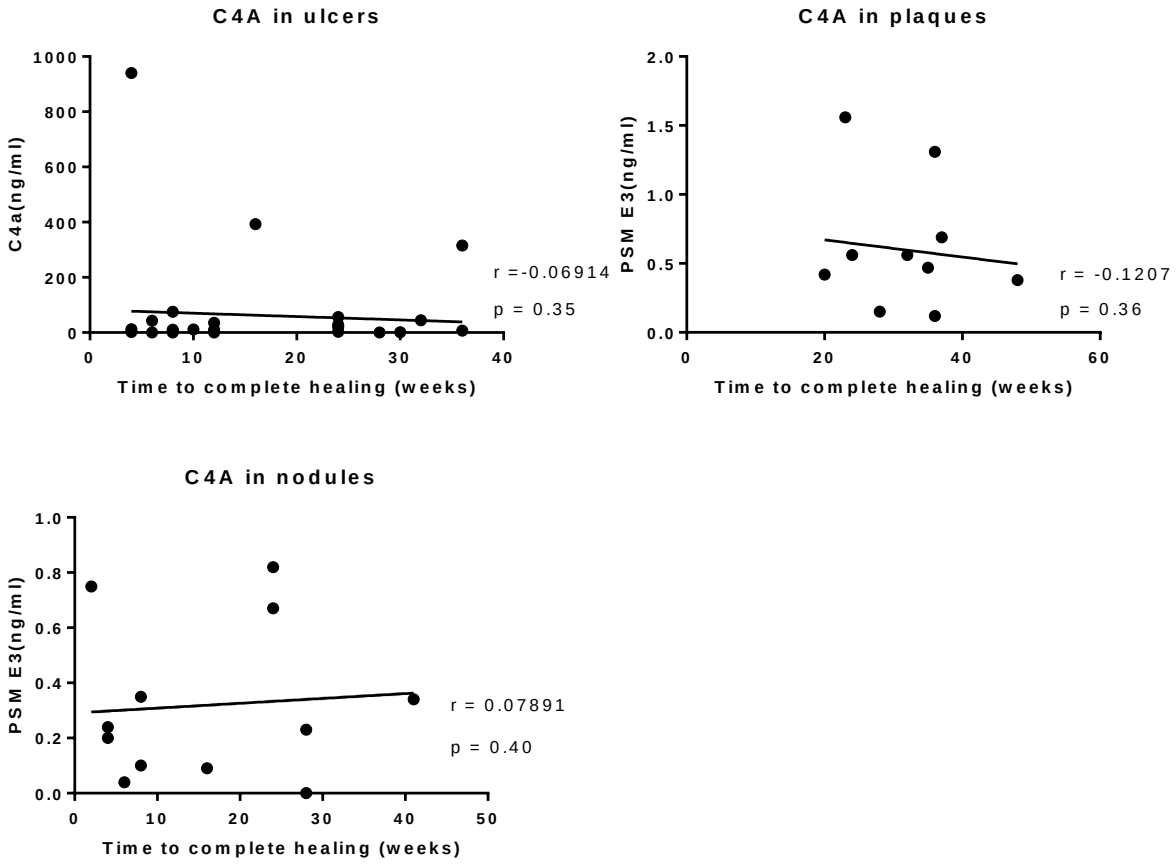

Figure 4.11: Correlation of C4A protein expression and time to healing in various lesions. Each dot represents protein concentration versus time to healing for a participant. The line represents linear regression.

4.4. The level of expression of IFI30, CD74, PSME3 AND C4A Buruli ulcer disease patients and controls from endemic and non-endemic areas.

The expression of baseline concentrations of IFI30, CD74, PSME3 and C4A proteins was compared in Buruli ulcer cases and controls from endemic and non-endemic areas. Figure 4.12 shows that IFI30 protein expression was higher in controls from non-endemic areas (1.34 (1.0 – 2.3) ng/mL), compared to those from cases (1.32 (0.59 – 2.1) ng/mL). This was statistically significant ($p = 0.02$). The expression of CD74 was higher in controls in endemic controls (2.3 (0.24 -6.23) ng/mL), compared to those of non-endemic individuals (1.42 (0.12 – 3.84) ng/mL) and cases (0.85 (0.12 – 3.89) ng/mL). The differences were not statistically significant ($p > 0.5$). The expressions of PSME3 protein were similar in cases (0.35 (0.2 – 0.61) ng/mL), endemic controls (0.32 (0.21 – 0.54) ng/mL) and controls from non-endemic areas (0.28 (0.2 – 0.37) ng/mL). C4A protein expression was significantly higher in non-endemic controls (67.71 (16.1 – 114.5) ng/mL), compared to those of cases (5.35 (0.20 – 28.32) ng/mL) ($p < 0.01$).

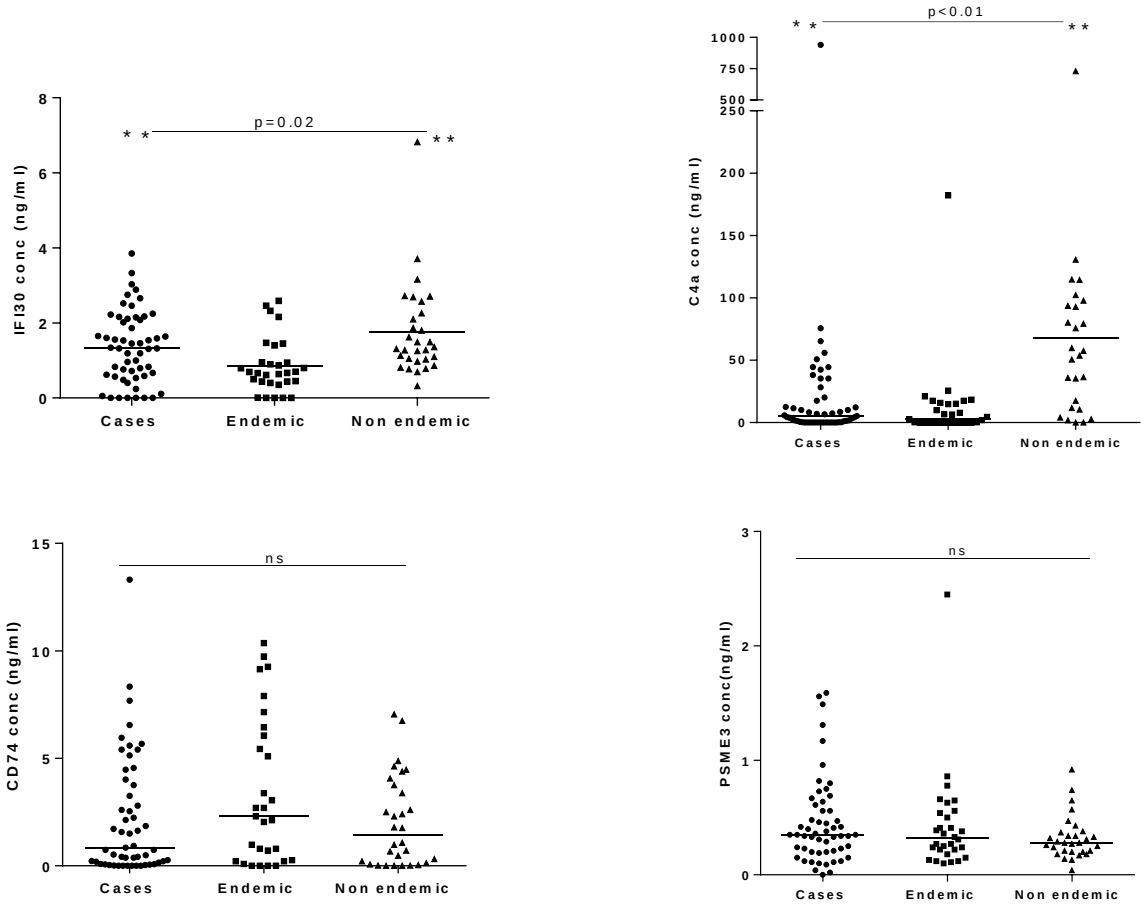

Figure 4.12: Differential expression of proteins by various groups of participants. Each dot represents baseline protein concentration for each participant. The horizontal line represents the median for each group.

4.5. The level of expression of IFI30, CD74, PSME3 AND C4A in the various lesions.

The expression of baseline concentration of IFI30, CD74, PSME3 and C4A protein was compared in patients presenting the various forms of Bu. Figure 4.14 shows that, IFI30 protein expression was significantly higher in nodule (1.60 (0.82 – 2.84) ng/mL), compared to those of ulcers (1.44 (0.63 – 2.16) ng/mL) and plaques (0.83(0.0 – 1.32) ng/mL) (p=0.03).

Similarly, CD74 protein expression of the protein were significantly higher in plaques (3.26 (0.85 – 4.55) ng/mL), compared to nodular forms (0.42 (0.04 – 1.50) ng/mL) (p = 0.04). The level of expression of PSME3 protein was higher in plaques (0.47 (0.38 – 0.69) ng/mL), compared to ulcers (0.34 (0.22 – 0.58) ng/mL) and nodules (0.24 (0.09 – 0.59) ng/mL) but their differences were not statistically significant $p > 0.05$.

C4A protein expression was higher in ulcers (7.15 (0.11 – 33.55) ng/mL), compared to nodules (4.28 (0.59 – 30.85) ng/mL) and plaques (3.87 (0.12 – 12.60) ng/mL), but their differences in expression levels were not statistically significant ($p > 0.05$).

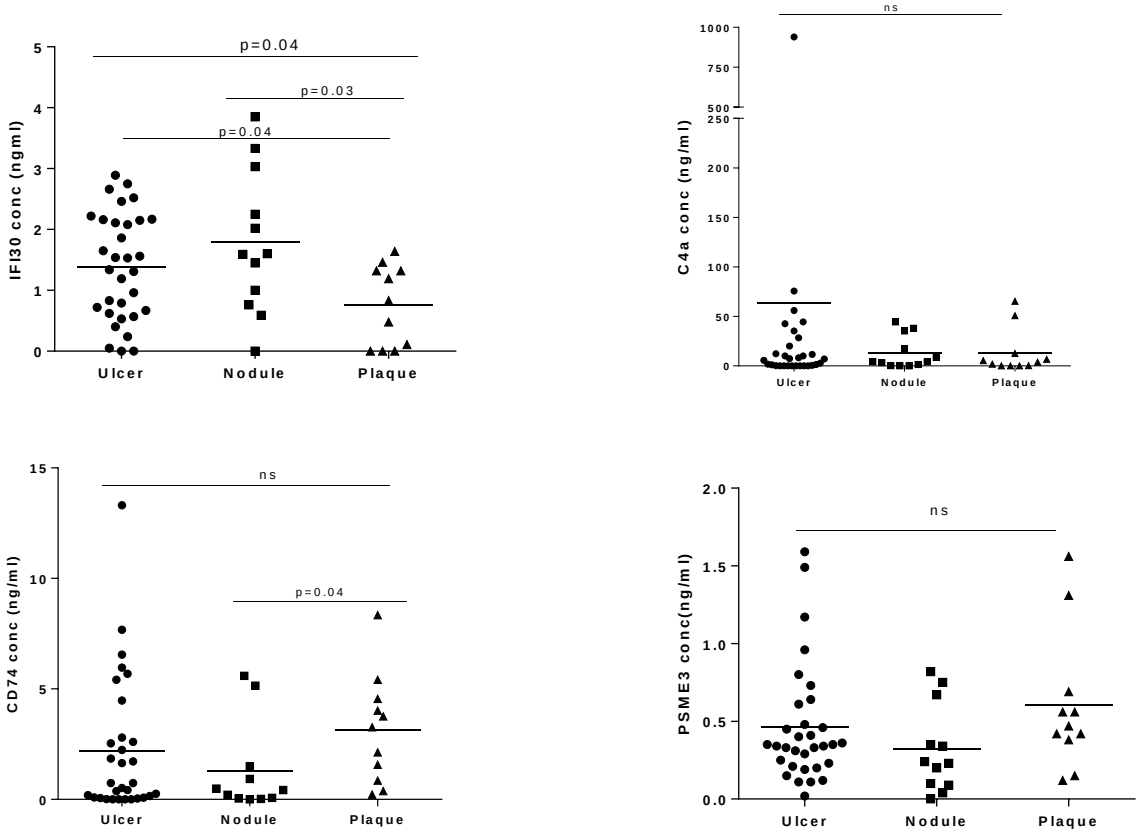

Figure 4.13: Differential expression of selected proteins by various lesion forms. Each dot represents protein concentration of each participant. The horizontal line represents the median for each group**.**

CHAPTER FIVE

DISCUSSION

5.1. Selection of novel protein(s) with potential for predicting fast and slow healing in Buruli ulcer.

Healing is achieved typically through haemostasis, inflammation, proliferation and synthesis of extracellular matrix (ECM), followed by a remodeling of the ECM (Phillips *et al.*, 2014). During haemostasis, blood platelets play a role in releasing cytokines, chemokines and hormones associated with healing. Inflammation phase causes debridement of tissues by inflammatory cells for angiogenesis, fibroplasia and epithelisation to occur. During the later stage, proliferation occurs and finally crosslinking of collagen (Simon *et al*., 2016).

Studies have suggested that mycolactone which is a toxin produced by *M.ulcerans* may have certain controls on proteins like growth factors needed for wound healing (Coutanceau *et al*., 2007; Sarfo *et al*., 2009).

Studies of serum proteins in Bu patients and controls indicated that in Bu disease there can be down-regulation of the circulating levels of a large array of proteins. Several proteins were noted to contribute to acute phase reaction, lipid metabolism, coagulation and tissue remodeling which are involved in healing (Phillips *et al.*, 2014a).

Through a collaboration with University of Southampton, a large array of proteins was generated from tissue biopsy samples from fast healing and slow healing Bu patients, using mass spectrometry and bioinformatics. The expression levels of some proteins were found to be higher in fast healers compared to those that healed slowly. In fact, as shown in Figures 4.1, 4.2 and 4.3, nineteen (19) proteins could be identified, to be involved in three key immunologic pathways.

However, due to cost, technical and time constraints, four of the proteins were selected for assay and quantification, as has been indicated in the Materials and Methods section.

IFI30, PSME3, CD74, C4A were selected among all other proteins expressed by Buruli ulcer patients because these proteins were identified in immunological pathways such as MHC class I, MHC class II and Complement cascade, hence it is anticipated that these proteins have the potential of predicting healing in Bu disease. Their level of expression was higher in tissue biopsies collected, compared to other proteins expressed. These proteins have the potential to throw more light on the biochemical pathways and processes underlying healing in Buruli ulcer.

5.2. Predictive capability/potential of IFI30, CD74, PSME3 and C4A for fast or slow healing Buruli ulcer patients.

5.2.1. Predictive capability/potential of IFI30 for fast and slow healing Buruli ulcer patients.

IFI30 plays a key role in antigen processing and MHC class I- restricted cross presentation. It does this by the reduction of disulphide bonds present in endocytic proteins, enhancing the unfolding of endocytosed protein, leading to degradation. (Hastings and Cresswell, 2011). Several studies indicate that IFI30 plays a key role in antigen processing and hence its influence on peptide repertoire, can change immune response characteristics and affect central tolerance. In melanoma studies, IFI30 regulates T-cells production. CD4+ T Cell activation is enhanced by presentation of shed tumour antigens by professional antigen presenting cells (APC) coupled with display of similar antigenic epitope by MHC II on malignant cells (Haque *et al*., 2002). Its expression in melanomas is supposed to result in improved presentation of melanoma antigens and more effective antimelanoma T-cells response. IFI30 expression in melanoma cells could prove to be very promising for direct antigen presentation and CD4+ cell recognition. In wound healing, the

specific role of T-cells is not completely understood but studies have suggested that decrease in levels of T-cells causes a delay in T-cell infiltration and hence impaired healing. Also, the skin gamma-delta T-cells regulate several aspects of wound healing which include maintaining tissue integrity, defending against pathogens and regulation of inflammation (Simon *et al*., 2016).

In this study, fast healing Bu expressed higher levels of interferon-γ inducible protein (IFI30) compared to slow healing Bu (Figure 4.4). Although the levels were higher in fast healers, it was not statistically significant. Higher levels of IFI30 could have meant improved presentation of *Mycobacterium ulceran* antigens and regulation of T-cells production and CD4+ cells recognition. Mycolactone which is involved in the pathogenesis of the disease has been shown to impair the capacity of T-cells to produce cytokines when stimulated *in vitro*. The dynamics of these cytokines during treatment shows that they were not positively regulated during treatment (Phillips *et al.*, 2009b).

The study also suggests that mycolactone probably impairs T-cells production by suppressing the level of expression of IFI30 capable of regulating the production of T-cells for proper wound healing. Correlation analysis of IFI30 protein baseline concentration and the time to complete healing in the various lesion (Figure 4.8) shows that in nodules, the higher the concentration of IFI30 protein, the longer the time to complete healing, but, in plaques, the higher the concentration of IFI30 protein at baseline, the shorter the time to complete healing. What this suggests is that the pathophysiological features of nodules and plaques are different.

5.2.2. Predictive capability/potential of CD74 for fast and slow healing Buruli ulcer patients.

CD74 is highly expressed in inflammation. It is a receptor for binding macrophage migration inhibitory factor of *H. pylori*. NF-KB Erk1/2 activation occurs, along with induction of proinflammatory cytokine secretion (Mun *et al*., 2013). CD74 has recently emerged as an integral

component of a receptor complex for macrophage migrating factor which is a cytokine-like protein that is very versatile. It mediates both innate and adaptive immunity. It plays a key role in inflammation (Mun *et al*., 2013).Studies suggest that the protein complexes with CXCR2 which is an IL8 receptor and functions to recruit leukocytes to sites of infection.

CD74 is expressed in positive MHC class II cells, including monocytes, activated T-cells and fibroblasts. Healing is therefore associated with level of expression of CD74 cells because of its relation with activated T-cells and fibroblasts, involved in healing. In this study, expression level of CD74 in both fast healers and slow healers were almost the same (Figure 4.5). This indicates that the protein may not be a suitable marker to predict fast healing or slowing healing of Buruli ulcer disease.

5.2.3. Predictive capability/potential of PSME3 for fast and slow healing Buruli ulcer patients.

PSME3 plays a vital role in haemostasis and cellular regulation (Rechsteiner and Hill, 2015). Studies suggest that PSME3 kills T-cells *in vitro* but the role of the protein on T-cells *in vivo* is not clearly understood (Yi *et al*., 2017). T-cells assume a dendritic morphology in normal skin and constitutively produce low levels of cytokines that contribute to epidermal haemostasis. When there is a wound, an unknown antigen is expressed on damaged keratinocytes. Neighbouring T-cells then round up and contribute to wound healing by local production of epithelial growth factors and inflammatory cytokines (Yi *et al*., 2017).

In this study, PSME3 levels showed a higher trend in slow healers, compared to fast healers (Figure 4.6). Although this difference was not statistically significant, it somehow supports observations from *in vitro* studies where T-cells were killed by PSME3 and hence impaired healing (Yi *et al*., 2017).

5.2.4. Predictive capability/potential of C4A for fast and slow healing Buruli ulcer patients.

C4A chain provides an interaction surface for antigen-antibody complex. The alpha chain subunit may cleave to release C4 anaphylatoxin which is a mediator of local inflammation. Neutrophils are activated by C4A which increases inflammation (Beltrame *et al*., 2015).

C4A activates neutrophils which are involved in inflammation, except that high levels of C4A causes prolonged inflammation which slows down healing (Beltrame *et al*., 2015). In this study, fast healing Buruli ulcer patients expressed non-significantly higher level of C4A, compared to slow healing Bu (Figure 4.7). This may suggest that neutrophils needed for inflammation phase of healing are well activated in fast healing Bu.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This study showed that;

- IFI30, CD74, PSME3 and C4A proteins were generally expressed by Buruli ulcer cases and control. Higher median level of IPI30/IFI30/GILT protein was associated with fast healing and in agreement with initial pathway analysis (Figure 4.1). This may be suitable predictors, if confirmed in larger studies. However, CD74, PSME3 were lower in fast healing, whilst C4A was higher in fast healing which is in disagreement with the initial pathway analysis.
- Interestingly, IFI30 levels in plaques and nodules suggested a predictive capability of the protein for either fast healing or slow healing of plaques and nodules. In nodules, the higher the baseline concentration of IFI30, the longer the time to complete healing, but, in plaques, the higher the baseline concentration of IFI30, the shorter the time to complete healing. What this suggests is that the pathophysiological features of nodules and plaques are different. Each of them presents differently which may require a modification of the therapeutic regimen. This finding could be a useful guide for extending or shortening antibiotic therapy in nodules or plaques, based on their initial or baseline concentration of IFI30, if confirmed in larger studies.

6.2 Recommendations

From the results and discussions, the following recommendations are made;

1. Further analysis of IFI30 protein be carried out in larger sample size of Buruli ulcer patient cohorts.

2. Further studies to investigate the correlation of IFI30 and CD4+ T cells in the peripheral blood be carried out in fast and slow healing Buruli ulcer patients.

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