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Carlos Alberto Pereira Capela

IMMUNOGENETIC DETERMINANTS OF SUSCEPTIBILITY/RESISTANCE TO *Mycobacterium ulcerans* INFECTION: A POPULATION BASED STUDY – BENIN BIOLOGICAL BANK ON BURULI ULCER

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Tese de Doutoramento
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Trabalho Efetuado sob a orientação do
Doutor Fernando José dos Santos Rodrigues
e Doutor Jorge Manuel Rolo Pedrosa

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Declaração

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Immunogenetic determinants of susceptibility/resistance to *Mycobacterium ulcerans* infection: a population based study – Benin biological bank on Buruli ulcer

Determinantes imunogenéticos de susceptibilidade/resistência a infecções por *Mycobacterium ulcerans*: estudo populacional - banco biológico da Úlcera do Buruli no Benim

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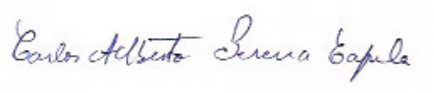

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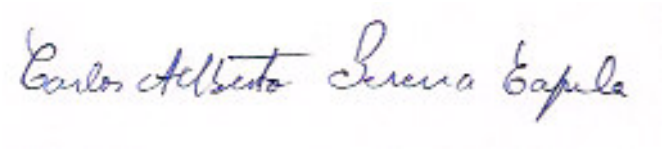
Signature: 

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ABSTRACT

Buruli ulcer (BU) is an infectious disease found in tropical regions of Africa, America, Asia, and Australia. Most of the cases are reported in West Africa and BU is considered a neglected tropical disease by the World Health Organization (WHO). This necrotising skin infection is caused by *Mycobacterium ulcerans* that secretes the exotoxin mycolactone as its main virulence factor.

There is emerging evidence for a major role of genetic factors contributing to either BU development or disease progression. Indeed, not all individuals, even after sustained exposure to *M. ulcerans* in endemic wetlands, develop BU and when patients present these lesions, a wide spectrum of clinical manifestations can be observed, including non-ulcerative and ulcerative lesions. In addition, spontaneous healing of BU lesions has been previously reported, as well as clustering of cases within specific families.

In that sense, it is clear that, to further evaluate the influence of host genetic variability in BU susceptibility and disease severity, new tools are needed. Therefore, we have built a bank of biological samples from a cohort of fully characterized BU patients from Benin, West Africa, as well as from a cohort of unrelated controls matched for age, gender and water contact habits. With this biological bank, the impact of several immune-related polymorphisms can be evaluated, not only for BU susceptibility or resistance, but also for differences in disease phenotypes.

Since a precise BU phenotype definition is critical to proceed to any genetic study, we first planned a clinical-based study, retrospectively examining records of patients in Benin, who had laboratory confirmed *M. ulcerans* infection. We started by evaluating the relationship between the type of lesion presentation and the time delay to seek medical attention, defined as the time between the first remembered signs of disease and attendance for medical care.

Of the 476 BU patients studied, 32% had a non-ulcerated form of skin lesion (nodule, oedema or plaque), while 67% presented ulcerated lesions. Only 1% of the patients had disease with bone involvement. In the non-ulcerated forms, the average time delay between symptoms and medical care was 32.5 days, while for ulcerated forms a statistically significant different value was observed (60 days), confirming disease progression from non-ulcerated to ulcerated skin lesions.

In addition to the classical forms of BU presentation, the WHO has recognized three levels of increasing severity of lesions. In our cohort, we registered that 66% of the patients

presented WHO categories 1 and 2, while 34% presented severe WHO category 3 lesions. Interestingly, time delay in seeking medical assistance did not contribute to the progression of less severe to more severe lesions, since the median time delay for WHO category 1 and 2 lesions (60 days) and category 3 severe lesions (60 days) was the same. The same behaviour was observed restricting analysis to multi-focal lesions and larger lesions, with a diameter superior to 15cm. These observations indicate that, rather than lesions becoming progressively more severe over time, severe lesions present a stable form and therefore should represent a separate phenotype of disease. These results also suggest that host genetic variability can have a strong contribution to the different disease presentations.

Taking into consideration the knowledge obtained with this retrospective analysis, we took advantage of the population-based biological bank, composed of DNA samples from 208 BU patients and 300 unrelated healthy individuals. In the first immunogenetic study, we focused on Tumour Necrosis Factor-alpha (TNF-α), a relevant immune-mediator involved in the innate branch of the immune response, known to play a key role in controlling *M. ulcerans* infection in animal models. Based upon the functional role of the *TNFA* [-308 $G^{low} \rightarrow A^{high}$ (rs1800629)] single nucleotide polymorphism (SNP) on protein production levels, we evaluated the genetic frequency of each allelic variant in cases and controls. We observed that -308 A allele was associated with increased risk susceptibility to BU, considering a recessive model of transmission. This data further reinforces the relevance of TNF-α in BU development, and is in line with data from a previous meta-analysis showing an increased susceptibility to *Mycobacterium tuberculosis* in individuals carrying this SNP with impact on promoter activity of *TNFA*.

In our second immunogenetic-based study, we evaluated genetic variants in Nucleotide-binding Oligomerization Domain-containing protein 2 (*NOD2*), Parkinson disease protein 2 (*PARK2*) and Autophagy-related protein 16-1 (*ATG16L1)* genes and their impact in susceptibility to BU, given the relevance of autophagy in the host response to mycobacteria. Our data show that the rs1333955 SNP in *PARK2* is significantly associated with increased susceptibility to BU. In addition, both the rs9302752 and rs2066842 SNPs in *NOD2* genes significantly increased the predisposition of patients to develop category 3 lesions, respectively, whereas the rs2241880 SNP in *ATG16L1* was found to significantly protect patients from presenting the ulcer phenotype. In this case, our findings indicate that specific genetic variants in autophagy-related genes influence susceptibility to BU and its progression to severe phenotypes. Although

further studies are needed, our data suggest that disturbances on microtubules and dynein constitution (integrative part of the cytoskeleton of the autophagosome), a process that is known to be affected by mycolactone, might potentially impair the autophagy process and impact the risk and progression of *M. ulcerans* infection.

Overall, our results reinforce the relevance of host genetic variability in the susceptibility/resistance to BU, as well as in the evolution of lesions to distinct phenotypes. Next steps are crucial in functionally validating the relevance of the associated variants on the mechanisms of host susceptibility or resistance to BU, through functional studies with particular emphasis on the immunological function.

RESUMO

A úlcera do Buruli (UB) é uma doença infeciosa das regiões tropicais de África, América, Ásia e Austrália. A maioria dos casos ocorre na África Ocidental e esta doença é classificada pela Organização Mundial de Saúde (OMS) como uma doença tropical negligenciada. Esta infeção cutânea necrotizante é causada por *Mycobacterium ulcerans*, que tem como principal fator de virulência a exotoxina micolactona.

Evidências recentes apontam para um papel da variabilidade genética do hospedeiro como contributo relevante para a suscetibilidade na aquisição da UB, bem como para a sua progressão. De fato, nem todos os indivíduos igualmente expostos a *M. ulcerans* nas zonas húmidas endémicas desenvolvem infeção e os doentes que desenvolvem a doença podem apresentar um largo espectro de manifestações clínicas, incluindo lesões não ulcerativas e ulcerativas. Adicionalmente, pode ocorrer resolução espontânea da infeção e está descrito uma concentração de casos de UB em determinadas famílias.

Neste sentido, fica evidente que, para avaliar a influência da variabilidade genética do hospedeiro na suscetibilidade e/ou agressividade da UB, são necessárias novas abordagens. Assim, construímos um banco de amostras biológicas de doentes do Benim, África Ocidental, com caracterização clínico-epidemiológica completa, em associação a uma coorte de controlos endémicos não relacionados, ajustada para idade e género e mantendo contacto e atividades semelhantes com os meios aquáticos. Com esta ferramenta, pode-se avaliar o impacto de diversos polimorfismos genéticos, na sua maioria relacionados com a imunidade inata, não só na suscetibilidade ou resistência à UB, mas também nos distintos fenótipos das manifestações clínicas.

Sendo fundamental uma definição clara de fenótipos associados à UB, primeiro planeámos um estudo clínico retrospetivo com pacientes do Benim com diagnóstico de infeção confirmado laboratorialmente. Começámos por avaliar a relação entre a forma clínica de UB e o tempo decorrido entre os primeiros sinais recordados pelos pacientes e a observação clínica (e terapêutica) inicial. Dos 476 pacientes selecionados, 32% apresentavam formas não ulceradas (nódulo, edema ou placa), 67% apresentavam úlcera e apenas 1% apresentava osteomielite. O grupo de doentes com formas não ulceradas teve uma mediana de 32.5 dias de atraso entre os primeiros sintomas e a procura de cuidados médicos, enquanto que o grupo que apresentava úlceras registou um período de atraso significativamente distinto, com uma

mediana de 60 dias. Estes dados confirmam que a doença, tipicamente, progride de formas não ulcerativas para úlceras cutâneas.

Para além das lesões clássicas de UB, a OMS reconheceu 3 níveis de severidade crescente das lesões de UB. No presente estudo, foram registadas lesões de categoria 1 e 2 em 66% dos doentes, enquanto que 34% dos doentes apresentavam lesões severas de categoria 3. Numa análise subsequente, mostrou-se que o atraso no tempo de procura de cuidados médicos não foi fator contributivo para apresentação de formas mais severas da doença, uma vez que a mediana do atraso para as lesões menos severas de categoria 1 e 2 (60 dias) e para as lesões severas de categoria 3 (60 dias) era a mesma. O mesmo comportamento foi observado quando a análise foi restrita a lesões múltiplas ou lesões de maior diâmetro, acima de 15cm. Mostrámos, assim, que o tempo de progressão natural da doença não determina formas clínicas mais agressivas de UB, sustentando a hipótese de que a variabilidade genética do hospedeiro pode contribuir para manifestações clinicas distintas (fenótipos) da doença.

Tendo em conta o conhecimento adquirido com a análise retrospetiva, recorremos ao banco biológico constituído por amostras de DNA de 208 pacientes de UB e 300 controlos saudáveis. No primeiro estudo imunogenético desta tese, investigámos o papel do Fator de Necrose Tumoral-alfa (TNF-α), dado tratar-se de um imunomediador relevante, previamente implicado na resposta inata e com um papel protetor na infeção por *M. ulcerans* em modelos animais. Com base na alteração funcional resultante do polimorfismo *TNFA* [-308 $G^{low} \rightarrow A^{high}$ (rs1800629)], com impacto nos níveis de TNF-α, avaliámos a frequência alélica da variante nos casos e nos controlos. Observámos que o alelo A estava associado a aumento do risco de suscetibilidade para UB, de acordo com um modelo recessivo de transmissão alélica. Este achado reforça o papel do TNF-α no desenvolvimento da UB e está de acordo com o verificado recentemente numa meta-análise que associa o mesmo alelo ao aumento de suscetibilidade de infeção pelo *Mycobacterium tuberculosis*.

Num segundo estudo imunogenético, avaliámos variantes genéticas nos genes *Nucleotide-binding Oligomerization Domain-containing protein* 2 (*NOD2*), *Parkinson disease protein* 2 (*PARK2*) e *Autophagy-related protein* 16-1 (*ATG16L1)*, uma vez que as proteínas que codificam fazem parte, de uma forma complementar, do processo de autofagia. De facto, a maquinaria da autofagia tem vindo a ser implicada, de forma crescente, no controlo e eliminação intracelular de micobactérias, levando-se a hipótese de que variantes

genéticas/funcionais tenham impacto na suscetibilidade à UB. Utilizando as mesmas coortes, demonstrámos que o polimorfismo rs1333955 no *PARK2* está significativamente associado a um aumento da suscetibilidade a UB. Associámos ainda os polimorfismos rs9302752 e rs2066482 do gene *NOD2* ao aumento de predisposição para desenvolvimento de fenótipos mais agressivos de UB (categoria 3 da OMS), respetivamente. Mostrámos também que o polimorfismo rs2241880 do gene *ATG16L1* protege os pacientes de desenvolverem o fenótipo de úlcera. Desta forma, demonstrámos que variantes específicas em genes relacionados com o processo de autofagia influenciam, quer a suscetibilidade para o desenvolvimento de UB, quer a progressão para fenótipos distintos da doença. Apesar de serem necessários mais estudos funcionais, os nossos resultados sugerem que interferências na constituição dos microtúbulos e da dineína (parte integrante do citoesqueleto dos auto-fagossomas), processo que é sabido ser afetado pela micolactona, podem ter impacto no risco de infeção e na progressão do processo infecioso por *M. ulcerans*.

Globalmente, os nossos resultados reforçam a importância do papel da variabilidade genética do hospedeiro na suscetibilidade/resistência à UB, assim como na determinação da sua evolução para os diferentes fenótipos característicos desta doença negligenciada. Os próximos passos para validar a relevância destes achados, no que respeita aos mecanismos da suscetibilidade/resistência do hospedeiro à UB, passarão por estudos funcionais, com ênfase na função imunológica.

TABLE OF CONTENTS

ABREVIATIONS

Ag85A	Antigen 85A
AFB	Acid-fast bacilli
ATF-2	Cyclic AMP-dependent transcription factor
ATG16L1	Autophagy-related protein 16-1
BCG	Bacille Calmette–Guérin
BU	Buruli ulcer
CBP/p300	CREB-binding protein/p300
CCL5	Chemokine (C-C motif) ligand 5
CI	Confidence interval
CMI	Cell-mediated immunity
CTDUB	Centre de Dépistage et de Traitement de l'Ulcére de Buruli
CR	Complement receptors
DC	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non- integrin
Egr-1	Early growth response protein 1
Ets-1	Protein C-ets-1
GCF2/LRRFIP1	GC-binding factor 2/Leucine-rich repeat flightless-interacting protein 1
GFP	Green-fluorescent protein
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
HWE	Hardy-Weinberg equilibrium
ICVS	Life and Health Sciences Research Institute
IP	Incubation period
IFN-γ	Interferon-gamma
IFNGR	Interferon-gamma receptor
IL	Interleukin
IL12R	Interleukin-12 receptor
Ig	Immunoglobulin
iNOS	Inducible nitric oxide synthase

IRIS	Immune Reconstitution Inflammatory Syndrome
LD	Linkage disequilibrium
MALDI-TOF	Matrix assisted laser desorption ionization – time of flight
MBL2	Mannose-binding lectin 2
MCP-1	Monocyte chemotactic protein-1
MDP	Muramyl dipeptide
MIP2	Macrophage inflammatory protein 2
MSMD	Mendelian Susceptibility to Mycobacterial Disease
NF-κB	Nuclear factor κB
NOD2	Nucleotide-binding oligomerization domain-containing protein
NLR	NOD-like receptors
NO	Nitric oxide
NRAMP1	Natural resistance-associated macrophage protein 1
OR	Odds ratio
PACRG	Parkin co-regulated gene protein
PAMPs	Pathogen-associated molecular patterns
PARK2	Parkinson disease protein 2 (Parkin)
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PNLLUB	Programme National de Lutte contre la Lèpre et l'Ulcère de Buruli
PRR	Pattern recognition receptor
RANTES	Regulated on activation, normal T cell expressed and secreted protein
ROS	Reactive oxygen species
SLC11A1	Solute carrier family 11 (Proton-coupled divalent metal ion transporter), member 1
SNP	Single nucleotide polymorphism
TB	Tuberculosis
Th1/2	T-helper 1/2
TLR	Toll-like receptors
TIR	Toll-interleukin-1 receptor
TNF-α	Tumour Necrosis Factor- alpha
VDR	Vitamin D receptor

WASP	Wiskot-Aldrich syndrome proteins
WHO	World Health Organization
WT	Wild-type
YRI	Yoruba in Ibadan

OBJECTIVES AND OUTLINE OF THE THESIS

Over the past years, emphasis has been given on acquiring a strong structured knowledge on pathogens and their interaction with the host immune system. Nowadays, host genetic variability is becoming a new focus for a better understanding on disease expression. Similar to other mycobacterioses, mainly to infections by pathogenic *Mycobacterium tuberculosis* and/or *Mycobacterium leprae*, those working in the field of Buruli ulcer (BU) question the major determinants behind the evidence that: just a few people exposed to *Mycobacterium ulcerans* develop disease; BU has a wide spectrum of clinical manifestations with different degrees of severity; self-healing is known to occur; and history of BU in the family is a risk factor.

Disease expression, as defined by a phenotype, is a crucial concept for the dissection of host genetic variability, including in infectious diseases. In the case of BU, while the characterization of ulcerative/non-ulcerative forms is well established, the severe clinical forms such as large or multifocal lesions are less understood. In light of this fact, there are questions that remain to be answered: Represent the more severe lesions an evolution of previous classical forms? Or are they distinct phenotypes? From this uncertainty, we intend to clarify the stability of BU phenotypes, particularly the more severe clinical forms, based on the time patients wait to seek medical care and treatment is established.

Additionally, given the low bacterial diversity among *M. ulcerans* strains in endemic regions of Africa, a possible effect of pathogen variability on the course of disease is unlikely. This observation poses host genetic variability as a putative determinant factor for resistance/susceptibility to BU and related distinct clinical phenotypes. This issue is far from being clarified, and therefore, in the present thesis, a cohort of BU cases and controls with full clinical characterization was built. In this context, genetic association studies were conducted to determine if/how immune-genetic variability is associated to BU susceptibility/resistance as well as with the phenotypes of the disease.

THESIS PLANNING

In **Chapter 1** of the thesis, a general introduction is presented, launching the fundamentals on the present knowledge and the unanswered questions on Buruli ulcer (BU). Initially, we review the disease, the etiologic agent – *Mycobacterium ulcerans*, and the basis for *M. ulcerans* virulence, namely in what regards the exotoxin mycolactone. This is followed by a review on the host-mycobacteria immune interaction. Then, a summary of the literature on the distinct clinical manifestations of BU is presented, focusing on the classical as well as on the severe forms of the disease. Data from cohort/cross-sectional studies published in the last three decades are reviewed and systematically analyzed, addressing classical and severe BU features. Specific known risk factors associated to pathogenesis and progression of the disease are also presented, followed by a detailed review on the effects of time delay to seek medical care on disease presentation. Finally, a general overview on immunogenetics to infectious diseases is made, with emphasis on genetic variability associated to resistance/susceptibility to mycobacterioses, namely tuberculosis (TB) and/or leprosy. Finally, the basis for BU genetic susceptibility related studies are presented.

In **Chapter 2**, an epidemiologic study with laboratory-confirmed BU cases, performed in Allada (Benin), focusing on classical and severe clinical forms and their major characteristics is presented. In addition, time delay to seek medical care is analyzed and related to the distinct clinical forms.

Chapter 3 is based on the role of Tumour Necrosis Factor-alpha (TNF-α) in BU pathogenesis and its association with susceptibility/resistance to development and progression of the disease. In this chapter, we describe the constitution of a Biological Bank (208 prospectively collected cases of BU in Allada – Benin, and 300 healthy endemic controls), used in order to carry out a case-control genetic variation study on the contribution of selected TNF-α single nucleotide polymorphisms for the risk of disease development, as well as progression for distinct phenotypes.

In **Chapter 4**, based on reports of genetic variants involved in autophagy regulation, as well as our own published data suggesting a central role for autophagy in the intracellular control of *M. ulcerans* infection through mycolactone-induced impairment of cytoskeleton-dependent cellular function, we dissect the contribution of selected autophagy-related genes to the risk of disease and its distinct phenotypes.

Finally in **Chapter 5**, the general discussion of the developed work is presented. In addition, we briefly present the identification of three multiplex polygamous families from Lalo (Benin), whose pedigree points to an autosomic recessive pattern of mendelian inheritance, and future investigations are proposed, specifically consisting in performing whole-exome sequencing for subsequent family-based associations and linkages.

CHAPTER 1

GENERAL INTRODUCTION

1. Buruli ulcer: the pathogen and the host immune response

1.1. Buruli ulcer

Buruli ulcer (BU) is a chronic skin and soft tissue infectious disease that can lead to permanent disability if untreated. It was first described by Sir Albert Cook in patients from the Buruli County in Uganda [1]. The causative organism – *Mycobacterium ulcerans* – was isolated in 1948 by MacCallum in the Bairnsdale region of Victoria, Australia [2]. The most distinctive characteristic of *M. ulcerans* is the production of mycolactone, a lipidic exotoxin that induces cellular apoptosis and local immunosuppression [3].

In the spectrum of human mycobacterial diseases – BU is the third most common in the context of immunocompetent hosts, after tuberculosis (TB) and leprosy [1]. BU generally begins as a painless dermal papule or subcutaneous nodule, which, over a period of weeks to months, can break down to form a necrotic ulcer with extensively undermined edges [4]. Less common clinical features, such as oedema or osteomyelitis can also be present [4]. Severe clinical presentations, such as larger or multiple lesions, are less understood in what regards their physiopathology, epidemiology, prevalence and associated risk factors.

At least 33 countries with tropical and subtropical climates have reported BU in Africa, South America and Western Pacific regions (Figure 1), with 2200 new cases reported in 2014 [5]. In Africa, most patients are children under the age of 15, from rural areas nearby swamps, lakes and slow-flowing rivers. These children mostly present affected limbs, which are probably related to skin injuries during swimming activities in aquatic environmental niches contaminated with *M. ulcerans*, facilitating infection and disease development.

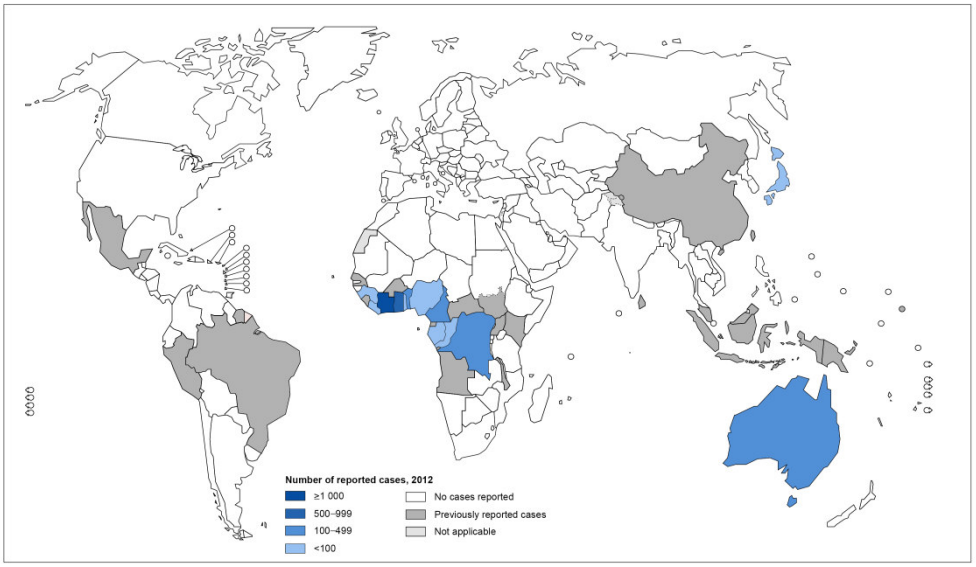

Figure 1. Distribution of Buruli ulcer, worldwide, 2012 (from WHO, 2015).

1.2. The etiologic agent of Buruli ulcer

1.2.1. *Mycobacterium ulcerans*

M. ulcerans is genetically related to the pathogenic mycobacteria, *Mycobacterium tuberculosis* and *Mycobacterium leprae*. Similar to other mycobacteria, *M. ulcerans* is characterized by low genetic diversity [6, 7]. In terms of phylogeny, *M. ulcerans* presents a high nucleotide sequence similarity with *Mycobacterium marinum* (around 98%), suggesting that this bacterium is an ancestor of *M. ulcerans* [8]. However, over the course of its evolution, *M. ulcerans* acquired the pMUM giant virulence plasmid that encodes for the enzyme complex responsible for the production of the exotoxin mycolactone [9] – the main pathogenic virulence factor of *M. ulcerans*. Similar to other pathogenic mycobacteria, *M. ulcerans* grows at a slow rate, but presents a distinctive low optimum growth temperature between 30 and 33ºC [2, 10]. The lower optimal temperature of this mesophilic pathogen may explain its preference for the skin and soft tissues of infected hosts.

1.2.2. *Mycobacterium ulcerans* virulence – the exotoxin Mycolactone

Mycolactone, the exotoxin produced by *M. ulcerans*, is a polyketide derived macrolide [3]. Distinct strains of *M. ulcerans* isolated from patients from diverse regions of the globe produce different congeners of mycolactone [11]. For instance, mycolactone A/B was

identified in *M. ulcerans* strains with origin in Africa; mycolactone C has origin in Australian isolates; and mycolactone D was found in strains from Asia [11]. These congeners, which can exist in the *trans* and *cis* form, only differ in the side chain, resulting in distinct levels of cytotoxicity [12].

The role of mycolactone in *M. ulcerans* survival in its natural aquatic environment is not yet completely understood, however, there is evidence pointing to its relevance in colonization of new hosts and new environmental niches due to its involvement in the structure of the *M. ulcerans* extracellular matrix [13].

Mycolactone is required for virulence [3] and was shown to be cytotoxic for several mammalian cell types, including monocytes, macrophages, neutrophils, lymphocytes, fibroblasts, dendritic cells, epithelial and adipose cells [3, 14-17], being described as an inducer of cell cycle arrest, apoptosis, necrosis and immunosuppression [3, 14-16, 18-20]. Some studies have shown that this lipidic molecule is found primarily in the cytosolic compartment of host cells, which can be an indicator that mycolactone's target is present in the cytosol [21]. In fact, recent evidence pointed out the Wiskot-Aldrich syndrome proteins (WASP) as a target for mycolactone [22]. Mycolactone disrupts the auto-inhibition of WASP, leading to an uncontrolled assembly of actin in the cytoplasm, which in turns leads to cell detachment and death by anoikis [22]. Our group further has demonstrated that mycolactone induces alterations in the cytoskeleton, which is fundamental to maintain cell structure homeostasis and autophagosomes synthesis, ultimately contributing to bacilli survival [19].

1.3. The host immune response against *Mycobacterium ulcerans*

Mycobacterial mechanisms of virulence evolved to hinder the development of host protective immunity and have been described for TB, leprosy and BU. As an example, in *M. tuberculosis,* macrophage activation to a protective phenotype depends on Tumour Necrosis Factor-alpha (TNF-α) and Interferon-gamma (IFN-γ) produced by activated T helper 1 (Th1)-type lymphocytes, in contrast to the activation of macrophages through IL-4 and IL-13. Indeed, these latter cytokines, produced during the Th2-type response, appear to deactivate phagocytes, leading to impaired cellular immunity [23]. In the case of *M. leprae*, a failure to induce the production of IL-1β and IL-18 by *M. leprae*-exposed monocytes is likely due to the delay in caspase-1 activation [24]. Monocytes stimulated with *M. leprae* also showed reduced

activation of the transcription factor NF-κB [25]. Considering that activation of NF-κB is a key event to produce a protective pro-inflammatory response, *M. leprae* bacilli are able to block the activation of an effective program of immune response. Thus, those virulent mycobacteria are capable of subverting the immune response as a strategy for survival in the infected host.

Similarly, *M. ulcerans'* exotoxin mycolactone has been reported to suppress the host innate immune response by interfering with activation of NF-κB [26], disrupting immune cell trafficking [16] and inhibiting cytokine production [14], particularly in the center of progressive BU lesions in which mycolactone accumulates.

1.3.1. Host cell - *Mycobacterium ulcerans* initial recognition: triggering of innate immune responses

Phagocytic uptake of mycobacteria has been shown to occur mostly by binding to receptors on the plasma membrane of phagocytic cells. These receptors include complement receptors (CRl, CR3, CR4); mannose receptors that bind mannosylated structures on the bacterial surface for opsonized and non-opsonized entry; Fcγ receptors that internalize IgG – opsonized bacteria; and scavenger receptors [27-29]. Toll-like receptors (TLR) are also involved in cellular recognition of mycobacteria [30]. TLR2, TLR4 and, more recently, TLR1/TLR6 that heterodimerize with TLR2, have been implicated in the recognition of mycobacterial antigens, such as the glycolipids lipoarabinomannan, mycolic acid, lipopeptides, and phosphoinositol of the mycobacterial cell wall. Comparatively, phenolic glycolipids are present in *M. ulcerans*' cell wall [31], but their role in the initial recognition and interaction with resident immune cells is yet underexplored. It is becoming clear that phagocytosis does not lead to innate immune activation in the absence of functional TLRs [30]. In the case of *M. ulcerans* infection, it has been described that TLR2 and TLR4 and Dectin-1 of keratinocytes actively participate in the internalization of *M. ulcerans*, the production of reactive oxygen species (ROS), and the expression of chemokines and human cathelicidin [32]. Dectin-1 is also involved in the internalization of bacilli by keratinocytes [32].

Additional microbial pattern recognition receptors have been recognized. One such receptor is nucleotide-binding oligomerization domain-containing protein 2 (NOD2), via recognition of muramyl dipeptide (MDP) [33]. The activation of this receptor triggers a distinct network of innate immune responses, including the production of IL-32, which culminates in

the differentiation of monocytes into dendritic cells [34] and in the activation of the autophagy machinery, crucial for the control of mycobacterial growth and spread [35]. Autophagy is a regulated process contributing to the innate control of intracellular pathogens by triggering the autodigestion of cytoplasmic components and driving pathogen clearance. This process is known to be dependent on microtubule cytoskeleton and dynein-driven transport, with dynein playing a role in the delivery of autophagosome contents to lysosomes during autophagosome-lysosome fusion [36]. Since microtubules and dynein are affected by mycolactone, cytoskeleton-related changes might potentially impair the autophagic process and impact the risk and progression of *M. ulcerans* infection [19]. Indeed, disturbances in the activation of NOD2, and consequently the autophagic process, have been implicated in the pathogenesis of human leprosy [34] and TB [37].

1.3.2. The intracellular lifestyle of *Mycobacterium ulcerans*

Mycobacterium spp are successful pathogens able to grow intracellularly in macrophages and induce the production of various chemokines (e.g. RANTES and Monocyte chemotactic protein-1 (MCP-l)) and cytokines (e.g. TNF-α and IL-lβ). This molecular environment is required for recruitment and activation of leukocytes and the triggering of innate immune mechanisms to control infection through cell-mediated immunity (CMI) [38]. This is also the case for *M. ulcerans* infection. *In vitro* and *in vivo* studies show that a characteristic feature of *M. ulcerans* infection is the continuous colonization of macrophages that arrive to the site of infection, followed by the intra-macrophagic multiplication of bacteria, which in turn induces cell lysis and the consequent release of *M. ulcerans* into the extracellular compartment (Figure 2) [39]. Indeed, in progressive lesions, *M. ulcerans* mainly accumulates in the extracellular compartment of the necrotic area [20]. This leads not only to a continuous invasion of healthy tissues, with enlargement of the necrotic areas due to mycolactone secretion, but also to a constant influx of leukocytes, to the periphery of the necrotic region [20].

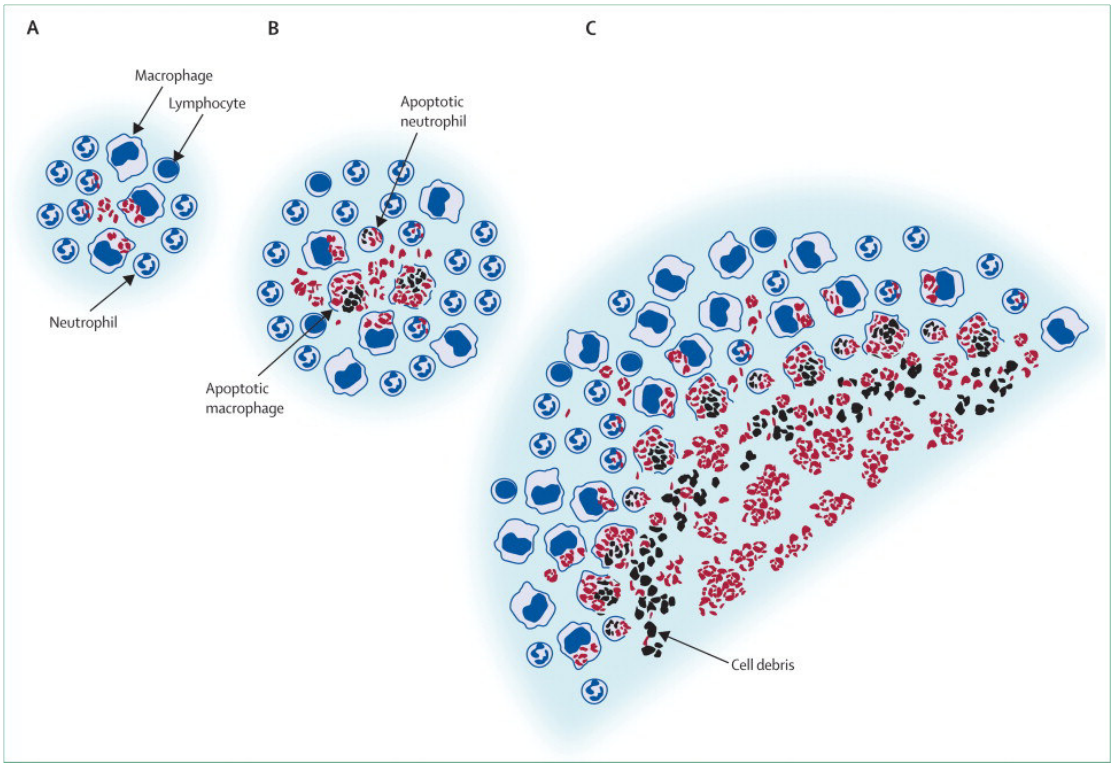

Figure 2. Initiation and establishment of a Buruli ulcer lesion. Early stage of infection (A) with *M. ulcerans* (red bacilli) phagocytosed by neutrophils and macrophages in the acute inflammatory infiltrate. A more advanced stage (B) characterised by the presence of an area with inflammatory cellular infiltrate with intraneutrophil and intramacrophage bacilli, and apoptotic neutrophils and macrophages. The advanced stage of the lesion with extensive necrotic, acellular areas (C) with containing abundant clumps of extracellular bacilli, cellular debris, and neutrophils and macrophages, with intracellular bacilli at the edge of the necrotic areas (*from* Silva MT et al, 2009).

1.3.3. Adaptive cell-mediated immune response

The relevance of CMI, based primarily in T-helper 1 (Th1) immune responses, for the control and resistance to *M. ulcerans* infection has been previously established [38]. For adaptive immunity to be mounted, it is necessary that antigen-presenting cells, like macrophages and dendritic cells, transport antigens to the draining lymph nodes in order to activate naïve T cells [40]. It has been shown that T cells are involved in a transient control of virulent *M. ulcerans* strains at an initial phase of infection, when the concentration of mycolactone is still low [41]. However, in advanced stages, when bacterial loads of virulent *M. ulcerans* are high and the mycolactone levels are also increased, there is an impairment of cytokine responses [15, 42] and of dendritic cell maturation/migration [16], death of

infiltrating inflammatory cells [20], and ultimately colonization and destruction of the draining lymph node [41].

Regarding the production of antibodies, significant levels of immunoglobulin (Ig) G against *M. ulcerans* can be found in both patients and in unaffected household contacts from BU endemic areas [5, 43]. This antibody production in endemic unaffected contacts may be related to colonization with *M. ulcerans*, although, for TB and leprosy, it has been shown that only patients with advanced disease reveal significant antibody production against these facultative intracellular pathogens [44].

1.3.4. The role of cytokines during *Mycobacterium ulcerans* infection

Several cytokines were shown to be expressed in human BU lesions, depending on the load of *M. ulcerans* and the stage of the infectious process [45-49]. Among the detected cytokines, IFN-γ and TNF-α have important functions in macrophage activation [44]. This is of major relevance, taking into account the key role of macrophages in the immune response against intracellular pathogens [15]. The significance of IFN-γ in controlling *M. ulcerans* was verified during *in vivo* infections of IFN-γ-deficient mice [42]. These mice showed an increased susceptibility to *M. ulcerans* when compared to wild-type (WT) counterparts. However, the differences in susceptibility between IFN-γ-deficient and WT mice diminished when the virulence of the *M. ulcerans* strain was higher. Accordingly, nitric oxide (NO) production, an IFN-γ-induced macrophage bactericidal mechanism, was shown to be impaired *in vitro* by mycolactone, in a dose-dependent manner [42]. Studies on human ulcerative lesions also illustrate the importance of IFN-γ, since the presence of granulomas was associated with a higher expression of this cytokine and with a lower bacterial load, while non-granulomatous showed a more anti-inflammatory profile [46, 47].

TNF-α also plays a critical role in macrophage activation during *M. ulcerans* infection by inducing the production of NO, through inducible nitric oxide synthase (iNOS) activity [50]. The involvement of TNF-α in BU was shown by the detection of mRNA of this cytokine in BU lesions [45, 46]. However, mycolactone also inhibits TNF-α production [15], further controlling/suppressing the immune response. A similar protective effect mediated associated with the production of TNF-α has been shown to be an essential requirement to initiate timely

chemokine-induced cell recruitment and the subsequent establishment of protective granulomas during infection with *M. tuberculosis* [51].

Other pro-inflammatory cytokines, such as IFN-β, IL-12p35, IL-12p40, IL-15, IL-8, and IL-6, IL-1β, have also been detected in *M. ulcerans*-infected human tissues, and the positive correlation among them suggests the relevance of a protective Th1 immune response [45, 47, 48]. Indeed, higher mRNA levels of these cytokines/chemokines were detected in granulomatous lesions, suggesting their involvement in the containment of *M. ulcerans* infection [45, 47, 48].

Overall, *M. ulcerans* is an intracellular pathogen that induces immunologically relevant inflammatory responses. The balance between initial immune recognition and granulomatous CMI may result in the control of mycobacterial growth. On the other hand, the severity of BU lesions seems to result mostly from the cytolysis and local immunosuppression induced by mycolactone. In this sense, an integrated research plan that takes into consideration the complexity of BU pathogenesis and its distinct morpho-histological features and clinical phases/presentations would allow a better understanding of the disease and shed light on mechanisms of host resistance/susceptibility. As such, the identification of host genetic variability that may result in increased susceptibility/resistance to BU is crucial for the better understanding of the disease.

2. Clinical presentation and disease progression: defining phenotypes

2.1. Buruli ulcer incubation period and mode of transmission

Most cases of BU occur in patients who live permanently in endemic areas, and because the exact mode of transmission is unknown, it is generally impossible to estimate with precision the incubation period [52]. Few published studies have addressed this issue. The Uganda Buruli Group estimated that the normal incubation period (IP) was 4–13 weeks [53]. The shortest IP described was 2–3 weeks, in a new-born from Papua New Guinea [54]. Recently, in Australia, five patients living outside endemic areas that had travelled to known endemic regions had reported an IP ranging from 2 to 5 months [55]. Other reports of travelers to endemic regions have also provided information, such as a minimum IP of 6 weeks in the case of a Nigerian physician working in New York City [56] and 5 months in a traveler from Papua Nova Guinea [57].

The most accepted mode of BU transmission is thought to occur by the inoculation of mycobacteria through minor skin injuries when in direct contact with mycobacteria-contaminated water [58, 59]. Another proposed mode of transmission is through traumatic introduction of *M. ulcerans* into the subcutaneous tissue by insects [59]. This hypothesis is supported by the detection of *M. ulcerans* DNA in insects in many endemic areas in West Africa [60, 61] and also in mosquitoes in an Australian BU focus [62].

2.2. Clinical forms and pathology

2.2.1. Classical presentations

BU mostly presents as a progressive necrotizing infection that mainly affects the skin and subcutaneous tissue. Classical clinical presentations of BU are characterized by different types of lesions (Figure 3), starting with a painless pre-ulcerative skin nodule, papule, plaque and/or oedematous lesion, which can progress overtime into the most common clinical form, an ulcer with undermined edges, as a result of the continuous destruction of subcutaneous tissue [44].

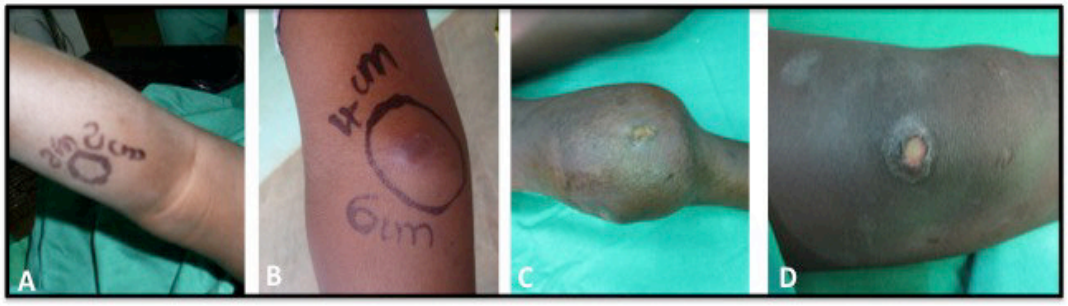

Figure 3. Cassical Buruli ulcer clinical forms. Representative images of (A) nodule, (B) plaque, (C) oedema and (D) ulcer.

In western and central Africa, BU (Table 1 and Table 2) traditionally affects children with less than 15 years of age, although people of all ages can be affected [63]. The majority of the lesions appear on the lower limbs, but the trunk and upper limbs are also affected [64]. This preference for limbs is most likely related to the probable mode of transmission of *M. ulcerans* in association to aquatic-related activities and/or vector insect bites [59-61, 65].

Clinically, all pre-ulcerative lesions are firm, painless and associated with colour changes. Papules are characterized by raised skin lesions with a diameter inferior to 1cm,

while nodules are lesions that extend into the subcutaneous tissue within a diameter of 2cm [44]. Plaques are considered when both previous lesions extend to more than 2cm, with the characteristic undermined and irregular edges becoming clearer [44]. Oedematous forms are characterized by diffuse swelling, regardless the size of the lesion [44]. The oedematous clinical form represents a distinct, often more severe, pre-ulcerative clinical form [66, 67]. Indeed, tissue necrosis can progress to an ulcer, even after the commencement of appropriate antibiotic treatment. This ongoing ulceration, despite appropriate antibiotherapy, is associated with tissue swelling and secondary ischaemia of skin and superficial tissues, analogous to the paradoxical enlargement and suppuration of tuberculous lymphadenitis on treatment [68]. The typical ulcerative form of BU is easier to recognize, with the characteristic undermined edges associated to a white slough at the base of the ulcer [44]. Remarkably, even at these advanced stages, lesions are still painless.

Histologically, early BU lesions are characterized by the presence of *M. ulcerans* in the intracellular compartment, mixed inflammatory infiltrates (neutrophils and mononuclear cells), apoptosis, necrosis, and vasculitis. In advanced BU lesions, bacilli are predominantly extracellular and extensive necrosis associated to minimal inflammation is observed, although inflammatory cells with intracellular bacilli can still be found in the periphery [20, 39, 69]. Comparing pre-ulcerative to ulcerative lesions, the former type tends to present more epidermal hyperplasia, more acute inflammation and much less features of chronic inflammation, such as granulomas [70].

Table 1. Cohort/Cross-sectional studies (1993-2015) published on PubMed® with Buruli ulcer classical cases. Among the data systematically reviewed, general epidemiological information such as gender and age distribution, risk factors and relative distribution of classical and severe clinical forms was included.

First Author and Year (Reference)	Country (Continent)	Number of Patients	WHO Criteria	General Epidemiological Data						Classical Clinical Forms			Severe Clinical Forms			
		(n) **Analysed specific phenotype**	Clinical Based; Lab + (%) (§1); PCR + (%)	Gender distribution	Age	Age <15years old	Time delay to seek medical care	Relapsing cases (%); Risk factors and interaction analysis; etc (§3)	Outcome analysis (mortality; sequealae; lengh of hospitalization, etc) (§4)	Ulcerative dominant form	Lower Limb	Clinical forms distribution (Nodule, Oedema, Plaque) (§5)	Osteo-myelitis	Multifocal lesions	Large Lesions (>15 cm)	WHO cat. 3
				(Female %)		(%)	(d/w/m/y) (§2)			(%)	(%)	(%)	(%)	(%)	(%)	(%)
Amofah G et al, **2002**	Ghana (Af)	5619	Clinical Based (active and inactive cases)	49.0	Median 25 [12-50]	_	_	Age-gender and age-site interaction	_	48.5	65.7	_	_	_	_	(head and neck - 3.8)
Kanga Jm et al, **2001**	Cote D`Ivoire (Af)	4642 (44.7%)	Clinical Based	M>F	_	57.0	_	Exposure risk	S	89.5	_	N-4.0; Oe-6.5	_	_	_	_
Sopoh GE et al, **2007**	Benin (Af)	2598	Lab + 50%	50.3	_	51.0	_	Relapsing 6.0%	_	72.0	63.3	_	6.0	6.2	_	_
Debacker M et al, **2004**	Benin (Af)	1630	Lab + 55.6%	_	_	_	Median (d) 20-213 (§6)	Relapsing 3.5%	LOH	_	_	_	13.0	_	_	_
Vicent QB et al, **2014**	Benin (Af)	1227	PCR + 100%	52.0	Median 12 [7-28]	_	_	Age-gender and age&gender-site lesion interation	S	66.0	60.0	N-2.0; Oe-25.0; P-54.0	7.0	4.0%	36.0	_
Marion E et al, **2014**	DR Congo (Af)	573	PCR + 19%	56.0	_	56.0	_	_	_	86.0	_	_	_	_	_	_
Mavinga Phanzu et al, **2013**	DR Congo (Af)	259 (33.4%)	Lab + 27.8%	50.2	Median 27 [11-44]	_	_	Relapsing 6.0%	_	74.0	_	_	_	_	_	19.7
Phanzu DM et al, **2011**	DR Congo (Af)	254 (Interventional study: Pre-64/Post-190)	Clinical Based	29.4	Pre-Median 19.4; Post-Median 21	_	Pre - 6 w Post - 8 w	Pre - Relapsing 32.8%; Post - Relpasing 11.6%	_	Pre - 95.3 Post - 85.8	Pre - 65.6 Post-68.5	_	_	_	_	_
Noeske J et al, **2004**	Cameroon (Af)	202 (46% of active cases)	PCR + 66.8%	43.1	Median 14.5	50.0	Mean (m) 10.5 [1w-8y]	Relapsing 12.4%	BCG protect from multifocality	92.8	62.0	N-2.0; Oe-2.0; P-3.5	14.9	correlation with BCG	average diameter 10.3cm	_
James K et al, **2003**	Togo (Af)	180	Clinical Based	14.9	Mean 20.7	20.6	_	Exposure risk related w/ water	_	_	88.9	_	_	9.4	11.1	_
Boyd SC et al, **2012**	Australia (Au)	180	PCR + 99%	51.0	Median 61 [1-94]	_	Median (d) 42 [2-270]	_	Age increase risk of multifocality	87.0	61.0	_	_	5.0	_	_
Johnson RC et al, **2005**	Benin (Af)	160 (21.3% of active cases)	Clinical Based	_	_	57.5	_	_	_	59.9	30.0	N-16.9; Oe-2.5; P-11.9	8.8	5.6	_	_
Debacker M et al, **2004**	Benin (Af)	151	Clinical Based	_	_	46.4	_	_	_	_	_	_	_	_	_	_
Marion E et al, **2015**	Benin (Af) (§7) (nigerian patients)	127	PCR + 100%	52.1	_	53.5	24% patients delay > 1y	Comparative time delay w/ local patients	_	78.0	56.7	_	20.0	_	_	58.8

Table 1 Cont. Cohort/Cross-sectional studies (1993-2015) published on PubMed® with Buruli ulcer classical cases. Among the data systematically reviewed, general epidemiological information such as gender and age distribution, risk factors and relative distribution of classical and severe clinical forms was included.

First Author and Year (Reference)	Country (Continent)	Number of Patients	WHO Criteria	General Epidemiological Data						Classical Clinical Forms			Severe Clinical Forms			
		(n) **Analysed specific phenotype**	Clinical Based; Lab + (%) (§1); PCR + (%)	Gender distribution (Female %)	Age	Age <15years old (%)	Time delay to seek medical care (d/w/m/y) (§2)	Relapsing cases (%); Risk factors and interaction analysis; etc (§3)	Outcome analysis (mortality; sequealae; lengh of hospitalization, etc) (§4)	Ulcerative dominant form (%)	Lower Limb (%)	Clinical forms distribution (Nodule, Oedema, Plaque) (§5) (%)	Osteo-myelitis (%)	Multifocal lesions (%)	Large Lesions (>15 cm) (%)	WHO cat. 3 (%)
Saka B et al, **2012**	Togo (Af)	119 (54.8%)	PCR positive 100%	43.7	Median 14	56.3	Mean (m) 11.4±4 [4d-7m]	Age-site lesion interaction	S	71.4	50.4	N-12.6; Oe-7.6; P-4.2	_	10.1	_	_
Bretzel G et al, **2011**	Togo (Af)	109 (54%)	PCR positive 100%	47.7	Median 12	_		_	_	74.8	_	_	_	_	_	22.9
Porten K et al, **2009**	Cameroon (Af)	105	Clinical Based	44.4	Median 15.5 [11-34]	_	Median (w) 12 [3-30]	_	_	86.7	64.8	Oe-7.1	_	11.4	median diameter 4 cm [2-7]	_
Steffen CM et al, **2010**	Australia (Au)	92	Lab positive 100%	42.4	Median 42	_	_	Remember insect bite 21.0%	M; S; Surgery	90.0	79.3	N-5.4; Oe-3.3; P-1.1	_	_	_	_
Amofah G et al, **1993**	Ghana (Af)	90	Clinical Based	_	_	49.0	_	Age-gender lesion interaction; sazonality	BCG protect from ulcer size progression	_	_	_	_	_	_	_
Bratschi et al, **2013**	Cameroon (Af)	88	Lab positive 100%	41.0	Median 12.5 [8-30]	59.1	_	Site lesion-insect biting relation	_	_	55.7	_	_	2.3	_	_
Quek TY et al, **2007**	Australia (Au)	85	Lab positive 100%	47.0	Median: Male 63; Female 81	_	_	Sazonality	Time delay endemic vs non endemic zones	22.4	60.0	P-48.2	_	_	_	_
Ngoa UA et al, **2012**	Gabon (Af)	77	PCR positive 68.4%	54.6	_	52.0	_	_	LOH	73.0	53.0	_	_	_	lesions > 5cm = 37.0	_
Phanzu DM et al, **2006**	DR Congo (Af)	51	Lab positive 70.5%	25.0	Median 17	44.4	Median (d) 60 [7-840]	Relapsing 38.9%	Superinfection 52.8%	94.4	63.9	P-2.8; Oe-2.8	16.7	15.7	_	_
Burchard GD et al, **1986**	Gabon (Af)	23	Clinical Based	_	_	mainly children	_	_	_	_	mainly limbs	_	_	_	_	_
Kollie K et al, **2014**	Liberia (Af)	21 (35%)	PCR positive 100%	47.6	_	35.0	D	_	_	71.4	81.0	Oe- 9.5	14.3	_	_	52.4
Nakanaga K et al, **2011**	Japan (Au)	19	Lab positive 100%	73.5	Mean 39,1 [11-81]	_	_	_	Recurrences (=0%)	100.0	_	_	_	_	_	_
Chukwuekezie O et al, **2007**	Nigeria (Af)	14	PCR positive 44%	57.1	_	64.3	_	_	_	50.0	71.4	Oe-11.0	11.0	_	_	_
Guerra H et al, **2008**	Peru (sA)	8	Lab positive 100%	62.5	[18-58y]	_	[1-8m]	_	_	_	_	_	75.0	_	12.5	12.5

§1 – Lab positive: At least 2 laboratory results being positive (*WHO recommended*); **§2 – Time delay to seek medical care: d** – days; **w** – weeks; **m** – months; **y** – years; **§3 – D**: regional distribution; **Relapsing**: relapsing cases; **§4 – Outcome analysis: S** – sequelae; **M** – mortality; **LOH** – length of hospitalization; **§5 – Clinical forms distribution: N** – Nodule; **Oe** – Oedema; **P** – Plaque; **§6 – Non-ulcerative lesions:** Median (d) 30-46; ulcerative lesions: Median 61 (d) [30-122]; bone lesions: median (d) 91 [30-213]; **§7** - Focus on Nigerian patients treated in a Beninese medical centre. **Af** - Africa; **Au** - Australia/Asia; **Sa** - South America.

Table 2. Cohort/Cross-sectional studies (1993-2015) published on PubMed® with Buruli ulcer specific phenotypes clinical cases: Among the data systematically reviewed, general epidemiological information such as gender and age distribution, risk factors and relative distribution of classical and severe clinical forms was included.

First Author and Year (Reference)	Country (Continent)	Number of Patients	WHO Criteria	General Epidemiological Data						Classical Clinical Forms				Severe Clinical Forms		
		(n) **Analysed specific phenotype**	Clinical Based; Lab + (%) (§1); PCR + (%)	Gender distribution (Female %)	Age	Age <15years old (%)	Time delay to seek medical care (d/w/m/y) (§2)	Relapsing cases (%); Risk factors and interaction analysis; etc (§3)	Outcome analysis (mortality; sequealae; lengh of hospitalizatio n, etc) (§4)	Ulcerative dominant form (%)	Lower Limb (%)	Clinical forms distribution (Nodule, Oedema, Plaque) (§5) (%)	Osteo-myelitis (%)	Multifocal lesions (%)	Large Lesions (>15 cm) (%)	WHO cat. 3 (%)
Mensah-Quainoo et al, **2008**	Ghana (Af)	99 **Phenotype: 1 lesion**	Lab + 94% PCR + 72.9%	57.5	–	62.6 (§8)	–	Relapsing 7.1%	–	52.0	50.9	–	–	11/110 (not subjected to analysis)	–	–
Kibadi K et al, **2010**	DR Congo (Af)	92 **Phenotype: diameter ≥10cm**	PCR + 66.3%	53.2	–	–	–	Treatment Trial	Treatment efficacy	–	52.5	–	–	2.2	mean diameter (cm) 13,8±16,2	2.2
Pommelet et al, **2014**	Benin (Af)	81 **Phenotype: osteomyelitis**	PCR + 100%	33.3	Median 11 [7-16]	–	–	BCG associated w/ risk	LOH; S	–	–	–	–	–	–	–
Portaels F et al, **2003**	Benin (Af)	73 **Phenotype: osteomyelitis**	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Agbenorku P et al, **2011**	Ghana (Af)	38 **Phenotype: head and neck site** (§10)	Clinical Based	40.0	Mean 14.4	65.8	–	–	LOH; S	76.3	–	–	–	–	–	–
Lagarrigue V et al, **2000**	Benin (Af)	33 **Phenotype: osteomyelitis**	Lab + 100%	–	–	–	–	Relation w/ primary skin lesion	–	–	–	–	–	–	–	–
O`Brien DP et al, **2013**	Australia (Au)	32 **Phenotype: paradoxical reactions** (§11)	Lab + 100%	–	–	–	–	Risk factors; time evolution	Rate of ocurrence 23%	–	–	–	–	–	–	–
O`Brien DP et al, **2014**	Australia (Au)	17 **Phenotype: oedema lesions**	Lab + 100%	29.0	Median 70 [17-82]	–	Median 20 d [28-73]	Assoiated risk w/ Diabetes, cancer, imunossup.	–	–	–	–	–	–	–	41.0
Phanzu DM et al, **2011**	DR Congo (Af)	13 **Phenotype: head and neck site**	PCR + 62%	38.5	Median 11	69.2	Median 12 w [3-52]	–	S	76.9	–	Oe-23.1	15.4	23.1	–	–
Ouattara D et al, **2002**	Cote D`Ivoire (Af)	11 **Phenotype: multifocal lesions**	Clinical Based	–	–	–	–	–	Healing time; surgery; S	–	–	–	–	all, varies between 3-7	–	–

§8 – < 14 years old; §9 – Paradoxical reactions: were considered a specific phenotype; **§10 – Atypical sites of lesion:** were considered a sever phenotype – usually included inside category 3 WHO. . **Af** - Africa; **Au** - Australia/Asia; **Sa** - South America.

2.2.2. Aggressive severe clinical forms and osteomyelitis

For patient management purposes, there are three main categories in which BU lesions can be divided, according to the severity of the clinical presentations [71]. Category 1 accounts for the presence of a single small lesion (nodule, papule, plaque or ulcer with less than 5cm in diameter), and is considered the less severe form of BU; category 2 accounts for non-ulcerative plaques, oedematous forms or single large ulcers with a diameter between 5 and 15cm; and finally, the most severe forms of BU presentation are grouped in category 3, which accounts for the presence of lesions in atypical regions of the body (e.g. head, face), multiple lesions, osteomyelitis and large lesions with a diameter higher than 15cm. These more aggressive category 3 lesions are probably underreported and, in terms of pathogenesis, less understood (Table 2 and Figures 4 and 5).

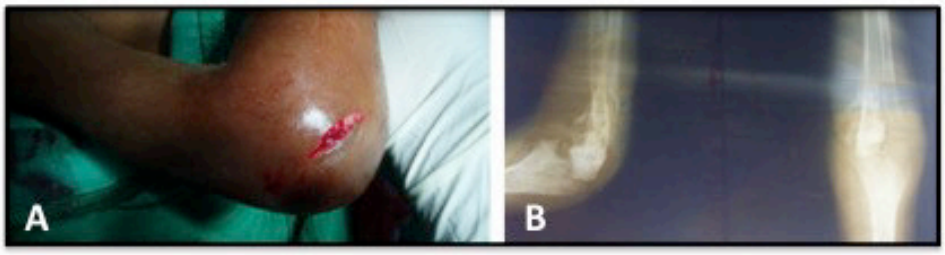

Figure 4. Buruli ulcer clinical form: osteomyelitis. Representative image of (A) osteomyelitis, *presumptive*; (B) X-ray confirmed.

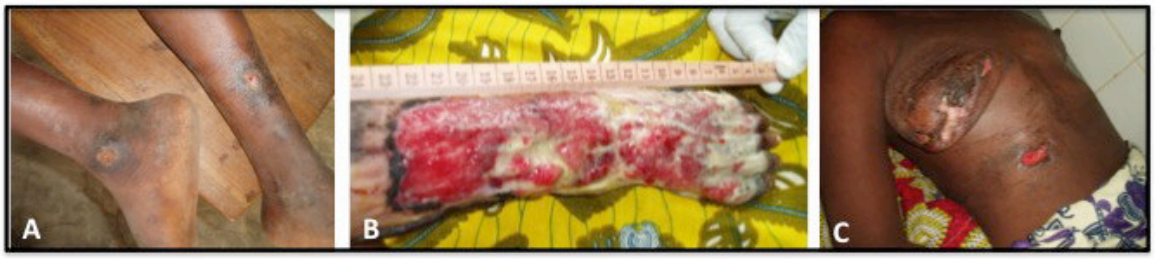

Figure 5. Severe Buruli ulcer clinical forms. Representative images of (A) multifocal lesion, (B) large lesion (>15cm) and (C) WHO Category 3 lesion, representing an atypical affected area.

Concerning BU multiple lesions, they are mostly localized on limbs, distal joints and small bones [72-76]. The number of foci typically ranges between two and seven [73, 75]. A

few reports have addressed the mechanisms behind *M. ulcerans* dissemination and the rationale for multifocality. The occurrence of bacteraemia with extracellular and/or intra-phagocytic localization could contribute to the dissemination of infection to distant sites in the body through lymphohematogenous spreading [76]. On other hand, the emergence of new lesions could be linked to an excessive inflammatory immune response. The histopathological observations revealed neutrophil-containing inflammatory infiltrates, vasculitis and vascular thrombosis, indicating an intensive inflammatory response [70] – similar to occasional exacerbated inflammatory responses following effective anti-mycobacterial therapy [77, 78], considered a paradoxical reaction or Immune Reconstitution Inflammatory Syndrome (IRIS). This is also described in patients with HIV under treatment who are co-infected with other mycobacteria, such as *M. tuberculosis* [79] or *M. leprae* [80].

M. ulcerans infection can also affect bone, evolving to osteomyelitis [72, 73], another severe clinical form of BU. Osteomyelitis is one of the most puzzling clinical forms of BU, due to the *M. ulcerans* optimal growth temperature (32°C), which is lower than those registered in the bone *milieu*. Although traditionally bone lesions have been documented beneath skin lesions (contiguous osteomyelitis) [4], a recent systematic description of BU osteomyelitis cases in patients with no identifiable present or past BU skin lesions, suggest a metastatic development of osteomyelitis [81]. These observations reinforce that osteomyelitis is not necessarily the result of an uncontrolled multiplication of bacteria in its elective tissue (skin) and that some individuals are susceptible to bone invasion by a small load of bacteria without contiguous tissue destruction.

2.2.3. Epidemiological studies review: distinct methods and their contribution for understanding Buruli ulcer and its distinct clinical forms

Several common features of BU are consistently described in different studies (Table 1). Specifically, studies report BU as mainly being a pediatric disease in Africa (median age: 12 years old) [82], while higher median ages are reported in Austro-Asiatic and South American cohorts (median age of diagnosis: 61 years old) [83]. Some studies proceed with a gender to age interaction, pointing males to be overrepresented in <15 years old age group [81, 84, 85].

Lesions tend to predominate on the lower limbs in adults, regardless the continent (30%) [86] – ranging between 48.5% [85] and 88.9% [87]. Other studies confirm that younger

age groups tend to express more lesions in upper body parts [81, 85, 88]. This difference in the preponderance of global presentations is most probably explained by the mode of exposure, discussed in next section.

The ulcerative clinical form of BU is by far the most commonly reported in African cohorts, ranging between 50% (Nigeria) [89] and 95.3% (DR Congo) [66]. In Australian cohorts, a preponderance of non-ulcerative clinical forms is reported, which is mostly explained by the precocity of medical recognition. Concerning non-ulcerative clinical forms, some studies report their relative frequencies. Oedema could represent a maximum of about 10% in African cohorts [81, 89, 90] and a minimum of 2%, as reported in Cameroon [91].

Concerning severe clinical forms, 10 of the 28 studies analysed (Table 1) reported frequencies of osteomyelitis ranging between 6% (Benin) [92] and 20% (Nigeria) [93]. Another 10 in 28 studies reported frequencies of multi-focal lesions, ranging between 2.3%, in Cameroon [94], and 15.7% in DR Congo [95]. Larger lesions, as defined by diameters greater than 15cm, were taken into account in 3 of the 28 analysed studies, which report variability between 11.1% [87] and 36% [81]. Considering a more broad report on severity, WHO category 3 relative frequencies were reported in 5 of the 28 selected studies, 4 in Africa and 1 in South America. In African studies, the frequency of WHO category 3 lesions was reported to be between 19.7% [96] and 58.8% [93].

Few studies address specific phenotypes (Table 2) such as mono/multifocality lesions strictly (2 studies), larger lesions (1 study), oedematous lesions solely (1 study), osteomyelitis (3 studies) and affecting atypical sites, mainly head and neck (2 studies).

Although these studies retrieve complex information on BU, such as relapsing cases and outcome issues, accounting numbers of disease-related deaths, length of hospitalization and sequelae, most of them missed to establish an association between BU severity and time delay to seek medical care. Moreover, considering the sum of the relevant studies published in the last three decades on BU, it is evident the limited analysis of severe clinical forms of the disease. As such, more robust studies are needed, for which epidemiological data can also be explored to provide stronger evidence on the existence of stable BU phenotypes (common or rarer; classical or severe).

2.3. Risk factors for Buruli ulcer acquisition and disease progression

2.3.1. Buruli ulcer acquisition associating risk factors

Exactly how *M. ulcerans* infects human hosts remains elusive; however, unlike TB or leprosy, infection is acquired from the environment (directly and/or indirectly), rather than through contact with infected humans. As previously referred, the most accepted *M. ulcerans* modes of transmission are the inoculation of mycobacteria through minor skin injuries when in direct contact with mycobacteria-contaminated ecosystems, including water, and insect biting. Most biting arthropods selectively feed at specifics sites based on visual, physical or chemical cues, such as distance of the ground, breath and skin temperature of the bait [97]. The resulting feeding patterns are often focused either on the feet and ankles or the head of the human subject [97]. Furthermore, wearing protective clothing and of the use of mosquito repellent are considered protective against the development of BU [98]. It was also possible to isolate *M. ulcerans* DNA in distinct niches such as freely in the air [99] and in water-parasites amoebae [100, 101]. In that sense, *M. ulcerans* is becoming a paradigmatic case study on a multi-host pathogen dynamic inhabitant [58] – with insect-vector and aquatic ecosystems as the basis for its survival and human infection (Figure 6 and Figure 7). Given the lack of knowledge on the definitive mode of transmission for BU, it is important to investigate what factors could increase the risk of developing the disease.

Figure 6. Aquatic environment with a fisherman in a highly endemic BU region. Lake in Zé Region, Atlantique Department, Benin.

Figure 7. Washing of clothes in a highly endemic BU region. Zé Region, Atlantique Department, Benin.

A unique combination of climate and landscape conditions found in BU endemic areas has been shown to provide an optimal environment that supports *M. ulcerans* colonization and transmission to humans [102, 103]. Indeed, many publications have reported an association between BU and humid environments [59]. An inverse correlation has been shown between the prevalence of BU and the distance from the Couffo River in Benin [104] and a direct association with increased contact with a natural water sources [105]. Further support of this association comes from the isolation of a virulent strain of *M. ulcerans* from an environmental aquatic sample from a BU endemic region in Benin by Portaels and collaborators [106]. Altitude has also been pointed out as a risk factor for *M. ulcerans* acquisition, since villages with higher BU prevalence rates are frequently located in areas of low elevation [103, 107]. Given that lowlands tend to be wetter than higher grounds, they provide more favourable conditions for the proliferation and spread of the etiologic agent.

In line with these previous observations, studies have also associated the high prevalence of BU with farming activities that occur primarily in terrains with lower elevation [107]. Indeed, individuals are more prone to frequent these wetter lowlands to plant and tend their crops, thus becoming vulnerable to infectious agents in the area. In addition to farming, several other water-related activities have been described as risk factors for the development of BU, namely fishing, bathing, swimming, and washing of clothes [108-110]. The pattern of distribution of BU lesions supports the association of disease development and water-related activities, given that, as referred above, in adults BU lesions occur mostly on the lower limbs

and are equally distributed between the left and right side of the body. Children, on the other hand, tend to present a more dispersed distribution of their lesions on the trunk, since their upper body is often exposed [111, 112]. Additionally, it has been described, in females, which are more likely to cover their upper body with clothing, fewer lesions on the trunk [111, 112].

Contrary to environmental conditions, gender and socioeconomic status do not seem to be consistently associated as risk factors for BU development [98]. However, age (5-14 years old and elderly people) has been identified as a risk factor [108, 110, 111, 113]. These epidemiological data are mostly explained by:

(a) younger children are, comparatively to adults, in more prolonged/frequent contact with water due to recreational purposes and more subjected to multiple self-injuries [114];

(b) children are more often subjected to medical care attention, when compared to adults that resort to traditional-based initial treatments [114];

(c) the fact that immune function reduces with senescence. Moreover, delayed health-seeking behaviours in older people may contribute to neglect skin lesions [115].

Other risk factors described to increase the risk of BU development include poor hygiene and Human Immunodeficiency Virus (HIV) infection [116].

2.3.2. Buruli ulcer disease progression associating risk factors

The factors determining differential BU disease progression are lesser understood. The full knowledge towards the determinants of disease evolution will be of a great relevance when considering the operational definitions for timely treatment. On one hand, differences in the virulence of *M. ulcerans* strains have been suggested as having an impact on disease evolution, although data have shown low genetic variability between isolates from across Central and West Africa [117]. On the other hand, physical and/or immunological host mechanisms are also likely to be involved.

Gender is frequently associated with human susceptibility to infectious diseases [118]. In the context of human mycobacterioses, infections with *Mycobacterium avium intracellulare*, *M. marinum*, *M. leprae*, and *M. tuberculosis* are more frequent in adult men than in adult women [118], which could be caused by distinct exposure and/or differential host immune responses. Regarding BU, gender effect on disease progression is a subject of debate and distinct data have been reported (Table 1).

In some studies, prevalence of HIV infection among BU patients was shown to be significantly higher [116, 119], although others failed to prove this association [120]. Nonetheless, the impact of immunosuppression induced by HIV co-infection on BU disease progression to severe clinical forms, has been demonstrated in those with a higher level of immunosuppression [121]. Additionally, hypoproteinaemia and anaemia were recognized as being associated with severe BU, given that low tissue oxygen levels could promote the haematogenous spreading of *M. ulcerans* [122]. Accordingly, this association has also been described for disseminated clinical forms in other mycobacterioses. Indeed, disseminated TB is associated with weight loss [123], as well as with severe hematologic disorders [124]. Disseminated leprosy was also observed in rats submitted to a protein-free diet [125].

Of relevance, most of the 10 selected studies (Table 2), selecting populations ranging between 11 and 99 patients, were mostly accomplishing laboratory confirmation criteria, as defined by WHO [126].

2.3.3. Time delay to seek medical care: disease presentation effect

In addition to the above described risk factors, one factor that might influence BU disease progression is the time delay in seeking medical care for the establishment of therapeutics. Late diagnosis usually occurs and can be attributed to the level of accessibility and quality of health care systems in endemic regions [127, 128]. Several other factors lead to late diagnosis of BU, when considering the African context. Studies in Benin [127] and Ghana [128] have investigated the obstacles to medical care, which include community stigma, mistaken beliefs about the cause of disease, fear of recurring infections after surgical treatment, anxiety about the outcome of surgery, fear and concern about scarring and disabilities after treatment, neglected perceived seriousness of infection and local beliefs of spiritual causes that require the intervention of traditional healers, particularly herbalists. So, promoting awareness and knowledge on the disease outcomes and the effect of the available therapeutics may prompt timely and appropriate medical care [128].

Time delay to seek medical care has been addressed in several BU epidemiologic studies (Table 1 and Table 2), not only under a perspective of measuring health-services local capacity and population-awareness on BU, but also to better understand the natural evolution of BU and to measure the added-risk for sequelae and/or mortality.

Eleven out of 38 analysed studies, at some point, measure this parameter, although distinct methods were applied. First, as an objective question about a subjective personnel dimension such is the remembering of a first sign or symptom related to a later evidence of a disease – reproducibility could be difficult to ensure. Moreover, being BU a predominantly paediatric disease, the subjectiveness is even more pronounced, although the only method resides in the classic medical interview. None of the reports discuss specific methodologies to overcome such limitations. Second, this time to seek medical care could be measured in days, weeks, months or years. Distinct studies summarize data in different time-units, using distinct statistic variability (median or mean). Third, even less studies take into account the effect of distinct time delays on distinct BU phenotypes.

Considering African studies, reported values for time delay range between 42 [129] and 84 days [130], taking into consideration all clinical forms. Specifically, a unique Beninese study [127] reported distinct clinical forms relating to time elapsed since first remembered symptoms. Time delay was shorter for non-ulcerated clinical forms (median 30 to 46 days) then for ulcerated forms (median 61 days) and larger for osteomyelitis (median 91 days). In this study, ulcerative lesions were associated to longer time delay periods in seeking medical attention [127]. In Australian studies, due to more advanced health-services and patient-awareness, time lapsed until medical care was reported to be much shorter – between 20 days median [131] and 42 days (ranging 2 and 270 days) [83], considering all clinical forms.

On the other hand, the effect of time delay on the development of severe clinical presentations (i.e. multiple lesions, osteomyelitis and large lesions) has never been specifically addressed. It is still not clear whether severe clinical lesions are a result of continuous growth (e.g. larger lesions), evolution to more complex clinical forms over time (*e.g.* osteomyelitis or multi-focal disseminated forms), or whether these severe lesions are time-independent, raising the possibility of stable distinct phenotypes determined by host specific mechanisms.

In general, the physiopathology of classical clinical forms of BU is more well-defined than that of severe lesions. Indeed, as referred above, progression of pre-ulcerative to ulcerative lesions seems to be due to a natural time evolution of the pathologic process; however, fewer identifiable factors are known to be associated to the development of severe clinical forms. In fact, there is a need to clarify whether there is any effect of time-delay to seek medical care on the development of severe BU clinical phenotypes. Such study could lead not

only to a better phenotype definition, but also to increased knowledge on host susceptibility to BU development and distinct disease phenotypes.

2.4. Buruli ulcer diagnosis, prevention, treatment, and prognosis

In most BU endemic settings, the working conditions are difficult and the diagnosis of the disease is often made on clinical and epidemiological grounds. However, BU disease presents with a diverse range of clinical symptoms and, due to possible confusion with other tropical skin diseases, the added value of microbiological confirmation is becoming more appreciated [132, 133]. BU diagnosis can be challenging, mainly because the most sensitive laboratorial tests are difficult to be applied in the field, and require qualified staff and specific equipment [126].

There are four tests commonly used for the laboratory diagnosis of BU [126, 134]: direct examination of acid-fast bacilli through Ziehl-Neelsen staining of a smear made from a swab; *M. ulcerans* culture at 32°C; histopathology; and Polymerase Chain Reaction (PCR) for the IS2404 fragment of the *M. ulcerans* genome. Among these tests, the most specific and sensitive technique is the PCR for the IS2404 fragment.

A case of *M. ulcerans* infection is defined by the presence of a lesion clinically suggestive of BU plus any of the following [126]:

(a) a positive culture of *M. ulcerans* from the lesion;

(b) a positive PCR result from a swab or biopsy of the lesion;

(c) histopathology of an excised lesion showing a necrotic granulomatous ulcer with the presence of acid-fast bacilli (AFB).

Some preventive strategies, like the use of clothing while farming and the decontamination of skin injuries, have been indicated as being protective [108, 109]. The WHO efforts have been focused on diminishing morbidity associated with BU, by implementing strategies for early diagnosis and treatment, which include education and awareness activities in the communities, the training of village health workers and the improvement of health centres infrastructures [135].

There is no specific vaccine available against BU, and Bacille Calmette–Guérin (BCG) vaccination only provides a short-term transient protection against the disease [136]. Some authors have reported in the mouse model of infection that, although BCG vaccination [50] or

vaccination with Ag85A from *M. ulcerans* or *M. tuberculosis* [137], delays the start of *M. ulcerans* replication and the appearance of lesions, it does not avoid the later progression of disease.

BU treatment generally involves antibiotic administration. The WHO recommends eight weeks of antibiotic treatment composed by a combination of rifampicin (10 mg/kg once daily) and streptomycin (15mg/kg once daily) [71]. For non-ulcerative lesions and small ulcers, this antibiotic treatment is usually efficient to resolve the infection. Nevertheless, in the case of large lesions, the efficacy of the antibiotic treatment is variable, and surgery might also be necessary [71, 138].

WHO guidelines define three categories of treatment based on:

(a) clinical form (ulcerative or non-ulcerative);

(b) lesion size (lesions less than 5cm and lesions of 5cm or more in diameter) and;

(c) disseminated or mixed forms [71].

Antibiotic treatment of 8 weeks is recommended for all three categories [71]. For lesions ≥5cm, surgery is additionally recommended, if necessary, after at least 4 weeks of antibiotic treatment. For very large lesions, antibiotic treatment may be administered for up to 12 weeks [71]. The clinical outcome of the treatment with the WHO recommended regimen has been classified as a:

(a) "success" (10 to 30% reduction of the size of the ulcer and/or absence of new necrotic tissue);

(b) "clinical *status quo"* (no change in the size of the lesion or presence of necrotic tissue);

(c) "failure" (increase in the size of the lesion and presence of new necrotic tissue) [139].

In Africa, since BU does not cause pain and there is a misperception for the disease consequences, affected individuals usually delay seeking professional medical care and instead prefer to follow the treatment prescribed by village healers [127, 128]. Consequently, when affected people decide to seek hospital treatment, lesions are mostly in advanced stages, implying that the majority of patients need to be submitted to surgery and to long-term hospitalization [127, 128]. Although BU is not usually a direct cause of mortality, it is related with high morbidity rates and stigmatization, since self-healing and surgical treatment of

lesions can lead to disabling contractures and scars, and amputation can also be required [135].

Spontaneous healing has been described in a minority of BU cases. There is scarce knowledge concerning the relevance of this process, mostly due to the lack of epidemiological studies addressing this issue. In a recent work, 26 (4.7%) of total considered BU patients presented a spontaneous healing process [140]. In most of reported cases, spontaneous healing was a lengthy process observed in old extensive BU lesions.

3. Host genetic variability

3.1. Host genetic variability to infectious diseases

For many years, epidemiological studies have shown that individuals may markedly differ in their susceptibility to infectious diseases and, as such, clinical development of disease occurs in just a restricted number of individuals after similar contact with a given pathogen (*reviewed in* [141]). In this line, throughout human evolution, pathogenic agents, due to their deleterious effects, have exerted a strong selective pressure in shaping human genome. Evolutionary advantages of a variety of immunological responses to a wide range of infectious pathogens have been highlighted hereafter [142]. The host-pathogen interplay that results in differential activation of a gene cascade and/or cell recruitment/differentiation process during infection may lead to the development or persistence of disease and/or to differential clinical outcomes. In fact, the susceptibility to a certain infectious agent and the severity of infection are dependent on the interaction of environmental and host genetic factors – specifically immunological players [143].

The majority of the human genome is identical between individuals and only around 0.01% accounts for inter-individual variability [144]. Single nucleotide polymorphisms (SNPs) are common genetic variants within a population that reach frequencies higher than 1% [145]. These single-base pair changes account for a great part of DNA differences among individuals [144]. Briefly, a SNP can:

(a) be "*silent*" if within the exonic region and does not change the protein sequence (synonymous SNP) [146];

(b) result in a change of an amino acid (non-synonymous SNP) [146];

(c) modify the entire amino acid sequence of the protein, if it alters the initiation codon site or an edited nucleotide [146];

(d) affect gene expression, by altering, among others, the RNA decay or the binding of transcription factors to the promoter region [146] and;

(e) affect the mRNA splicing, if it results in new splice variants [147].

Overall, it is estimated that from the known 1.42 million SNPs distributed throughout the human genome, only 60,000 fall within exon regions, both coding and untranslated regions [146]. Specifically, SNPs may have strong functional consequences, at expression and functional levels, within the cellular networks. Thus, the functional genetic diversity of the immune response has long been considered as having a dominant role on inter-individual variation in susceptibility/resistance to infectious diseases. The impact of genetic variations in the susceptibility to disease or in disease progression is mostly evaluated by association studies [147-150].

There are two main study designs for genetic association studies, the case-control or population-based study, which is carried out in a population composed by unrelated cases and controls and compares the frequency of a SNP allele between the affected and the unrelated control population; and the family-based study, which tests if a SNP allele is transmitted more frequently than the normal allele from the parents to the affected offspring [141].

Population-based association studies can be performed using two major approaches: candidate-gene or genome-wide studies. Candidate-gene association studies are performed by evaluating the frequency of chosen polymorphisms in candidate genes in the cohort of cases and controls. The choice of SNPs panels is based on the possible biological relevance of protein function/expression or based on previous description of polymorphisms associated with other diseases sharing common biochemical or immunological pathways [141, 145, 150]. Conversely, genome-wide studies consist in evaluating, in cases and controls, the allelic frequency of hundreds of thousands of SNPs across the whole genome and thus with an unbiased approach, grounded on the hypothesis that common phenotypes result from a group of common polymorphisms in a specific population with a relatively low effect size [151]. In this type of studies, a large sample size is necessary to obtain statistical power to detect disease associations [150, 151]. Genome-wide studies are especially valuable for discovering genetic associations that otherwise would be ignored [141].

Among genetic variability associated to infectious diseases, innate immune related genes are, by far, the most explored, due to their essential role in the natural host defenses against microorganisms [152]. Pathogen recognition involves macrophages, dendritic cells, natural killer cells, granulocytes, and monocytes, which act as sentinels of the innate immune system (Figure 8). This complex process is coordinated by several families of pathogen recognition receptors (PRRs), such as TLRs; NOD-like receptors (NLRs); RNA helicase-containing proteins and the C-type lectins [152].

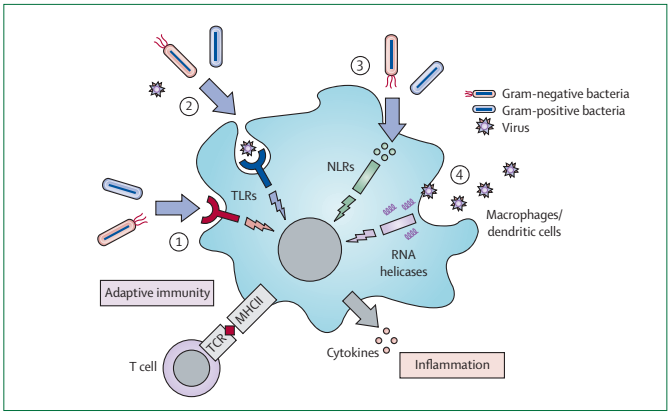

Figure 8. Recognition of microbial pathogens by the innate immune system. Microbial associated molecular patterns are recognised by transmembrane receptors (e.g.Toll-likereceptors [TLRs]), which trigger the activation of several signal-transducing pathways, leading to the production of cytokines and expression of costimulatory molecules. Cytokines induce and regulate the inflammatory response and orchestrate the adaptive immune response. By contrast with other TLRs, TLR3, TLR7, TLR8, and TLR9 are expressed mainly in the endosomal compartment (2), where local acidification is required for recognition of microbial products by their cognate receptors. Intracellular pathogens or microbial products released intracellularly after lysis of ingested microorganisms may also interact with intracytoplasmic receptors, such as nucleotide-binding oligomerisation domain-like (NOD2) proteins(3),or the RNA helicase- containing molecules (4:RIG-I or MDA5). TCR=T-cell receptor (extracted *from* Bochud PY et al, 2007).

3.2. Genetic susceptibility to tuberculosis and leprosy

From the beginning of the last century, the differential susceptibility to develop mycobacterioses has been associated with differential effects on the interaction of environmental, pathogen and host factors, although the relative contribution of each factor remains to be elucidated (Figure 9) [143]. The inter-individual genetic variability to mycobacterioses susceptibility/resistance has been largely reported [153, 154]. As such,

human infection with less virulent mycobacteria, like environmental-related mycobacteria (e.g. *M. avium*) or *M. bovis* BCG, could result in disseminated disease due to Mendelian Susceptibility to Mycobacterial Disease (MSMD). In this case, the inherited disorders are associated with dysfunctional functions of the IL-12-IFN-γ axis [155] Regarding the TB and leprosy, there is evidence that they belong to the spectrum of complex multigenic diseases, indicating that the differential susceptibility is influenced by multiple loci variants. The resistance to *M. tuberculosis* infection is correlated with the geographical origin, with the most vulnerable individuals tending to have ancestors from areas of low TB prevalence [156]. Accordingly, the incidence of TB is particularly high during outbreaks in populations with no ancestral contact with the infection, such as native Americans [156]. Familial aggregation studies with twin studies have shown much higher concordance rates for monozygotic than dizygotic pairs, for both clinical TB and leprosy [157, 158]. More globally, although one-third of the world's population has been exposed to *M. tuberculosis*, only 10% develop clinical disease [143, 151]. Additionally, epidemiologic-genetic studies have also focused on gene variability associated to patterns or phenotypes displayed by TB and leprosy patients, such as pulmonary TB, extrapulmonary TB, TB in children, paucibacillary or multibacillary leprosy [143, 159].

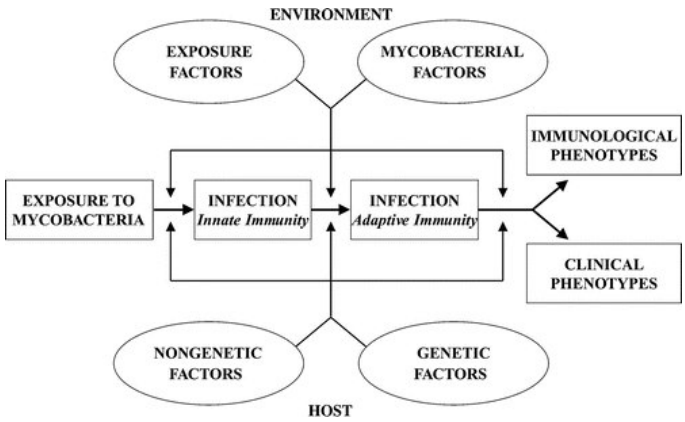

Figure 9. The various steps in the interaction between humans and mycobacteria. Exposure to mycobacteria does not always result in infection. Whether or not an established infection further develops depends on innate immunity, alone or in conjunction with adaptive immunity. Immunological and clinical phenotypes may be detectable once mycobacterial infection is established and adaptive immunity to mycobacteria is involved. Each of the three steps in this process is under host and environmental control. Host factors may be genetic (e.g., mutation in a gene involved in immunity to mycobacteria) or nongenetic (e.g., skin lesion) and may have an impact at each stage of the interaction. Environmental factors may be mycobacterial (e.g., virulence factors) or related to the mode of exposure (e.g., direct inoculation) and may have an impact at each stage of the interaction (*extracted from Casanova JL et al, 2002*)

Immunogenetic studies in mycobacterial infections are naturally focused on functional genetic diversity of the immune response, emphasizing the innate immune system, as it has been considered to have a dominant role in the development/outcome of the disease [152, 160]. Indeed, genetic variants for PRRs, cytokines, chemokines, among other molecules known to be important for the host immune response to mycobacterial infections, have been described and tested for association with disease, that will be briefly reviewed hereafter.

3.2.1. Pattern Recognition Receptors

Regarding PRRs, the molecules responsible for the recognition of PAMPs and for the initiation of the immune response, there are several studies showing the association of SNPs in genes encoding for TLRs, Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and NOD2 with TB and/or leprosy.

TLRs are type I transmembrane proteins that function as homodimers or heterodimers that are implicated in the selective recognition of a vast range of Pathogen Associated Molecular Patterns (PAMPs) [30, 161-163]. Activation of TLRs, together with several co-receptors, are differentially activated by PAMPs and can be co-stimulated by other extracellular (e.g. CD14, CD36, *etc.*) or intracellular (e.g. NOD2) molecules and lead to differential immune responses. Lipopolysaccharide of Gram-negative bacteria is mostly recognized by TLR4 [164], as well as mannan [40]. TLR2 has also been proposed to sense lipoteichoic acid, peptidoglygan, lipoarabinomannan, and phospholipomannan [40]. TLRs intracellular activation, through Toll-interleukin-1 receptor (TIR) domain, leads to the activation of several specific signal-transducing pathways and transcription factors such as nuclear factor-κB (NF-κB), among others, responsible to transcription of pro-inflammatory molecules. The observation that different TLRs may activate different signaling pathways with different biological consequences shows that the innate immune system can produce pathogen-specific defensive responses and subjecting it to distinct genetic variability when considering distinct infection disease [162].

Particularly in mycobacterial studies, most have tested associations of variants in innate immune genes with clinical TB and leprosy. Specifically, the scientific interest in *TLR4* gene was notorious due to its involvement in mycobacterial recognition [32, 40, 160]. The non-synonymous SNPs Asp299Gly (rs4986790) and Thr399Ile (rs4986791) in the *TLR4* gene

were associated with protection against leprosy [165], although no association was observed between these SNPs and TB [166-168].

NOD2 is a major intracellular PRR which binds peptidoglycans, such as peptidoglycan-derived muramyl dipeptide (MDP) [169] and has been recognized as being important to regulate the immune response to *M. tuberculosis* [160]. Indeed, some genetic variants of *NOD2* have been under study for association with both leprosy and TB. Three non-synonymous polymorphisms in the *NOD2* gene, Pro268Ser (rs2066842), Arg702Trp (rs2066844), and Ala725Gly (rs5743278), were found to be associated with TB [170]. While the mutant alleles of Pro268Ser and Arg702Trp were shown to decrease the risk of TB, the mutant allele of Ala725Gly was shown to confer susceptibility [170]. The Pro268Ser polymorphism resulted in amino acid change to serine, contributing to structural alterations to a more stable NOD2, enhancing its capacity to recognize MDP. The same probable effect is expected in Arg702Trp, where the highly hydrophobic tryptophan contributes to the stability of NOD2 structure. Inversely, in rs5743278, a decrease in amino acid hydrophobicity from alanine to glycine contributes to a lesser stable structure, increasing susceptibility to mycobacteria [170]. In addition, a genome-wide association study reported that rs9302752 in the 5' prime region of *NOD2,* was associated with leprosy [171], resulting in an altered production of interleukin-10 and a consequent Th1–Th2 switching [172]. Other *NOD2* variants, rs2287185, rs8044354, rs8043770, rs13339578, rs4785225, and rs751271, were also reported as being associated with leprosy susceptibility [173]. Furthermore, this study also demonstrated an association risk with specific phenotypes: in its reversal reaction and in erythema nodosum leprosum.

Genes encoding for other transcription factors, such as Vitamin D receptor (VDR), that transcriptionally control the production of bactericidal molecules such as cathelicidin [174] in response to mycobacteria, have also been investigated and several SNPs have been associated with TB and leprosy susceptibilities and disease outcomes [175, 176]. The mutant homozygous genotype of *VDR* polymorphism rs10735810, also known as FokI, was shown to be associated with a higher risk of developing TB [177] and the association of this SNP with extra-pulmonary TB was also reported [178, 179]. Additionally, the mutant homozygous genotype of rs7975232 (ApaI) was described as being significantly related with a slower response to TB treatment [180]. Others showed that, although rs731236 (TaqI) polymorphism was not associated with TB itself, the heterozygous genotype of this SNP was related with a faster response to TB treatment [181]. Another report described the association of rs1544410

(BsmI) heterozygous genotype and FokI WT homozygous genotype with spinal TB [182]. Regarding leprosy, the TaqI variant was associated with leprosy phenotypes [183]. While the mutant homozygous genotype was found more frequently in patients with tuberculoid leprosy, the WT homozygous genotype was associated with lepromatous leprosy [183]. It is reported that 3' end of VDR gene, where TaqI, BsmI and ApaI are located, contains several polymorphisms in different degrees of Linkage Disequilibrium (LD) [184]. Haplotypes were also tested, and the *VDR* f-b-a-T (FokI f, BsmI b, ApaI a, TaqI T) haplotype was significantly associated to a protective effect in TB [185].

3.2.2. Cytokines

Several SNPs in cytokine genes and in genes encoding for cytokine receptor molecules have also been previously assessed for association with mycobacterial diseases, including IFN-γ, IL12-receptor, IL-10, Parkinson Disease Protein 2 (PARK2), among others.

Because IFN-γ has been implicated in the host defense against mycobacterial infections [186, 187], common genetic variants within this cytokine were proposed to cause increased susceptibility to both TB and leprosy. IFN-γ is produced upon the activation of immune defense mechanisms early in infection or by antigen-specific T cells following the induction of specific immunity. It has been demonstrated by several studies that the A allele and AA genotype for the intronic IFN-γ polymorphism, rs2430561, increase the risk of TB [188, 189], while the T allele [189-191] and TT genotype [189] have a protective effect. Indeed, TT and AT genotypes were reported as being associated with a decreased probability of treatment failure comparing with AA genotype [192]. Another study also shows that the T allele is associated with less severe forms of TB [193]. Besides, as in the case of TB, the T allele of rs2430561 confers protection to leprosy development [194]. In addition to *IFNG* as well as Interferon-gamma receptor (*IFNGR*) genetic variants also seem to be related with TB, namely, rs1059293 and rs2834213 at *IFNGR2* (both in LD) were associated with resistance to TB [195]. Previously, CC genotype at the -56 C/T SNP (rs2234711) of *IFNGR1* was repeatedly associated with TB in African populations [196]. More recently, *IFNGR1* rs9376269 was found to increase TB risk after robust multiple testing in a large study with 673 patients [197]. Experimental data have shown that *IFNGR2* is a key regulator for IFN-γ-STAT1 signaling in T cells. During the development of Th1 cells, *IFNGR2* transcription is reduced in the IFN-γ rich

condition and this reduction alleviates a potentially harmful anti-proliferative action of IFN-γ-STAT1 signaling pathways [198].

IL-12 receptor (*IL12R*) variants were also tested for association, due to the role of IL-12 in driving the differentiation and expansion of Th1 cells. *IL12RB1* non-synonymous SNPs, rs11575934, rs375947 and rs401502, were described to be in LD, and the R214-T365-R378 mutant homozygotes were shown to have a higher risk of TB development than WT homozygotes [199]. Another genetic variation, rs436857, in the promoter region of *IL12RB1*, was also reported to confer an increased risk of pulmonary TB [200]. Additionally, the mutant allele of the *IL12RB2* promoter polymorphism rs11810249 was portrayed to decrease the transcriptional activity of the receptor [201]. Indeed, TB patients carrying the mutant allele presented reduced *IL12RB2* mRNA expression [201].

The primary effector cells in mycobacterial infections are macrophages, which are the main producers of inflammatory cytokines such as TNF-α, a pivotal mediator of inflammatory responses. Due to the importance of this cytokine in mycobacterial infections, association of *TNFA* gene polymorphisms with leprosy and TB has been under study. *TNFA* promoter gene polymorphism rs1800629 revealed association with TB, since its mutant allele was shown to increase the risk of developing extrapulmonary forms of the disease [202]. A recent meta-analysis revealed that the mutant allele of this SNP is associated with an increased risk of TB in an ethnicity dependent base [202]. Two other *TNFA* SNPs, rs1800630 and rs1799724, have also been studied. The mutant genotype of rs1800630 was reported to be a risk factor for pulmonary TB, while the mutant genotype of rs1799724 was described to be protective for pulmonary TB [203]. Contrarily to the results obtained for TB, the mutant allele of rs1800629 SNP was associated with protection from leprosy [194, 204].

IL10 polymorphisms that would result in increased expression activity could also be risk factors for mycobacterial infections, due to the anti-inflammatory activity of this cytokine, and its role in down-regulating inflammatory responses. Indeed, genetic alterations in the IL-10 encoding gene have also been associated to mycobacterioses. The A allele and AA genotype in rs1800872 (-592C>A), present in the promoter region, were associated to leprosy susceptibility [205]. *IL10* -1082*AA (rs1800896) was significantly associated with pulmonary non-tuberculous mycobacteria [206].

Other molecules, which generally are not directly associated with immune responses, can also be somehow associated with increased susceptibility to mycobacterial infections. For

example, the *PARK2* gene, which was previously associated with Parkinson's disease, and its co-regulated gene *PACRG*, were found to be factors of leprosy susceptibility. In particular, the 5' prime region shared by these two genes was found to contain several genetic variations that associate with leprosy [207].

In conclusion, there are several genetic variants that associate with both TB and leprosy, while others only associate with one of these mycobacterial diseases. Interestingly, the same SNP can present different results, being associated with disease susceptibility or with resistance, depending on the mycobacteriosis and also on the population. Additionally, a SNP that shows no association with susceptibility or resistance to one specific disease can be implicated in the development of severe forms of this disease, or in the differential individual predisposition to develop diverse disease phenotypes.

3.3. Genetic susceptibility to Buruli ulcer

As mentioned throughout this introduction, several clinical and epidemiological reports support the role of host genetic factors in the development of BU. Among others previously discussed:

(a) self-healing of BU lesions has been reported to occur among patients [140, 208-210];

(b) history of BU in the family is a risk factor for developing the disease [105];

(c) a considerable number of individuals living in BU endemic areas never develop clinical disease, even after continuous exposure to a *M. ulcerans* enriched environmental [211, 212];

(d) within BU patients, the disease presents a wide spectrum of clinical manifestations with different degrees of severity, without a major variability within the genetics of *M. ulcerans* [4] and;

(e) frequencies of antibodies to *M. ulcerans* in serum samples from affected and unaffected subjects are similar, indicating that many of the control subjects had been exposed to this bacterium but never developed disease [5, 43].

Taking this into consideration, a previous study suggested that genetic host susceptibility factors that are important for other mycobacterioses, including innate immune

system role-players [e.g. TLRs, NLRs, VDR, IFN-γ, TNF-α, IL-12 and NRAMP1 protein (natural resistance-associated macrophage protein 1)], could contribute equally to BU susceptibility/resistance [212].

Contrasting with the numerous association studies performed with TB and leprosy, little is still known about the role of host genetic variability in BU. Only one genetic association study was carried out for BU [211]. The report described that the D543N polymorphism in the *NRAMP1*, which has been previously related with TB [213] and leprosy [214, 215], is associated with BU development [211]. NRAMP1 protein is encoded by the *SLC11A1* gene in humans [216]. NRAMP1 is present in the membrane of late endosomes/lysosomes in macrophages [217], and its main function is the pH-driven transport of bivalent cations, such as Fe^{2+}, Zn^{2+} and Mn^{2+} [218]. Stienstra et al. suggested that intraphagosomal concentration of Fe^{2+} could play a role in intramacrophage mycobacterial growth [211], due to the possible involvement of this cation in generating antimicrobial hydroxyl radicals within phagolysosomes of infected macrophages [217].

Therefore, to study the genetic susceptibility to BU, there is an urgent need of a biological bank (biobank) of patients and controls. A robust design of such bank should take into account the:

(a) equal exposure and accumulated risk of contact with the pathogen (cases and controls) taking into account protection measures, living area and activities associated to aquatic environment, as addressed previously;

(b) common and/or stable phenotypes within BU cases, with a large number of included patients;

(c) absence of population stratification and;

(d) use retrospective cases/controls, age and gender-adjusted, that definitely would develop/not-develop disease, certifying that they were subjected to long and continuous pathogen exposition.

With such a biobank, with DNA from patients and controls, one can study the implication of genetic variants on validated molecular/pathways previously associated to immune responses mounted during infection with *M. ulcerans*.

In conclusion, there is evidence for a major role of host genetic factors in BU development. In that sense, we hypothesized that several polymorphisms already associated with TB or leprosy can also be associated with BU, and be responsible for susceptibility or

resistance, as well as for the inter-individual differences of disease phenotypes. The dissection of those genetic variations, performed in the present thesis, taking advantage of a biobank established in Benin, provide news insights into mechanisms of host susceptibility or resistance to BU.

4. References

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CHAPTER 2

CLINICAL EPIDEMIOLOGY OF BURULI ULCER FROM BENIN (2005-2013): EFFECT OF TIME-DELAY TO DIAGNOSIS ON CLINICAL FORMS AND SEVERE PHENOTYPES

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ABSTRACT

Buruli ulcer (BU) is a neglected infectious disease caused by *Mycobacterium ulcerans*, responsible for severe necrotizing cutaneous lesions that may be associated with bone involvement. Clinical presentations of BU lesions are classically classified as papules, nodules, plaques and edematous infiltration, ulcer or osteomyelitis. Within these different clinical forms, lesions can be further classified as severe forms based on focality (multiple lesions), lesions size (>15cm diameter) or WHO Category (WHO Category 3 lesions). There are studies reporting an association between delay in seeking medical care and the development of ulcerative forms of BU or osteomyelitis, but the effect of time-delay on the emergence of lesions classified as severe has not been addressed. To address both issues, and in a cohort of laboratory-confirmed BU cases, 476 patients from a medical center in Allada, Benin, were studied. In this laboratory-confirmed cohort, we validated previous observations, demonstrating that time-delay is statistically related to the clinical form of BU. Indeed, for non-ulcerated forms (nodule, edema, and plaque) the median time-delay was 32.5 days (IQR 30.0-67.5), while for ulcerated forms it was 60 days (IQR 20.0-120.0) (p=0.009) and for bone lesions, 365 days (IQR 228.0-548.0). On the other hand, we show here that time-delay is not associated with the more severe phenotypes of BU, such as multi-focal lesions (median 90 days; IQR 56-217.5; p=0.09), larger lesions (diameter >15cm) (median 60 days; IQR 30-120; p=0.92) or category 3 WHO classification (median 60 days; IQR 30-150; p=0.20), when compared with unifocal (median 60 days; IQR 30-90), small lesions (diameter ≤15cm) (median 60 days; IQR 30-90) or WHO category 1+2 lesions (median 60 days; IQR 30-90), respectively. Our results demonstrate that after an initial period of progression towards ulceration or bone involvement, BU lesions become stable regarding size and focal/multi-focal progression. Therefore, in future studies on BU epidemiology, severe clinical forms should be systematically considered as distinct phenotypes of the same disease and thus subjected to specific risk factor investigation.

AUTHOR SUMMARY

Buruli ulcer (BU) is a neglected disease caused by *Mycobacterium ulcerans*. Clinical presentations of BU lesions are classically classified as papules, nodules, plaques and edematous infiltration, ulcer or osteomyelitis. Within these different clinical forms, lesions can be further classified as severe forms based on focality (multiple lesions), lesions size (>15cm diameter) or WHO Category (WHO Category 3 lesions). There are studies reporting an association between delay in seeking medical care and the development of ulcerative forms of BU or osteomyelitis, but the effect of time-delay on the emergence of lesions classified as severe has not been addressed. To address both issues, and in a cohort of laboratory-confirmed BU cases, 476 patients from a medical center in Allada, Benin, were studied. In our cohort, we validated previous observations, demonstrating that time-delay is statistically related to the clinical form of BU, namely ulcers and osteomyelitis. However, time-delay is not related with more severe phenotypes, implying that severe clinical forms of BU should be considered as distinct phenotypes of the same disease and subjected to specific risk factor investigation.

INTRODUCTION

Buruli ulcer (BU), caused by *Mycobacterium ulcerans*, is the third most common mycobacteriosis worldwide, after tuberculosis and leprosy [1]. BU pathogenesis is mediated by mycolactone, a potent polyketide-derived macrolide that triggers apoptotic cell death [2] and is associated with the necrotic nature of the disease [3]. BU mostly affects people in tropical countries in Africa [4], America [5], Asia [6] and Australia [7]. Although no official estimate of global incidence is available at present, West Africa is the main endemic area, with 1967 new cases reported by Côte d'Ivoire, Ghana, and Benin in 2013 [8]. BU is a devastating necrotizing skin infection characterized by pre-ulcerative lesions (papules, nodules, plaques and edematous infiltration), which commonly develop into ulcers with undermined edges and can spread to an entire limb [9] and can also affect the bone (osteomyelitis) [10] . Moreover, within these clinical presentations, more aggressive severe forms of BU, such as multiple lesions, larger lesions or higher World Health Organization (WHO) categories have been described [11], although underreported and less understood. Epidemiological studies on *M. ulcerans* transmission, on BU risk factors and on the host immune status, suggest that the variable frequency of BU and its distinct clinical forms are related to: i) age; ii) gender; iii) preferential anatomical site; iv) water contact; and v) regional occurrences [12-16].

To date, a reduced number of risk factors underlying the severe BU phenotypes had been reported. HIV co-infection is one of the few examples. Some studies revealed an increased BU prevalence among HIV patients, especially those presenting large lesions and osteomyelitis [17,18]. Specifically, low CD4 cell counts were significantly associated with larger lesions and patients with a CD4 cell count below 500 cell/mm^3 took twice as long to recover from BU when compared with individuals with a normal CD4 cell count [19]. Other risk factors, such as hypoproteinemia [11] and anemia [20] were also identified to be associated with severe forms of BU disease.

In addition, the delay in seeking medical care and the late medical diagnosis of BU have been proposed to account for the disease presentation [21-24]. In fact, in BU endemic regions the culture and beliefs are powerful factors that affect proper medical intervention, as patients preferentially seek treatment from traditional practitioners, or herbalists [22]. On top of this, the lack of knowledge on the available treatments and their effectiveness, the financial constraints during hospitalization, fear of treatment, and poor access to health facilities are

also important aspects delaying the pursuit of proper treatment [25, 26]. Indeed, delay in seeking medical care has been previously associated with the distinct BU clinical forms. Taking into consideration that the time from progression of a pre-ulcer to an ulcer is variable and can range from a few weeks to several months (e.g. estimated average time of 30–90 days) [27], it was established that individuals with non-ulcerated forms had a median delay of 30 to 45 days, while individuals with ulcers presented a 60-day delay and patients with osteomyelitis up to 90 days [28]. Thus, the more advanced and destructive ulcerated forms and osteomyelitis are associated with longer delay-periods, while non-ulcerated forms are more common in patients with recent infection [28], justifying the importance of early diagnosis and treatment for the disease.

Nonetheless, more aggressive, severe clinical presentations of BU, such as large lesions (>15cm in diameter) and multifocal lesions, have also been described [11], although the underlying pathological mechanisms are yet unclear [29]. While this can be associated with characteristics of the patient itself (genetic susceptibility/health status) or with the virulence of the infecting strain, it is also rational to question the influence of the delay in health seeking on the appearance of the more severe forms of BU. To our knowledge, the latter aspect is yet to be studied. Therefore, to uncover whether the time-lapse between the first remembered symptoms and clinical diagnosis is associated to disease severity, we retrospectively analyzed a cohort of 476 laboratory-confirmed BU treated cases discovered in a highly endemic area in Allada, Benin, between 2005 and 2013.

MATERIAL AND METHODS

Ethics statement

Ethical approval (clearance Nu 018, 20/OCT/2011) for integrating studies on BU was obtained from the National Ethical Review Board of the Ministry of Health in Benin, registered under the Number IRB0006860. The *Centre de Dépistage et de Traitement de l'Ulcère de Buruli* (CDTUB) - Allada and the national BU control program authorities approved access to the registry. All data analyzed in this study was anonymized.

Study setting, participants and design

We retrospectively collected clinical data from 476 laboratory-confirmed BU patients of CDTUB in Allada, Benin - between January 2005 and December 2013. At the moment of diagnosis, parameters such as age, gender, major clinical form (nodule, plaque, edema, ulcer or osteomyelitis) and multifocal presentations were registered. For mixed clinical forms, the most severe lesion was considered the major clinical form. Additionally, lesion size (cm, considering major diameter), WHO category [30] (Category 1: maximum lesion diameter <5cm; Category 2: maximum lesion diameter 5-15cm; and Category 3: minimum lesion diameter >15cm associated or not with osteomyelitis and/or multifocal lesions and/or at a critical site), lesion site (upper or lower limb, trunk, head and/or neck) and laboratory confirmation tests (culture of *M. ulcerans* from the lesion, histopathology with the presence of acid-fast bacilli, or highly specific IS2404 real-time PCR) were taken into consideration. The HIV status was also retained for the present study and excluded from analysis if positive.

Delay in seeking medical care (time between first symptoms or signs remembered and medical attendance) was also recorded. The time of seeking medical care was defined as the moment of diagnosis and treatment initiation. All included patients completed antibiotherapy according to the WHO recommendations and were treated with surgical procedures [30].

Statistical analysis

Explanatory and descriptive analysis of the study cohort was performed based on the following variables: age at the moment of the BU diagnosis; gender; clinical form (ulcer, plaque, edema, nodule and osteomyelitis); lesion site; and lesion severity. Severe phenotypes were defined as multifocal lesions (more than one lesion); large lesions (diameter >15cm) or

Category 3 lesions (minimum lesion diameter >15cm associated or not with osteomyelitis, multifocal lesions and/or at a critical site) as classified by the WHO recommendations. Median comparisons were performed through one-way ANOVA's (Brown-Forsythe and Welch, when applicable) using age, gender, site of lesion, clinical BU form; and lesion severity as explanatory variables and time-delay seeking medical care (days, using means and medians distribution in each group) as a dependent variable. Unadjusted and adjusted (for age -cutoff value 15 years of age- and gender binary or linear logistic regression models) odds ratios were then calculated to explore the effects of time-delay in diagnosis into the clinical form of BU lesions, and particularly into severe phenotypes of BU. We systematically fit the model, controlling age (dichotomized or ordinal) and gender with the considered time-delay (to seek medical attendance) as explanatory variables for each of the clinical lesions and severe phenotypes defined for BU. All the described analyses were obtained using IBM SPSS Statistic v. 22. A result was considered significant for $p<0.05$.

RESULTS

Cohort characterization

The BU cohort (CDTUB, Allada, Benin), comprising 476 cases, had laboratory BU confirmation by at least one laboratory diagnostic test, as recommended by the WHO. Results were positive for IS2404 RT-PCR in 430 (90.3%) cases and Ziehl-Neelsen staining in 327 (68.7%) cases. All cases were HIV negative. The median age at diagnosis was 12 years (IQR: 7—24 years; mean 17.9 ± 16.3 years), with 321 (67.4%) patients 15 years old or under. Although the overall gender ratio of the patients was balanced (245 [51.5%] male) (Table 1), a major distortion of this ratio was recorded as a function of age, with males being predominant in younger patients and females in older patients (OR 2.99, 95%CI 2.00-4.46, p=0.0001). Specifically, male patients accounted for 193 (60.1%) of the patients younger than 15, but only 52 (33.5%) of those were over 15 (Table 1).

Table 1. Age and gender distribution according to lesion location, lesion phenotype, and lesion severity in 476 laboratory-confirmed BU treated patients at CDTUB - Allada from 2005 to 2013.

	Patient n (%)	Age distribution (>15 | ≤15)	Gender distribution (F|M)
Gender			
Male	245 (51.5%)	52 | 193	..
Female	231 (48.5%)	103 | 128	..
Age			
> 15 years old	155 (32.6%)	..	103 | 52
≤ 15 years old	321 (67.4%)	..	128 | 193
Lesion location[a]			
Head and neck	6 (1.3%)	2 | 4	4 | 2
Thorax and abdomen	43 (9.0%)	9 | 34	18 | 25
Upper Limb	171 (35.9%)	51 | 120	88 | 83
Lower Limb	256 (53.8%)	93 | 163	121 | 135
Lower limb lesions vs. Upper limb lesions	256 (53.8%)	93 | 163	121 | 135
	220 (46.2%)	62 | 158	110 | 110

Clinical lesion			
Nodule [a]	4 (0.8%)	0 | 4	3 | 1
Edema [a]	24 (5.0%)	4 | 20	10 | 14
Plaque [a]	125 (26.3%)	44 | 81	62 | 63
Ulcer [a]	320 (67.2%)	105 | 215	155 | 165
Osteomyelitis [a]	3 (0.6%)	2 | 1	1 | 3
All	476 (100%)	155 | 321	231 | 245
Non-ulcerative vs. Ulcerative forms	156 (32.8%)	49 | 107	77 | 82
	320 (67.2%)	105 | 215	155 | 165
Edema vs. Other non-ulcerated forms	24 (5.0%)	4 | 20	10 | 14
	129 (27.1%)	44 | 85	65 | 64
Multifocal lesions			
Multifocal	22 (4.6%)	8 | 14	10 | 12
Unifocal	454 (95.4%)	147 | 307	221 | 233
Lesion size			
> 15cm	142 (29.8%)	47 | 95	62 | 80
≤ 15cm	334 (70.2%)	108 | 226	169 | 165
WHO Category			
Category 3	161 (33.8%)	55 | 106	71 | 90
Category 1 + 2	315 (66.2%)	100 | 215	160 | 155

[a] dominant clinical form

Considering the dominant clinical BU form per patient, 4 (0.8%) presented nodules (Figure 1A, 1F, and Table 1), 24 (5.0%) presented edema (Figure 1B, 1F, and Table 1), 125 (26.3%) presented plaques (Figure 1C, 1F, and Table 1), and 320 (67.2%) presented ulcers (Figure 1E, 1F, and Table 1). Osteomyelitis was diagnosed in 5 patients (1.1%), and was considered the most relevant form in 3 of the patients (0.6%) (Figure 1E, 1F, and Table 1).

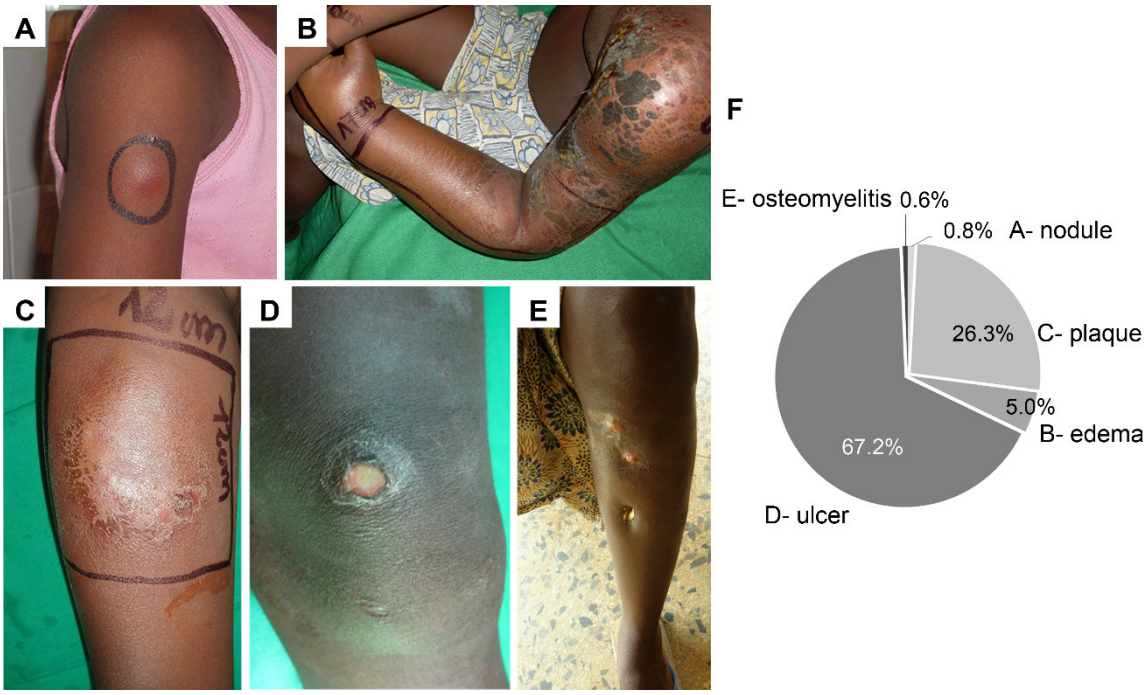

Figure 1. Prevalence of clinical BU lesions. Representative images of (A) nodule, (B) edema, (C) plaque, (D) ulcer, (E) osteomyelitis and (F) the percentage of each clinical presentation.

Concerning the site of lesions, 256 (53.8%) patients presented lesions on the lower limbs, while 171 (35.9%) had lesions on the upper limbs (Figure 2 and Table 1). Atypical sites (head, neck and/or trunk) accounted for 49 (10.3%) patients (Figure 2 and Table 1). Site of the lesion and relative age, gender and dominant clinical form distribution are represented in Figure 2.

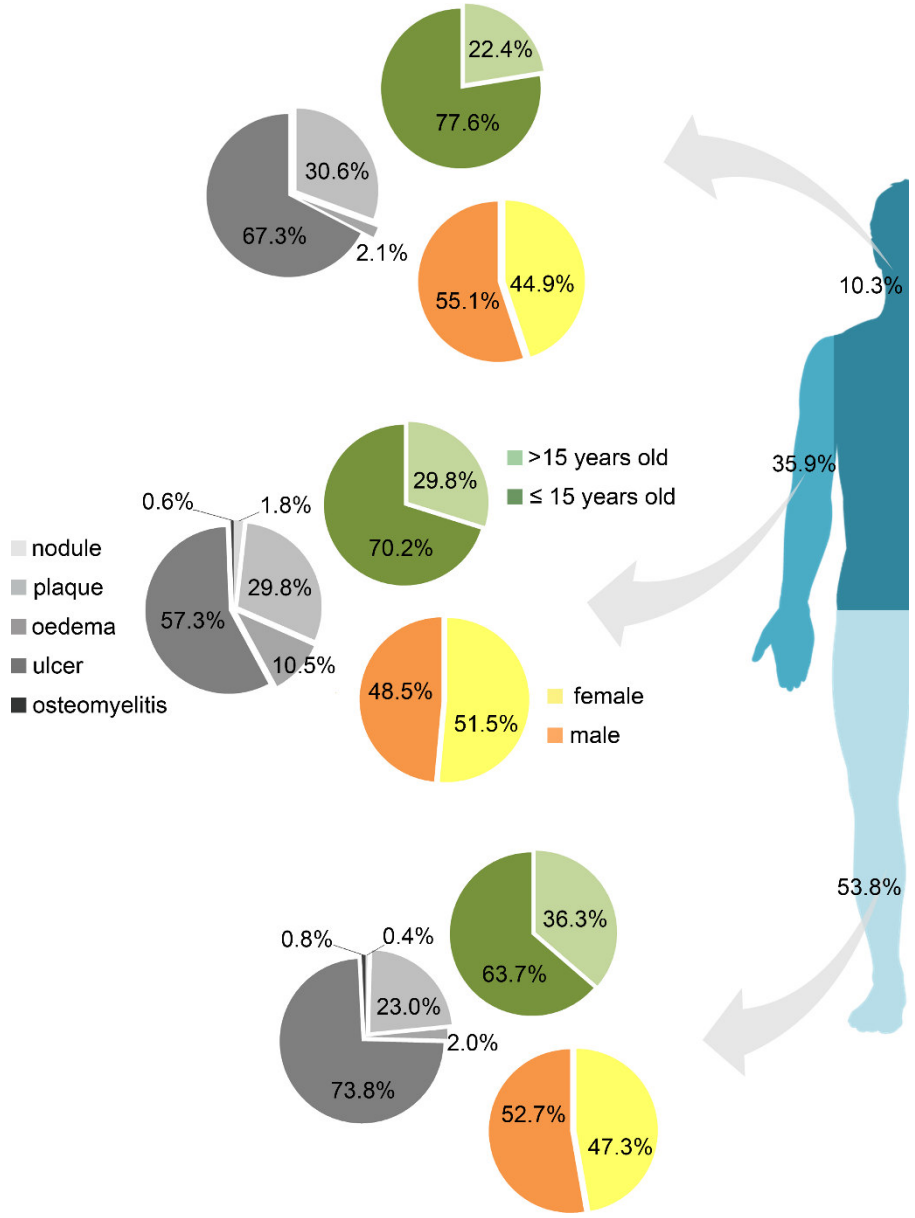

Figure 2. Age, gender, clinical BU lesion, and lesion location. Age (≤15 years old, >15 years old), gender, and clinical BU forms according to lesion distribution throughout the three major areas of the body (lower limbs, upper limbs, and head+trunk).

Regarding the observed severe forms of BU, 22 (4.6%) patients presented lesions in more than one localization (Figure 3A and Table 1), while 142 (29.8%) patients presented lesions larger than 15cm in major diameter (Figure 3B and Table 1). The WHO category 3 is a broader classification given that it comprises patients with multiple lesions, lesions with a diameter >15cm associated or not with osteomyelitis and/or lesions at a critical site. Taking into account these criteria, we recorded 315 (66.2%) patients in category 1+2 and 161 (33.8%) in category 3 (Figure 3C and Table 1).

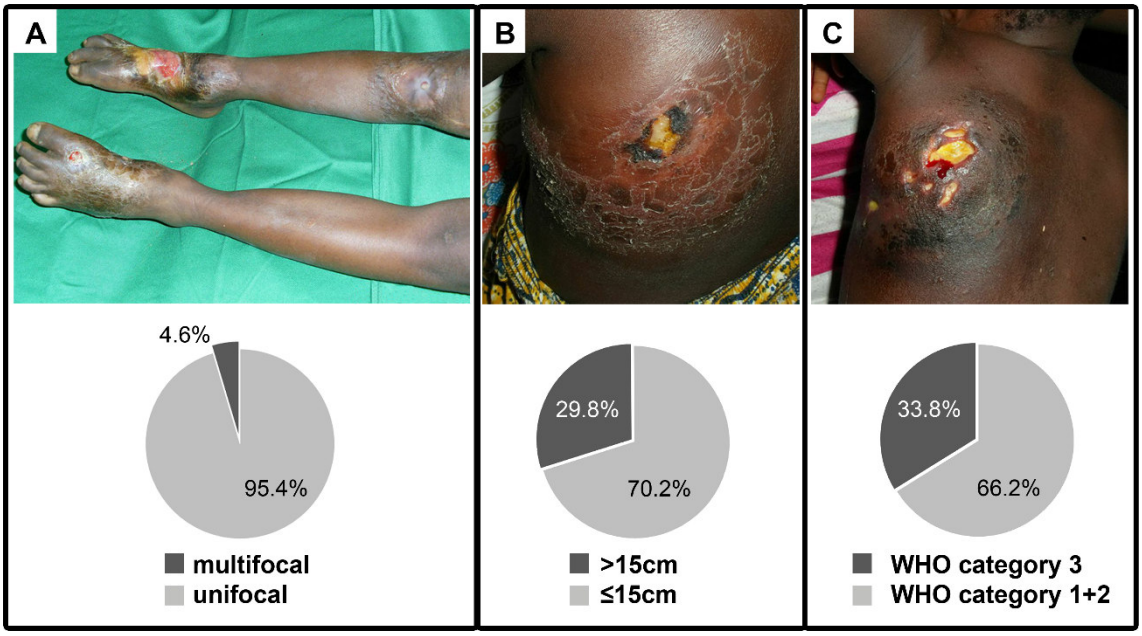

Figure 3. Prevalence of severe BU lesions. Representative images of (A) multifocal lesions, (B) large lesions (>15cm), (C) WHO Category 3 lesions, and the percentage of each clinical presentation.

The different clinical presentations, as well as the severe forms of these lesions, were subjected to age and gender adjustments (Table 2 and Table 3, respectively). No significant interference was recorded in the binary logistic regression, except for upper body lesions (upper limb, head or neck), for which there was an overrepresentation of younger ages (OR 0.986, 95%CI 0.974–0.998, p=0.018) (Table 2).

Table 2. Univariate analysis of the effect of age and gender on clinical BU forms and lesion location in 476 laboratory-confirmed BU treated patients at CDTUB - Allada from 2005 to 2013 (binary logistic regression).

	Coeff. (SE)	Odds Ratio (CI)	p-value
Male			
Age	0.006	0.972 (0.960-0.984)	**0.0001**
Upper body lesions [a]			
Age	0.006	0.986 (0.974-0.998)	**0.018**
Gender	0.200	0.811 (0.559-1.177)	0.271
Nodule [b]			
Age	0.105	0.867 (0.706-1.065)	0.174
Gender	1.164	0.218 (0.022-2.135)	0.191
Plaque [b]			
Age	0.006	1.003 (0.991-1.016)	0.624
Gender	0.213	0.965 (0.635-1.465)	0.866
Edema [b]			
Age	0.020	0.970 (0.933-1.008)	0.116
Gender	0.433	1.136 (0.486-2.654)	0.768
Ulcer [b]			
Age	0.006	1.004 (0.992-1.016)	0.534
Gender	0.200	1.039 (0.702-1.537)	0.850
Osteomyelitis [b]			
Age	0.042	0.972 (0.896-1.055)	0.501
Gender	0.934	1.220 (0.196-7.603)	0.831

[a] upper body lesions: head + neck + upper limbs + thorax + abdomen

[b] dominant clinical form

Table 3. Univariate analysis of the effect of age and gender on severe BU forms in 476 laboratory-confirmed BU treated patients at CDTUB - Allada from 2005 to 2013 (binary logistic regression).

	Coeff. (SE)	Odds Ratio (CI)	p-value
Multifocal lesions			
Age	0.012	1.010 (0.986-1.035)	0.433
Gender	0.450	1.224 (0.507-2.956)	0.653
Larger lesions (>15cm)			
Age	0.006	1.004 (0.992-1.017)	0.489
Gender	0.207	1.363 (0.909-2.043)	0.134
WHO category 3			
Age	0.006	1.004 (0.992-1.016)	0.550
Gender	0.200	1.342 (0.908-1.985)	0.140

Time-delay to seek medical care

The overall mean time-delay to seek medical care was 101.1 days (95%CI 86.3-117.0) (Table S1).

Table S1. Time-delay according to gender, age, lesion location and clinical lesion phenotype in 476 laboratory-confirmed BU treated patients at CDTUB - Allada from 2005 to 2013.

	Time-delay [b] days median [IQR]	Time-delay [b] days mean (95%CI)	p-value [c]
Gender			
Male	60 [30-90]	96.6 (78.1-114.5)	p=0.538
Female	60 [30-90]	106.04 (81.7-130.4)	
Age			
> 15 years old	60 [30-120]	140.1 (103.1-177.1)	**p=0.004**
≤ 15 years old	45 [30-90]	82.5 (69.8-95.2)	
Lesion location [a]			
Head and neck	52.5 [30-90]	68.0 (2.8-133.2)	
Thorax and abdomen	60 [30-120]	103.4 (64.6-142.2)	p=0.614
Upper Limb	60 [30-90]	89.2 (67.8-110.5)	

Lower Limb	60 [30-90]	109.5 (86.7-132.2)	
Lower limb lesions vs. Upper limb lesions	60 [30-90]	109.5 (86.7-132.2)	p=0.233
	60 [30-90]	91.4 (73.2-109.5)	
Clinical lesion			
Nodule [a]	30 [25-75]	50.3 (21.0-120.0)	
Edema [a]	45 [30-105]	84.2 (50.1-126.3)	
Plaque [a]	30 [30-60]	73.9 (50.1-109.5)	
Ulcer [a]	60 [30-120]	111.0 (92.9-130.9)	
Ostemyelitis [a]	365 [228-548]	395.0 (90.0-730.0)	
All	60 [30-90]	101.1 (86.3-117.0)	
Non-ulcerative vs. Ulcerative forms	32.5 [30-67.5]	81.1 (55.3-106.9)	**p=0.009**
	60 [30-120]	111.0 (92.9-130.9)	
Edema vs. Other non-ulcerated forms	45 [30-105]	84.2 (50.1-126.3)	**p=0.030**
	30 [30-60]	73.1 (44.7-101.5)	

[a] dominant clinical form
[b] time delay until seeking medical care
[c] comparison of median - time delay distribution between groups with Welch's t-test.

Since the variable time-delay does not follow a normal distribution (Kurtosis=48.2; Skewness=6.4), median variations were considered to compare the distinct behavior of dependent variables. Time-delay to seek medical care was indistinct for male and female gender (p=0.538) (Figure 4A and Table S1): median was 60 days [IQR 30-90] for both genders.

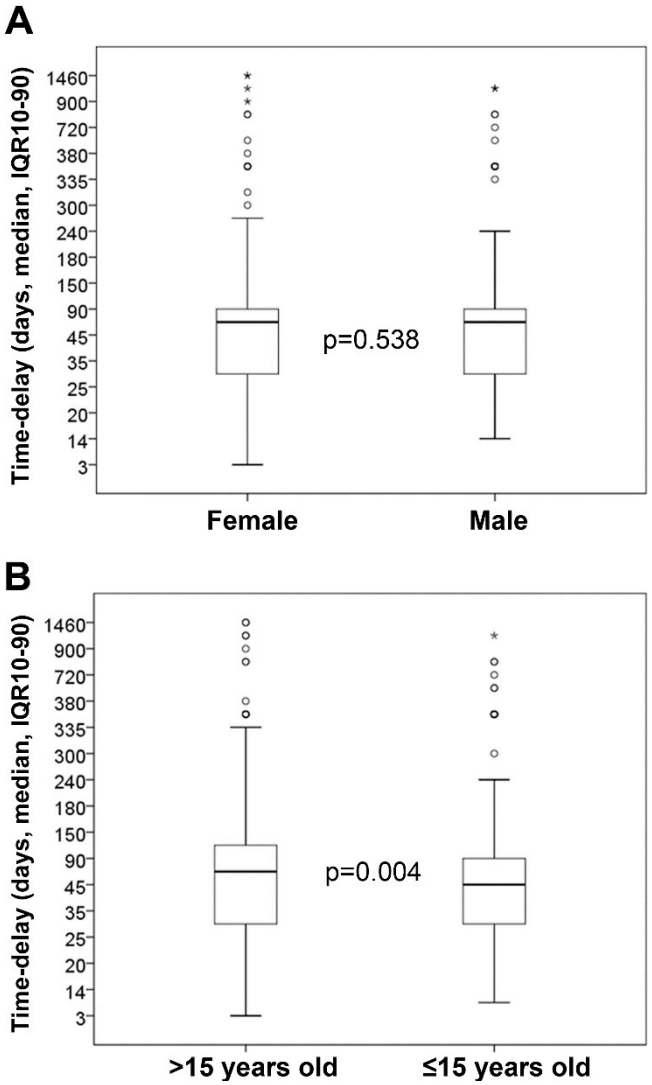

Figure 4. Time-delay to seek medical care related to gender and age. Time-delay to seek medical care related to (A) gender and (B) age. Circles represent the outliers and asterisks represent the extreme outliers. Statistical significance was calculated using Welch's *t*-test. Differences with a p-value of ≤0.05 were considered significant.

However, age was associated with significantly different delay times (p=0.004) (Figure 4B and Table S1). Median was 60 days [IQR 30-120] for patients over 15 years old at the moment of the diagnosis; while time-delay was 45 days [IQR 30-90] for patients with 15 years of age or under.

Time-delay was also related to the clinical form of the disease (Figure 5).

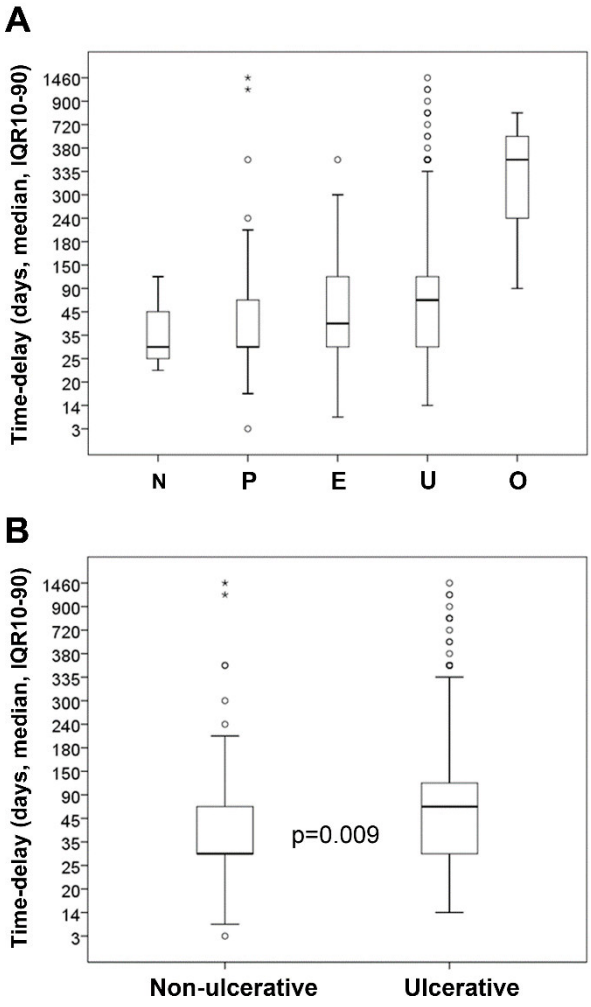

Figure 5. Time-delay to seek medical care related to clinical BU forms. Time-delay to seek medical care related to (A) clinical form N – nodule; P – plaque; E – edema; U – ulcer; O – osteomyelitis and (B) non-ulcerative vs. ulcerative lesions. Circles represent the outliers and asterisks represent the extreme outliers. Statistical significance was calculated using Welch's *t*-test. Differences with a p-value of ≤0.05 were considered significant.

Median was 32.5 days [IQR 30-67.5] for non-ulcerated forms (nodule, edema, and plaque); 60 days [IQR 20.0-120.0] for ulcerated forms; and 365 days [IQR 228-548] for bone lesions. When the time-delay among patients with non-ulcerated versus ulcerated forms was compared, we confirmed significant discrepancies (p=0.009) (Figure 5B and Table S1). In addition, among the non-ulcerated clinical forms, edema was significantly associated with longer time-delays when compared with others non-ulcerated forms (median 45 days, IQR 30-105 versus 30 days, IQR 30-60, respectively, with p=0.03) (Table S1). Even when age and

gender were adjusted in binary logistic regression, we observed an increased risk of developing ulcerative lesions as each day/month passed (Table 4).

Table 4. Multivariate analysis of the effect of time-delay on clincal BU forms in 476 laboratory-confirmed BU treated patients at CDTUB - Allada from 2005 to 2013 (binary logistic regression).

	Coeff. (SE)	Odds Ratio (CI) (days)	Odds Ratio (CI) (months)	p-value
Male				
Age	0.006	0.973 (0.961-0.985)		**0.0001**
Time delay [a]	0.001	1.000 (0.999-1.001)	0.999 (0.966-1.034)	0.799
Upper body lesions [b]				
Age	0.006	0.987 (0.975-0.999)		**0.028**
Gender	0.192	0.837 (0.574-1.218)		0.352
Time delay	0.001	0.999 (0.998-1.001)	0.980 (0.945-1.017)	0.299
Ulcerative lesions				
Age	0.006	1.002 (0.989-1.014)		0.793
Gender	0.204	1.067 (0.709-1.576)		0.786
Time delay	0.001	1.002 (1.000-1.004)	1.065 (1.005-1.129)	**0.025**

[a] time delay until seeking medical care; odds ratio for days and months

[b] upper body lesions: head + neck + upper limbs + thorax + abdomen

Considering severe forms of BU, none of the aggressive phenotypes were considered related to significantly different delay times to seek medical care: multifocal lesions (median 90 days, IQR 56.3-217.5, p=0.09) (Figure 6A and Table S2), larger lesions with diameter >15cm (median 60 days, IQR 30-120, p=0.92) (Figure 6B and Table S2) or category 3 WHO classification (median 60 days, IQR 30-150, p=0.20) (Figure 6C and Table S2), when compared with unifocal (median 60 days, IQR: 30-90), small lesions (diameter ≤15cm) (median 60 days, IQR 30-90) or WHO category 1+2 lesions (median 60 days, IQR 30-90), respectively (Figure 6A-C and Table S2).

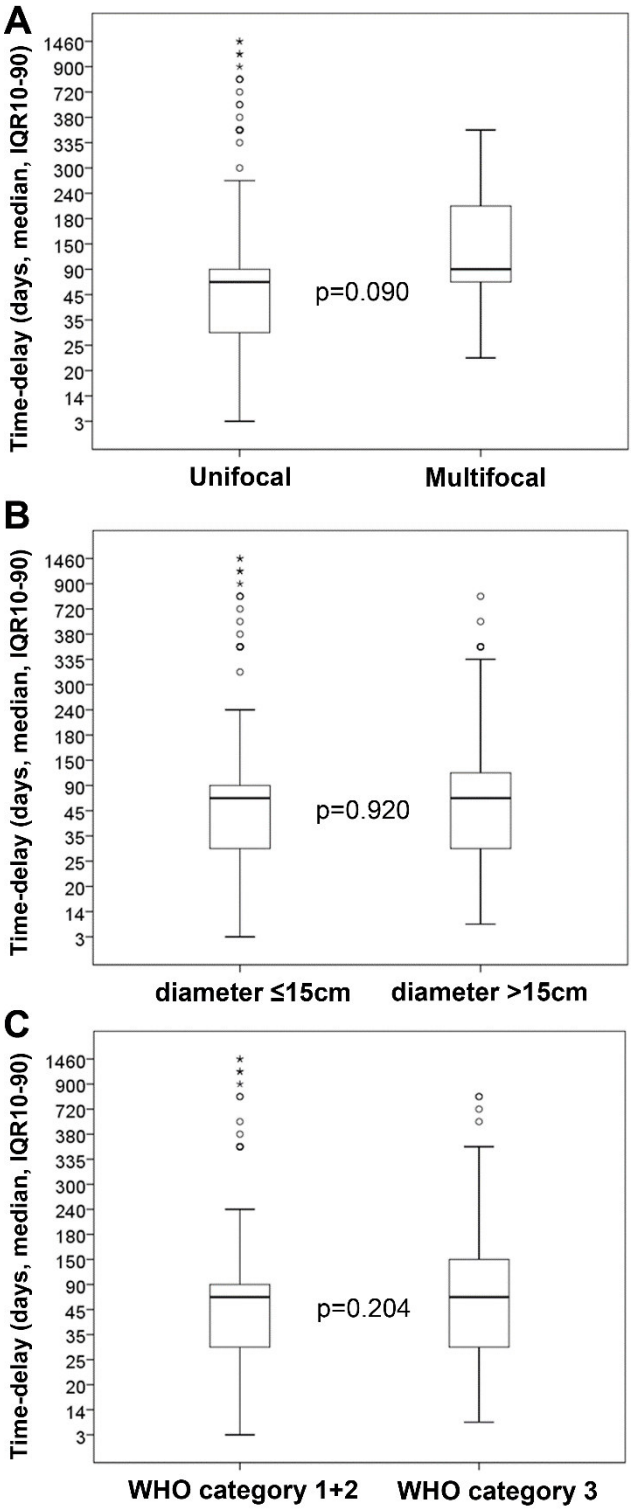

Figure 6. Time-delay to seek medical care related to severe BU forms. (A) multifocality (multifocal vs. unifocal lesions); (B) lesion size (≤15cm vs. >15cm); (C) WHO Category (Category 3 vs. category 1+2). Circles represent the outliers and asterisks represent the extreme outliers. Statistical significance was calculated using Welch's *t*-test. Differences with a p-value of ≤0.05 were considered significant.

Table S2. Time-delay according to gender, age, lesion location and severe lesion phenotype in 476 laboratory-confirmed BU treated patients at CDTUB - Allada from 2005 to 2013.

	Time-delay [a] days median [IQR]	Time-delay [a] days mean (95%CI)	p-value [b]
Multifocal lesions			
Multifocal	90 [56.3-217.5]	141.9 (94.1-189.7)	p=0.090
Unifocal	60 [30-90]	99.1 (83.7-114.5)	
Lesion size			
≥ 15cm	60 [30-120]	102.1 (83.8-120.4)	p=0.920
< 15cm	60 [30-90]	100.7 (81.1-120.4)	
WHO Category			
Category 3	60 [30-150]	113.4 (92.9-133.9)	p=0.204
Category 1 + 2	60 [30-90]	95.0 (75.2-114.8)	

[a] time delay until seeking medical care

[b] comparison of median - time delay distribution between groups with Welch's t-test.

Finally, when systematically fit within binary (dichotomized variables) (Table 5) or linear (Table 6) logistic regression models controlling for age and gender, time-delay to seek medical care remained statistically insignificant with respect to the occurrence of the most aggressive severe clinical forms.

Table 5. Multivariate analysis of the effect of time-delay on severe BU forms in 476 laboratory-confirmed BU treated patients at CDTUB - Allada from 2005 to 2013 (binary logistic regression).

	Coeff. (SE)	Odds Ratio (CI) (days)	Odds Ratio (CI) (months)	p-value
Multifocal lesions				
Age	0.013	1.009 (0.984-1.034)		0.48
Gender	0.452	1.203 (0.496-2.918)		0.68
Time delay [a]	0.001	1.001 (0.999-1.003)	1.032 (0.978-1.089)	0.26
Larger lesions (>15cm)				
Age	0.006	1.004 (0.992-1.016)		0.536
Gender	0.211	1.462 (0.967-2.208)		0.071
Time delay	0.001	1.000 (0.999-1.001)	0.980 (0.945-1.017)	0.925
WHO category 3				
Age	0.006	1.003 (0.991-1.015)		0.652
Gender	0.203	1.429 (0.960-2.128)		0.079
Time delay	0.001	1.001 (1.000-1.002)	1.020 (0.961-1.035)	0.247

[a] time delay until seeking medical care; odds ratio for days and months

Table 6. Multivariate analysis of the effect of time-delay on severe BU forms in 476 laboratory-confirmed BU treated patients at CDTUB - Allada from 2005 to 2013 (linear model regression).

	B and 95%CI	p-value
Lesion Size		
Age	0.001 (-0.070-0.072)	0.977
Gender	1.369 (-0.867-3.605)	0.229
Time delay [a]	(-)0.002 (-0.009-0.005)	0.598
Category 1, 2 and 3 (WHO)		
Age	0.001 (-0.002-0.003)	0.657
Gender	0.079 (-0.009-0.166)	0.079
Time delay	0.0001 (0.0001-0.0001)	0.244

[a] time delay until seeking medical care; odds ratio for days and months

DISCUSSION

BU pathogenesis is related with necrosis of the subcutaneous tissue associated with mycolactone, the potent cytotoxic/immunosuppressive toxin produced by *M. ulcerans* [3]. Initial pre-ulcerative lesions (papules, nodules, plaques and edematous infiltration) can evolve into ulcers and progressively spread over significant extensions of the body [9] or even affect the bone [10]. Large national studies in West African countries, namely Ghana [31], Benin [28, 29, 32] and Côte D`Ivoire [33], included the largest BU cohorts studied thus far and provided information about the age and gender of patients, site of lesions and the major clinical forms – providing further clues on the evolution of BU pathology. The majority of these studies used distinct methodologies (retrospective and/or prospective cohorts; cross-sectional) and a descriptive approach, with a large proportion of diagnoses being retrospective and scar-based. Here, we strictly consider laboratory-confirmed BU patients.

Concerning the BU clinical forms (papules, nodules, plaques, edematous infiltration, ulcers and osteomyelitis), the observations of our study globally fit the variances reported in those larger cohorts. Specifically, we confirm that BU is mainly a paediatric disease (median age of diagnosis 12 years with IQR: 7—24 years and mean of 19.7 years); with a predominance of lesions on the lower limbs (53.8%); a predominance of ulcerative forms (67.2%); and with an equilibrium between genders. In addition, there is a distinct distribution of gender when age is considered, with males being overrepresented in younger patients, reproducing data from previous studies [15, 29].

Osteomyelitis and edematous forms are classified as belonging to the spectrum of BU presentations, although some authors consider them to be more severe clinical forms [32, 34-36]. Regarding osteomyelitis, a great variance in prevalence is described and further complexity is added when suspected non-confirmed cases of bone involvement are included in the analysis. Indeed, reported prevalence values of bone disease related to BU were as high as 29.5% [37] and 36.1% [38]. However, when only confirmed osteomyelitis cases were considered, prevalence decreased with values ranging between 6% [29] and 20% [39] in Africa and only 1% in Australia [40]. Moreover, HIV infection seems to favour the occurrence of osteomyelitis [17]. In the present study, osteomyelitis lesions only occurred in 1.1% of the at-risk population, which could be related to the fulfillment of confirmed diagnosis criteria (e.g. x-ray or surgical evidence) and the absence of the HIV co-infection selection criteria.

Edematous lesions manifest as diffuse, extensive, usually non-pitting swelling with ill-defined margins involving part or all of a limb or other part of the body [41]. Cases of edematous *M. ulcerans* infection can be misdiagnosed as bacterial cellulitis leading to delays in diagnosis, progression of disease, increased morbidity and increased complexity and cost of treatment. Additionally, edema is often self-perceived as not being a relevant health problem, therefore delaying seeking medical attention. In previous studies, prevalence was determined to be between 2.5% [42] and 12.5% [31]. In our study, edematous forms accounted for 5% of the studied population, fitting with the prevalence reported in similar cohorts [31, 34-36, 42].

Within to the above described clinical BU presentations, more aggressive, severe clinical presentations have been described [11], although the underlying pathological mechanisms are yet unclear [29]. In our study, within the severe phenotypes, 33.8% of the patients were in WHO category 3; 4.5% presented multifocal forms; and 29.8% of the patients presented lesions >15cm in major diameter. Regarding multifocal lesions, previous studies describe highly variable prevalences (e.g. 2.0% -11.1%) [40, 42-46]. Moreover, in our African cohort, we verify that age does not associate with multifocal lesions, conversely to an Australian cohort [40]. Regarding lesion size, only a few studies report large lesions as a specified studied variable, since these lesions are usually included in category 3 lesions. However, when considered separately, their prevalence ranged between 11.1% [47] and 36.0% [29], while category 3 lesions have been reported to range between 19.7% [48] and 60.0% [39] – values replicated in the present study.

The effect of time-delay in seeking medical care for BU patients is a relevant issue for public health and patient management. Our observations in a cohort of laboratory-confirmed cases of BU show that gender was not related with distinct behavior in seeking specific medical care and that younger patients, mainly through their parents/legal tutors, spent less time seeking medical attention prior to diagnosis (median 45 versus 60 days, for the group ≤15 years old versus >15 years old respectively, p=0.004). In line with previous African studies, we found that more advanced ulcerative forms were related to the delay in seeking medical care. Remarkably, and contrary to what one would expect, we found that multifocal lesions, larger lesions or WHO category 3 lesions may be considered distinct clinical entities since the time-delay in seeking medical attention had no significant role in disease progression. As a matter of fact, in Africa, time-delay was seen as a marker of accessibility to medical care and, in fact, some studies compare time-lapse before and after interventional politics on health care

improvement. In West Africa, studies reported a time-delay between 42 [38] and 84 days [44, 49], taking into consideration all clinical forms. Specifically, a Beninese study reported distinct clinical forms relating to time-lapse since first symptoms were remembered [28]. Time-delay was shorter for non-ulcerated clinical forms (median 30 to 46 days), than for ulcerated forms (median 61 days) and larger for osteomyelitis (median 91 days).

In Australian studies, time-lapse until medical care was reported to be much shorter – between 14 days (IQR 0-6 weeks) [40] and 42 days (ranging from 2 and 270 days) [50]. In this distinct health-care reality, determinants for delay in seeking medical care were related to atypical sites of lesions, associated with an increased complexity in medical BU diagnosis. Interestingly, in Australian patients, ulcerated versus non-ulcerated clinical forms did not experience significantly different time lapses. Moreover, independently of the advances in diagnosis and clinical management, there was no variation in time-delay between 1998–2004 and 2005–2011.

In Southern America, the time-delay reported among Peruvian BU patients was between 1 and 8 months [51].

Overall, our observations in a cohort of laboratory-confirmed cases of BU, strengthening previous observations and show that the time-delay in seeking medical care is related to the more advanced ulcerative forms, further justifying early diagnosis and treatment. Notably, we additionally show that time-delay was not significantly associated with more severe phenotypes of BU, such as multifocal lesions, larger lesions or WHO category 3 lesions. Indeed, our results demonstrate that after initial progression lesions become stable regarding size and focal/multifocal progression. Therefore, in future studies on BU epidemiology, severe clinical forms should be systematically considered as distinct phenotypes of the same disease and therefore subjected to specific risk factor investigation. These results further highlight that intrinsic regulatory mechanisms, such as the host immune response and local biochemical and physical factors, most likely have relevant roles in determining severe phenotypes, justifying more structural immune-related and bacterial genetic studies.

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CHAPTER 3

TNFA -308G/A POLYMORPHISM IS ASSOCIATED TO BURULI ULCER SUSCEPTIBILITY

The results presented in this chapter were published:

(i) in conferences

Ana Rita Silva-Gomes, Carlos Capela, João Menino, Ange Dossou, Alexandra G. Fraga, Ghislain Sopoh, Fernando Rodrigues, Jorge Pedrosa, "The Impact of Immune Gene Polymorphisms in Human *Mycobacterium ulceran*s infection", XL Annual Meeting of the Portuguese Society of Immunology (poster presentation).

Carlos Capela, Ana Rita Silva-Gomes, João Menino, Ange Dossou, Alexandra G. Fraga, Ghislain Sopoh, Jorge Pedrosa, Fernando Rodrigues, "Susceptibility to Buruli ulcer: multiple immune-genetic polymorphism dissection through a Benin (Africa) population case-control study", XXXIX Annual Meeting of the Portuguese Society of Immunology (poster presentation).

(ii) in master thesis

Part of the experiments and result analysis were integrated in Ana Rita Silva de Araújo Gomes's master thesis, (http://hdl.handle.net/1822/35110).

ABSTRACT

Epidemiological studies in Buruli ulcer (BU) support the hypothesis that genetic components contribute to both susceptibility and clinical lesions/phenotypes. Moreover, individuals who develop BU have an inherent inability to generate a strong Th1 response to mycobacterial antigens. Thus, the establishment of a bio-bank of BU-focused cohort, comprising a set of patients and controls, offers a significant opportunity for advancing our understanding of the genetic variants that contribute to disease susceptibility and/or clinical presentation.

In this work we built a population based biological bank, composed of 300 healthy individuals and 208 BU patients, living in an endemic BU area in Benin. Patients were clinically characterized and unrelated controls were selected to match age, gender, ethnic background, co-morbidities, geographical origin, and water contact habits, known as individual/behavioral risk factors.

Taking into consideration the relevance of the TNF axis in mycobacterioses, we performed a case-control study in our cohort and used *TNF-α* (*TNFA*) gene as a candidate for polymorphism-screening. We have genotyped the variants rs1800630 (-863 A/C), rs1799724 (-857 T/C), and rs1800629 (-308 G/A) of *TNFA* in our cohort based upon their functional role of on the production levels of this cytokine, as well as their previous association to the susceptibility to other mycobacterioses. We observed that the -308 A allele was associated with increased risk susceptibility to BU (OR=3.98; 95% CI, 1.04–15.2; P=0.03), considering a recessive model of transmission. Our results showed that the analysed SNP in the *TNFA* gene play key role in susceptibility to BU development in Beninese population.

INTRODUCTION

Several cytokines have been shown to be expressed in human BU lesions, throughout the different stages of infection. Among the different cytokines that have been studied, interferon-gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) are known to have important functions in macrophage activation, specifically during other mycobacterial infections [1]. This is of major relevance, taking into account the key role of macrophages in the immune response to intracellular *M. ulcerans* [2]. Indeed, both cytokines have been shown to potentiate the microbicidal mechanisms of *M. ulcerans*-infected macrophages, namely through the induction of nitric oxide by inducible nitric oxide synthase (iNOS) [3], ultimately resulting in the control of *M. ulcerans* proliferation. However, increasing concentrations of mycolactone, the major pathogenic virulent factor associated to *M. ulcerans*, inhibits TNF-α protein production and also impairs IFN-γ-mediated protection In human BU lesions [2], *TNFA* mRNA has been detected [4, 5]. Specifically, high mRNA levels of TNF-α and IFN-γ, as well as the pro-inflammatory cytokines/chemokines IL-12p35, IL-12p40, IL-15, IL-8 and IL-1β, were detected in granulomatous lesions, suggesting that these cytokines could be involved in the containment of *M. ulcerans* infection [5, 6]. On the other hand, non-granulomatous lesions presented a more anti-inflammatory profile [4, 6, 7]. In brief, TNF-α plays a central role in the pathogenesis of BU and is a crucial component of the innate immune response against *M. ulcerans*.

In other mycobacterial diseases, there is evidence that host genetic factors influence the outcome of disease [8]. Albeit the known relevance of the TNF-α axis in the immunological responses to *M. ulcerans* in cellular and animal models [2], the impact of genetic variability in the *TNFA* gene has not been previously explored in the context of human BU. Indeed, there are several polymorphisms within the promoter region of *TNFA* that may impact the expression levels of this cytokine, namely rs1800630 (-863 A/C), rs1799724 (-857 T/C), and rs1800629 (-308 G/A), that have been previously associated to disease. The rs1800629 is associated with tuberculosis, in which the A allele was shown to increase the risk of developing extrapulmonary tuberculosis [9], although this effect varied with ethnicity [10]. Conversely, the A allele of rs1800629 SNP was associated with protection from leprosy [11, 12]. The rs1800630 allele variants have also been shown to be implicated in the susceptibility to pulmonary tuberculosis, being the C allele associated to this disease in a recessive model [13]. Additionally, data have

shown that the C allele of rs1799724 (-857 T/C) associates with increased susceptibility to tuberculosis [13, 14].

Similar to other mycobacterial diseases, several clinical and epidemiological reports support the role of host genetic factors in the development of BU: (i) a considerable number of individuals living in BU endemic areas never develop clinical disease, even after continuous exposure to environmental *M. ulcerans* [15, 16]; (ii) frequencies of antibodies to *M. ulcerans* in serum samples from unaffected endemic individuals were similar to those affected by BU, indicating that many of the control subjects had been exposed to this bacterium but never express disease [17]; (iii) within the infected population, the disease presents a wide spectrum of clinical manifestations with different degrees of severity and stable phenotypes throughout time [18]; (iv) self-healing of BU lesions has been reported to occur among patients [19-23]; and (v) an epidemiological study in Benin reported that history of BU in the family was also a risk factor for developing the disease [24].

Thus, in this work we aimed at building a population based bio-bank of BU patients and corresponding unrelated healthy controls. For this, a collection of DNA from 300 healthy individuals and 208 BU patients was achieved, where both cases and controls were fully clinically characterized. Based on the central role of TNFA in BU pathogenesis, known also from the mice model [2], and its association with susceptibility/resistance to other mycobacterioses, we used *TNFA* as candidate gene for a case-control genetic study. Our data show SNPs within *TNFA* contribute to the risk of disease and its distinct phenotypes.

MATERIAL AND METHODS

Ethics considerations

Ethical approval (Clearance Nu 018, 20/OCT/2011) for the collection and analysis of human blood samples was obtained from the Provisional National Ethical Review Board of the Ministry of Health in Benin, registered under the Number IRB0006860. Before collection of the blood samples, written informed consent was obtained from the subjects or from the responsible for the underage subjects.

Biological Sample Bank

To investigate for genetic associated variability to BU development we constructed a unique bio-bank of biological samples from a BU-focused cohort in Benin, between 2005 and 2013. We established a strong interdisciplinary network involving a team of clinicians from the *Programme National de Lutte contre la Lèpre et l'Ulcère de Buruli* (PNLLUB) with a long-lasting experience in BU diagnosis and patient management. This collaboration allowed the prospective collection of clinical data and biological samples from BU patients at the Centre de Dépistage et de Traitement de l'Ulcère de Buruli (CDTUB) from Lalo and Allada in Benin (Figure 1).

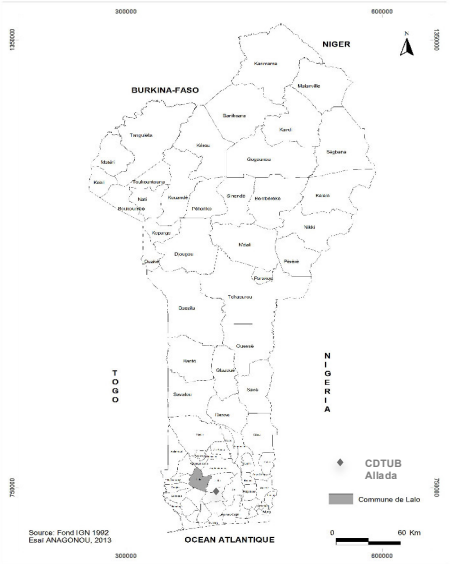

Figure 1. Lalo and Allada CDTUB location, inside Benin (West Africa).

Firstly, to define a precise clinical background for future integrated studies concerning epidemiology, microbiology, immunology, genetics or biochemistry on BU infection, we developed a robust structured clinical inquiry based on the state-of-the-art anamnesis modus operandi. In the first clinical visit, a thorough patient clinical report was done by a medical doctor. The clinical inquiry was created and adapted to Beninese socio–cultural reality, with guidance from local colleagues. At the moment of diagnosis, parameters such as age, gender, ethnic background, co-morbidities, geographical origin, and water contact habits were registered. A complete physical examination was carried out, during which the major clinical form (nodule, plaque, oedema, ulcer or osteomyelitis) and/or multifocal presentations were recorded. For mixed clinical forms, the most severe lesion was considered the major clinical form. Additionally, lesion size (cm, considering major diameter); WHO category (Category 1: maximum lesion diameter <5cm, Category 2: maximum lesion diameter 5–15cm, and Category 3: minimum lesion diameter >15cm associated or not with osteomyelitis and/or multifocal lesions and/or at a critical site); and lesion site (upper or lower limb, trunk, head and/or neck) were taken into consideration. This rigorous description of lesions was complemented with digital photographic support.

Laboratorial confirmation of BU was carried out by culture of *M. ulcerans* from the lesion, histopathology with the presence of acid-fast bacilli, and/or highly specific IS*2404* real-time polymerase chain reaction (PCR). The HIV status was also taken into consideration for the present study. All included patients completed anti-bacillary treatment according to the WHO recommendations, which was complemented with surgical procedures, when necessary (WHO, 2012).

During the first medical examination blood (quantity 10 cc) for DNA was stored by standard guidelines in the proximal facility (Allada Center or Lalo Center, Benin) and then duplicated at the Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Portugal.

For the control group, healthy, unrelated, age and gender matched individuals with the same exposure/living/habits conditions as the selected patients with BU were recruited. A thorough clinical interview was applied to randomized selected neighbours related to index patients included into our study. An annual follow up of the selected control individuals was performed in order to guarantee that they did not to develop disease. Blood samples were collected for DNA isolation.

Medical centre capacity, DNA extraction, storage and shipping of samples (blood)

CDTUB from Allada is a modernized medical and research centre with full capacity for diagnosis, treatment (medical and surgery) and physical rehabilitation for BU patients. Laboratory facilities are also fully equipped for processing samples, performing diagnostic testing and storing samples at a wide range of temperatures, both from Lalo and Allada Center. All blood samples collected from patients and controls were subjected DNA extraction at these facilities, using the NZY Blood gDNA Isolation kit (NZYTech) according to the manufacturer's instructions. Briefly, the method provided by this kit consists in a lysis step, followed by DNA extraction and purification using a silica-based column. Shipping samples to Portugal was performed according international rules with specific mandatory biologic risk identification and health service authority's agreement.

Samples genotyping

Genomic DNA from whole blood samples from patients and donors was isolated using the NZY Blood gDNA Isolation kit (NZYTech) according to the manufacturer's instructions. SNPs were selected based on previous published evidence of association with susceptibility to other mycobacterial diseases, with a particular emphasis on genetic variants with well-described functional consequences. Genotyping of *TNFA* (rs1800629, rs1800630, and rs1799724) SNPs was performed using the KASPar genotyping chemistry (LGC Genomics, UK) following the manufacturer's instructions.

Statistical analysis

A database of the samples containing the age, gender, characterization of the participants as BU patients or controls, lesion type (plaque, oedema, nodule, ulcer) and category (WHO criteria, categories 1, 2 or 3), atypical location of the lesion (e.g. face, thorax, abdomen, osteomyelitis), presence/absence of multiple lesions and genotype for all the tested SNPs was constructed using the IBM® SPSS® Statistics software, version 22.

A Kolmogorov-Smirnov test was applied to check for normality of the sample distribution on the variable "age", since the sample size was higher than 50. Given that data were not normally distributed, the Mann-Whitney U non-parametric test was used in the bivariate analysis of independency between the variables "age" (continuous variable) and " BU

or control'' (categorical variable). In the bivariate analysis of independency between the two categorical variables gender and BU or control the Pearson's $\chi 2$ test was used.

Goodness-of-fit x2 test was used to test for each SNP in control subjects to meet the Hardy–Weinberg equilibrium (HWE) or a Fisher's Exact test, if the expected counts for one of the genotypes is lower or equal to 5. The genotype frequencies were used to test the association of the selected SNPs with BU resistance or susceptibility, through the use of online software from the Institute of Human Genetics (Munich, Germany) (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Thus, the association of the SNPs with BU is evaluated using a Pearson's $\chi 2$ test with a 95% confidence level and can calculate the $\chi 2$ statistics and the Odds ratio (OR), the different genetic models were tested (50). Taking as an example the risk for the mutant allele, (i) the first approach considers the allele frequency of controls and cases, (ii) the second considers that risk of disease only occurs when the mutant homozygous genotype is present (recessive model), (iii) the third approach does not differentiate the individuals with one or two copies of the mutant allele (dominant model), and (iv) the last approach is the additive model, in which the genotypes are analyzed through Armitage's trend test, considering an r-fold increased risk for the heterozygous genotype and a 2r-fold increased risk for the mutant homozygous genotype (50). A p value lower or equal to 0.05 was considered significant.

RESULTS AND DISCUSSION

The aim of this study was to establish a bio-bank of BU-focused cohort, of an intensively-phenotyped cohort recruited from the general population across South Benin, as a resource for studying the genetics of BU (Figure 2).

The long term objective of the bio-bank organization was to build a population-based cohort from across BU endemic villages that would allow to study genetic susceptibility to *M. ulcerans*, both for risk stratification as well as for phenotypic presentation of the disease. In line with this main goal, the collaboration between the Portuguese and Beninese group also allowed to conduct a sustained public consultation programme to understand and explain the public reaction to genetics in healthcare, as well as their reaction to participation in research. Ultimately, this multi-institutional collaboration between Benin and Portugal will contribute to the dissemination of knowledge and best practice in human genetics research, allowing the development of a Benin-wide research platform in emerging technologies of health informatics in genetic research and the association of research data to routine healthcare data on an individual basis.

Construction of a Bio-bank of BU-focused cohort

We retrospectively collected clinical data from patients diagnosed and treated in Allada and Lalo (CDTUB) between January 2005 and December 2013 (Figure 1), with a total of 804 BU patients. Of these 804 patients, 704 (77.6%) had at least one of the considered diagnostic tests performed: culture of *M. ulcerans* from the lesion; histopathology of an excised lesion showing a necrotic ulcer with the presence of acid-fast bacilli (AFB) and/or IS*2404* real-time PCR (Table 1).

PCR-based IS*2404* detection is the most widely used method in BU diagnosis, as it is highly sensitive and specific. However, while the diagnosis is obtained reasonably rapid, it is expensive and requires trained personnel with specific equipment. The WHO recommends that PCR confirmation should be performed for at least 70% of the cases reported in any district or country – something accomplished and certified in the Allada medical centre (WHO, 2013). In our cohort (Table 1), IS*2404* was performed in 649 patients (80.7%), with positivity in 430 (66.3% performed) (Table 1).

Table 1. Laboratory confirmatory procedures among 804 selected patients diagnosed and treated at Allada CDTUB between 2005 and 2013 retrieved from Beninese Biological Specimens Bank. (+ = positive result; stain. = staining)

Laboratory procedure	N	% of 804 patients
IS2404 RT-PCR	**649**	**80.7**
Positive result	430	*66.3% of performed*
Culture	**285**	**35.4**
Positive result	41	*14.4% of performed*
Ziehl-Neelsen stain	**677**	**84.2**
Positive result	327	*48.3% of performed*
Any (at least 1)	**704**	**87.6**
Positive result	476	*90.3% PCR +* *68.7% Culture +*

Concerning direct smear examination to detect acid-fast bacilli, it is a costless test that is simple to perform but of low sensitivity [20]. AFB staining was performed in 677 of patients, being positive in 327 (48.3%). Culture on Lowenstein Jensen medium at 32°C is the most discriminatory method [20], but is not very sensitive and takes more than eight weeks, rendering it of little use to clinicians. Nevertheless, culture was universally performed, being positive in 41 patients (5.1%). Finally, histopathological examination is sensitive but expensive and requires a specialized laboratory with well trained personnel and invasive procedures (biopsy). In the present cohort, only residual cases were confirmed by histopathology (2.5%). The remaining cases (12.4%), for which no diagnostic test was performed, were from 2005 and 2006 – shortly after the WHO implementation of consensual diagnosis and treatment guidelines for BU (WHO, 2012).

According to previously published studies, in this cohort BU was confirmed as being a mainly paediatric disease (median age of diagnosis 12 years with IQR 7-28 years and mean 19.76 years) and was equally distributed among both genders (females 48.6%). The majority of BU patients presented ulcerative lesions (66%), while 2.4% had nodules and 22.8% has plaques. Osteomyelitis lesions were fewer than expected in present cohort with only 15 patients presenting bone lesions, representing 1.9% of the total population. These lesions were predominantly found the lower limbs (58.5%). Although there was a preponderance of unifocal

lesions (95.6%), 31.0% of the patients presented WHO category 3 lesions and 25.6% presented lesions larger than 15cm. All patients were treated according to WHO recommendations (WHO, 2012); however lesion reactivation occurred in 21 (2.6%) patients during or after the initial treatment.

For the construction of the biological bank for genetic studies of BU susceptibility, we took into the consideration only the cases that fulfilled the WHO laboratorial diagnostic criteria as defined by two positivity out of tree among histopathology, culture and PCR (WHO, 2015; Figure 2). As such, a total of 476 patients were selected for further studies, representing 59.2% of the total number of recruited patients. Of these 476 laboratory-confirmed patients, 208 were from the Zé District. Taking into consideration that the Zé District is a highly endemic BU region with low consanguinity rates and uniform ethnicity (*Adja ethnic group*) as previously described [24], besides presenting low prevalence of HIV [24], these 208 individuals were selected for inclusion in the bio-bank. An additional 300 unrelated, age and gender matched individuals living in the same Zé District, with the same water contact habits and ethnicity, were selected as controls.

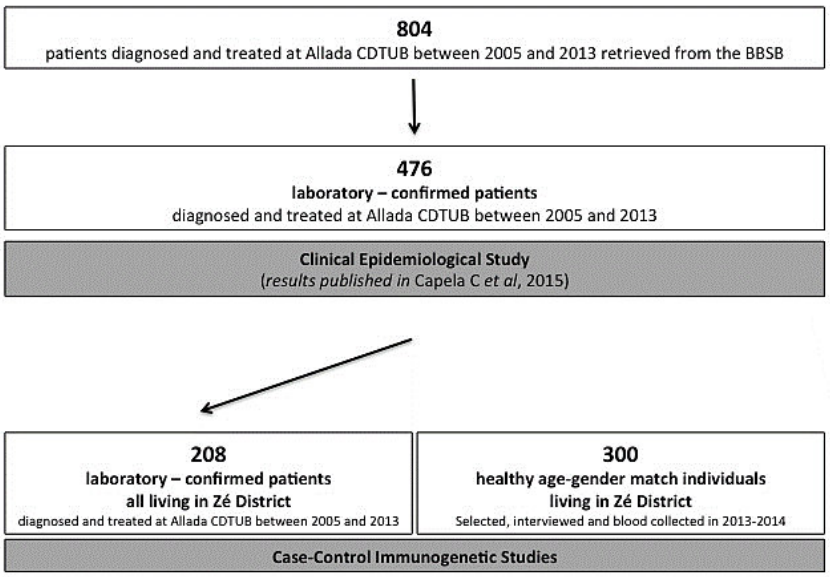

Figure 2. Algorithm for cohort selection to clinical and immunogenetic studies retrieved from Beninese Biological Specimens Bank.

The 208 BU cases were composed by 119 males (57.2%) and 89 females (42.8%), with a mean age of 22 (±18) years old, while the 300 controls were composed by 152 males (51.2%) and 145 females (48.8%), with a mean age of 24 (±19) years old. The Kolmogorov-

Smirnov test revealed that the occurrence of BU did not follow a normal distribution ($p<0.001$) using the age as variable. Thus, the Mann-Whitney non-parametric test was performed and the data showed that the age variable across cases and controls was not different ($Z=1.338$, $p=0.181$), confirming that the age was not determining the outcome of infection in our population. The association of gender and the occurrence of BU was also tested through a Pearson's $\chi 2$ test. The data obtained revealed that gender was also not influencing the outcome of infection ($\chi 2=1.79$, $p=0.181$, 1 degree of freedom).

BU cases were further categorized taking into account the distinct phenotypes of disease:

(a) type of lesions (non-ulcerative vs ulcerative lesions),

(b) severity of the lesion (category 1/2 vs category 3 lesions),

(c) number of lesions (single vs multiple lesions).

From the 208 patients, 146 had ulcerative lesions (70.2%), while 62 had non-ulcerative lesions (29.8%). Patients' lesions were also categorized by severity in accordance with the WHO parameters. In five cases there was missing information and therefore they were excluded from further analysis. From the remaining 203 BU cases, 38 had category 1 lesions (18.7%), 107 had category 2 lesions (52.7%) and 58 had category 3 lesions (28.6%). The number of lesions was missing in three patients. From the 200 cases, the majority of the patients presented a single lesion (94.6%), while only 11 patients (5.4%) had multiple lesions. Overall this cohort follows the epidemiological description of BU in other studies [18].

The rs1800630 of *TNFA* polymorphism associates with the risk for BU

The *TNFA* SNPs selected for this study were based on the literature under the criteria of having been previously associated with other mycobacterioses, namely tuberculosis and leprosy [9, 12]. To discard possible differential frequencies for each genetic variation under analysis, resulting from disturbing factors, we evaluated the Hardy-Weinberg equilibrium (Table 3). This rule allows to demonstrate that an allelic frequency remains constant from one generation to the next in the absence of disruptive circumstances, i.e. at equilibrium. The data shows that contrarily to rs1800629 and rs1800630, the rs1799724 is not in Hardy-Weinberg equilibrium in our population, thus further analysis of this SNP was hampered.

Table 2. Hardy-Weinberg equilibrium test results for the control population. The Hardy-Weinberg equilibrium is assessed through a $\chi 2$ test or a Fisher's exact test of the expected *vs* obtained genotype frequencies.

refSNP	Control population		Hardy-Weinberg equilibrium
	Genotype	Individuals (n)	P value
rs1799724	CC	271	0.45
	CT	25	
	TT	4	
rs1800629	GG	232	0.51
	GA	65	
	AA	3	
rs1800630	CC	240	0.45
	CA	58	
	AA	2	

The rs1800629 and rs1800630 were tested for association with BU, through a Pearson's $\chi 2$ test, comparing allele frequencies and genotype frequencies of the patients with the control population (Table 3).

Table 3. Association of receptor encoding *TNFA* gene SNPs with BU. Minor Allele - Association of the mutant allele with BU (comparison with WT allele). Recessive model - Comparison between mutant homozygous and WT homozygous plus heterozygous genotype frequencies. There is association with disease only when two copies of the mutant allele are present. Dominant model - Comparison between mutant homozygous plus heterozygous with WT homozygous genotypes. There is association with disease when one or two copies of the mutant allele are present. Additive model - Genotypes are analyzed through Armitage's trend test. If the heterozygous genotype confers an r-fold risk of disease, the mutant homozygous is associated with the double risk (2r-fold). OR - Odds Ratio; 95% CI - 95% confidence interval for the OR; $\chi 2$ - Chi-squared statistics for the association; P - p value for the association. The association was considered significant when the p value was lower than 0.05; NA - Not available.

	Study group (n)	Genotype n(%)			Minor Allele			Recessive model			Dominant model			Additive model		
					p value	$\chi 2$	OR (95% CI)	p value	$\chi 2$	OR (95% CI)	p value	$\chi 2$	OR (95% CI)	p value	$\chi 2$	OR (95% CI)
TNFA		G/G	G/A	A/A												
rs1800629	Control (300)	232 (77.3)	65 (21.7)	3 (1.0)	0.05	3.94	1.44 (1.00-2.06)	0.03	4.74	3.98 (1.04-15.2)	0.14	2.22	1.36 (0.91-2.04)	0.05	3.84	1.57
	BU patients (207)	148 (71.5)	51 (24.6)	8 (3.9)												
		C/C	C/A	A/A												
rs1800630	Control (300)	240 (80.0)	58 (19.3)	2 (0.7)	0.80	0.06	0.95 (0.63-1.44)	0.71	0.14	1.45 (0.20-10.4)	0.73	0.12	0.92 (0.59-1.45)	0.80	0.06	0.97
	BU patients (208)	169 (81.3)	37 (17.8)	2 (0.9)												

Our data shows that the rs1800630 was not associated to BU susceptibility, while the mutant A allele of rs1800629 was significantly associated with the development of disease ($\chi 2$=3.94, p=0.05), with an estimated 1.44 OR (95% CI=[1.00-2.06]) (Table 3). In addition, data from the genetic model analysis revealed that the susceptibility to *M. ulcerans* infection followed a recessive model ($\chi 2$=4.74, p=0.03, OR=3.98, 95% CI=[1.04-15.18]) and thus only homozygosity for the mutant allele renders an increased susceptibility to *M. ulcerans* infection. None of the analysed *TNFA* SNPs revealed associations with specific phenotypes (ulceration or more severe WHO category 3 and/or multiple lesions) (Supplementary Table 1).

In line with present results, a previous case-control study of tuberculosis in Tunisian patients showed that the A allele was associated with the development of extra-pulmonary tuberculosis [9]. Additionally, one meta-analysis also described that the A allele was related with a higher risk of tuberculosis development in Asians but not in Caucasians, suggesting racial differences may be associated with genetic risk [10]. In contrast, the same allele was associated with protection against leprosy in two different studies, in Nepalese [11] and Brazilian [8] populations. In this case, inclusion of distinct leprosy phenotypes in global

analysis (subjected to distinct *TNFA* expression profiles [25]), besides ethnicity diversity confounded-effect with linkage disequilibrium showing highly polymorphic major histocompatibility complex region on chromosome 6p21.3, is being pointed to explain this differences [11].

Several studies have evaluated the effects of the -308G/A promoter polymorphism on *TNFA* gene transcription and the mechanism underlying these effects [26-31]. While the majority of the studies report that A allele presents higher promoter activity than G allele [26, 28], others state that there is no transcriptional differences between the two alleles or even that the allele differences observed are cell type specific or stimulus specific [32]. Moreover, when the *TNFA* promoter was directly fused with the luciferase gene, differences in the transcriptional activity were only observed when the 3'UTR of *TNFA* gene was present [28, 30], suggesting that 3'UTR region could be interacting with the promoter to drive transcription in an allele dependent manner.

In addition, it seems that the A allele could also induce a chromatin conformation remodelling that leaves the binding site less available for the repressor or for the activator, therefore altering the transcription of the *TNFA* gene [27, 28, 30, 31]. Moreover such regulators, the transcriptional activator (Ets-1) and the transcriptional repressor (GCF2/LRRFIP1), can interact with other molecules to form larger protein complexes with differential affinity to each allele. In fact, the *TNFA* promoter activity is regulated by the formation of an enhanceosome [33, 34] and distinct enhancer complexes are recruited depending on the cell type and stimulus [34]. Interestingly, the enhanceosome generated after stimulation of macrophages with *M. tuberculosis* is composed by the transcription factors Ets-1, ATF-2, c-jun, Sp1 and Egr-1 and the co-activator proteins CBP/p300 [34]. Mutations in the DNA binding sites of some of these molecules were shown to decrease *TNFA* gene expression in a macrophage cell line infected with either *M. avium* or *M. smegmatis* [33].

CONCLUSION

Overall, our findings indicate that a specific genetic variant in the rs1800629 *TNFA* nucleotide alteration is significantly associated with the development of BU, highlighting the significant effect of single genetic factor towards the overall weight of the human immune response to *M. ulcerans*. Nonetheless, further functional analysis of this SNP within *M. ulcerans* infection models is of relevance to unravel its overall effects in immune response.

Ultimately, this study reinforces the applicability of host genomics and validate some of the objectives proposed with the construction of the biological bank. Here we validated the cohort of patients and relative controls in reaching significant association results. Next steps are crucial in fundament this gene in its proper immunological function through cellular stimulating-functional studies using patients histopathology/cells specimens or with knockout animal models.

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SUPPLEMENTARY DATA

Supplementary Table 1 - Association of *TNFA* encoding gene SNPs with BU non-ulcerative and ulcerative, WHO category 3 versus 1+2 type of lesions and multiple versus single lesions. Minor Allele - Association of the mutant allele with BU (comparison with WT allele). Recessive model - Comparison between mutant homozygous and WT homozygous plus heterozygous genotype frequencies. There is association with disease only when two copies of the mutant allele are present. Dominant model - Comparison between mutant homozygous plus heterozygous with WT homozygous genotypes. There is association with disease when one or two copies of the mutant allele are present. Additive model - Genotypes are analyzed through Armitage's trend test. If the heterozygous genotype confers an r-fold risk of disease, the mutant homozygous is associated with the double risk (2r-fold). OR - Odds Ratio; 95% CI - 95% confidence interval for the OR; $\chi 2$ - Chi-squared statistics for the association; P - p value for the association. The association was considered significant when the p value was lower than 0.05; NA - Not available.

	Study group (n)	Genotype n(%)			Minor Allele			Recessive model			Dominant model			Additive model		
					p value	$\chi 2$	OR (95% CI)	p value	$\chi 2$	OR (95% CI)	p value	$\chi 2$	OR (95% CI)	p value	$\chi 2$	OR (95% CI)
TNFA		G/G	G/A	A/A												
rs1800629	Non-ulcerative (62)	43 (69.4)	15 (24.2)	4 (6.4)	0.36	0.84	0.77 (0.44-1.35)	0.21	1.57	0.41 (0.10-1.71)	0.60	0.27	0.84 (0.44-1.62)	0.38	0.77	0.76
	Ulcerative (144)	105 (72.9)	35 (24.3)	4 (2.8)												
	Cat I or II (144)	105 (72.9)	34 (23.6)	5 (3.5)	0.62	0.24	1.16 (0.65-2.06)	0.58	0.31	1.52 (0.35-6.56)	0.75	0.10	1.12 (0.57-2.19)	0.64	0.22	1.17
	Cat III (58)	41 (70.7)	14 (24.1)	3 (5.2)												
	Single (193)	139 (72.0)	46 (23.8)	8 (4.2)	1.01	0.09	0.83 (0.24-2.87)	0.49	0.47	0.95 (0.05-17.5)	0.96	0.00	0.96 (0.25-3.76)	0.77	0.08	0.93
	Multiple (11)	8 (72.7)	3 (27.3)	0 (0.0)												
rs1800630	Non-ulcerative (62)	51 (82.3)	10 (16.1)	1 (1.6)	0.94	0.01	1.03 (0.51-2.09)	0.53	0.39	0.42 (0.03-6.84)	0.81	0.06	1.10 (0.51-2.38)	0.94	0.01	0.99
	Ulcerative (146)	118 (80.8)	27 (18.5)	1 (0.7)												
	Cat I or II (145)	115 (79.3)	28 (19.3)	2 (1.4)	0.21	1.60	0.60 (0.27-1.34)	0.37	0.81	0.49 (0.02-10.4)	0.26	1.30	0.61 (0.26-1.43)	0.21	1.59	0.62
	Cat III (58)	50 (86.2)	8 (13.8)	0 (0.0)												
	Single (194)	156 (80.4)	36 (18.6)	2 (1.0)	0.20	2.51	019 (0.01-3.21)	0.74	0.11	3.35 (0.15-73.9)	0.10	2.64	0.18 (0.01-3.07)	0.11	2.51	0.84
	Multiple (11)	11 (100)	0 (0.0)	0 (0.0)												

Supplementary Table 2 - Genotype and allele frequencies for rs1800629 SNPs in the HapMap population YRI from Nigeria and for controls and cases of the Benin population in study. YRI - Yoruba in Ibadan, Nigeria; Benin Controls - control population in study from Zé District, Benin; Benin Patients - BU patients population in study from Zé District, Benin. (*Information obtained from Phase II+III of HapMap released in Feb. 2009*)

	TNFA (rs1800629)				
	Genotype (freq)			Allele (freq)	
Populations	**GG**	**GA**	**AA**	**G**	**A**
YRI	0.832	0.159	0.009	0.912	0.088
Benin Controls	0.773	0.217	0.010	0.882	0.118
Benin Patients	0.715	0.246	0.039	0.838	0.162

CHAPTER 4

GENETIC VARIATION IN AUTOPHAGY-RELATED GENES INFLUENCES THE RISK AND PHENOTYPE OF BURULI ULCER

The results presented in this chapter were published:

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ABSTRACT

Buruli ulcer (BU) is a severe necrotizing human skin disease caused by *Mycobacterium ulcerans*. Clinically, presentation is a sum of these diverse pathogenic hits subjected to critical immune-regulatory mechanisms. Among them, autophagy has been demonstrated as a cellular process of critical importance. Since microtubules and dynein are affected by mycolactone, the critical pathogenic exotoxin produced by *M. ulcerans,* cytoskeleton-related changes might potentially impair the autophagic process and impact the risk and progression of infection. Genetic variants in the autophagy-related genes *NOD2*, *PARK2* and *ATG16L1* have been associated with susceptibility to mycobacterial diseases. Here, we investigated their association with BU risk, its severe phenotypes and its progression to an ulcerative form. Genetic variants were genotyped using KASPar chemistry in 208 BU patients (70.2% with an ulcerative form and 28% in severe WHO category 3 phenotype) and 300 healthy endemic controls. The rs1333955 SNP in *PARK2* was significantly associated with increased susceptibility to BU [odds ratio (OR), 1.43; P=0.05]. In addition, both the rs9302752 and rs2066842 SNPs in *NOD2* gee significantly increased the predisposition of patients to develop category 3 (OR, 2.23; P=0.02; and OR 12.7; P=0.03, respectively, whereas the rs2241880 SNP in *ATG16L1* was found to significantly protect patients from presenting the ulcer phenotype (OR, 0.35; P=0.02). Our findings indicate that specific genetic variants in autophagy-related genes influence susceptibility to the development of BU and its progression to severe phenotypes.

AUTHOR SUMMARY

Buruli ulcer (BU) is a neglected tropical disease caused by *Mycobacterium ulcerans*. Because the exact trigger is still under investigation, current treatment options rely mostly on the surgical excision of the affected site. There is therefore a pressing demand for improved risk prediction and tailored treatment as well as for new drug targets. By resorting to the largest case-control study reported to date, we show that genetic variation in the autophagy-related genes *NOD2*, *PARK2* and *ATG16L1* influence the risk and course of BU disease. Thus, our results provide crucial insights into the role of autophagy in the pathogenesis of BU.

INTRODUCTION

Buruli ulcer (BU) is a severe necrotizing human skin disease caused by *Mycobacterium ulcerans*, representing the third most common mycobacteriosis worldwide [1]. At least 33 countries from Africa, South America and Western Pacific, with tropical, subtropical and temperate climates, have reported BU [1]. Moreover, in 2014, 2200 new cases were reported in 12 of those 33 countries [1]. BU initiates as a small, painless, raised skin papule, nodule, plaque or oedema. Later, destruction of the subcutaneous adipose tissue leads to collapse of the epidermis and formation of a characteristic ulcer with undermined edges [1]. Advanced lesions display massive tissue destruction induced by the action of the exotoxin mycolactone, a potent cytotoxic and immunosuppressive polyketide-derived macrolide released by *M. ulcerans* [2]. Clinically, presentation is a sum of these diverse pathogenic hits subjected to critical, mainly local, immune-regulatory mechanisms [3].

Among the many immunological mechanisms defining susceptibility to infection and its progression, autophagy has been demonstrated as a cellular process of critical importance to immunity to viral, bacterial and protozoan infections [4]. Autophagy is a regulated process contributing to the innate control of intracellular pathogens by triggering the autodigestion of cytoplasmic components and driving pathogen clearance. Autophagy is known to be dependent on microtubule cytoskeleton and dynein-driven transport, with dynein playing a role in the delivery of autophagosome contents to lysosomes during autophagosome-lysosome fusion [4]. Since microtubules and dynein are affected by mycolactone [5], cytoskeleton-related changes might potentially impair the autophagic process and impact the risk and progression of *M. ulcerans* infection.

The function of specific components of the autophagic machinery, namely nucleotide-binding oligomerization domain-containing 2 (NOD2), E3 ubiquitin-protein ligase parkin (PARK2) and autophagy-related protein 16-1 (ATG16L1), has been associated with resistance to several intracellular pathogens, including *M. tuberculosis* [4]. Based on reports linking variants in these genes with defective activation of autophagy as well as our own data (proposing a central role for autophagy in the intracellular control of *M. ulcerans* infection through mycolactone-induced impairment of cytoskeleton-dependent cellular functions [5]), we designed a case-control genetic association study involving 208 prospectively collected cases of BU to dissect

the contribution of selected autophagy-related genes to the risk of disease and its distinct phenotypes.

MATERIALS AND METHODS

Patients and study design.

The study population comprised 508 individuals from Zé District (Atlantique Department, Benin), with 208 newly diagnosed BU patients recruited at the Centre de Dépistage et de Traitement de l'Ulcère de Buruli d'Allada after 2005, and 300 unrelated, age and gender-matched controls, with similar water contact habits and the same ethnic background (healthy endemic controls) [1] (Table 1, results section). This area presents a high incidence of BU, low consanguinity and uniform ethnicity [6]. All the subjects enrolled were HIV-negative and BCG-vaccinated. Collection of patient-level data included age, gender, clinical form, number and location of lesions and World Health Organization (WHO) clinical classification – as a severity cataloguing. All the patients enrolled were diagnosed after 2005, were positive for at least two of the three WHO recommended diagnostic tests, and received appropriate treatment. The National Ethical Review Board of the Ministry of Health in Benin (IRB0006860) provided approval for this study (clearance Nu 018, 20/Oct/2011), and written informed consent was obtained from all adult participants. Parents or guardians provided informed consent on behalf of all child participants.

Genotyping.

Genomic DNA from whole blood samples from patients and donors was isolated using the NZY Blood gDNA Isolation kit (NZYTech) according to the manufacturer's instructions. SNPs were selected based on previous published evidence of association with susceptibility to other mycobacterial diseases (Table S1, results section), with a particular emphasis on genetic variants with well-described functional consequences. Specifically, genetic variants in the multi-step intracellular xenophagy recognition process of mycobacteria through the NOD2-ATG16L1 axis and the complementary parkin-mediated ubiquitination were selected, thereby reinforcing the probability to detect positive associations. Genotyping of *PARK2* (rs1333955, rs1040079,

and rs1514343), *NOD2* (rs13339578, rs2066842, rs4785225, rs9302752, and rs5743278), and *ATG16L1* (rs2241880) SNPs was performed using the KASPar genotyping chemistry (LGC Genomics, UK) following the manufacturer's instructions.

Statistical analysis.

The associations between SNPs and BU was performed using Pearson's $\chi 2$ test providing a value of odds ratio (OR) with a 95% confidence interval (CI) for different genetic models (co-dominant, dominant and recessive). A P value lower or equal to 0.05 was considered significant. The linkage disequilibrium (LD) and Hardy-Weinberg equilibrium (HWE) tests were assessed by using the Haploview 4.2 software. Genotype frequencies were used to phase the haplotype configurations by resorting to the same software.

RESULTS

A total of 208 newly diagnosed cases of BU and 300 unrelated controls were selected according to fulfillment criteria. Demographics and clinical features of cases and age- and gender-matched controls are summarized in Table 1.

Table 1. General characteristics of BU patients and healthy controls.

Variable	BU (N=208)	Controls (N=300)	P value
Age, median (range)	14 (10-25)	17 (11-28)	0.247[a]
Gender, no (%)			
Male	119 (57)	154 (51)	0.206[b]
Female	89 (43)	146 (49)	
Clinical form, no. (%)			
Ulcer (± osteomyelitis)	146 (70.2)	-	-
Plaque	48 (23)	-	
Oedema	12 (6)	-	
Nodule	2 (1)	-	
Site of lesion, no. (%)			
Lower or upper limbs	181 (87)	-	-
Head or trunk	27 (13)	-	
WHO category, no (%)[c]			
1	38 (18)	-	-
2	112 (54)	-	
3	58 (28)	-	

[a] P value is for Pearson's $\chi 2$ test.
[b] P value is for Mann-Whitney U test.
[c] The WHO category of BU lesions was defined according to standard criteria as follows: category 1, maximum lesion diameter <5cm; category 2, maximum lesion diameter 5–15cm; and category 3, minimum lesion diameter >15cm associated or not with osteomyelitis and/or multifocal lesions and/or at a critical site.

The median age of cases was 14 years [interquartile range (IQR): 10-25] and similar to that of controls [17 years (IQR: 11-28)]; P=0.25. The gender distribution of cases and controls was also not significantly different [89 (43%) females in 208 cases; and 146 (49%) females in 300 controls; P=0.21]. Clinical features were in concordance with general African characteristics of BU [1]. The dominant clinical form reported was the ulcer (70.2%, including 6 cases with osteomyelitis), the mainly affected site were the limbs (87%), and the WHO

categories 1 to 3 were displayed in 18.3%, 53.8% and 27.9% of the cases, respectively. The minor allele frequencies and HWE values for all SNPs are shown in Table S1.

Table S1. Description of *NOD2*, *PARKIN2* and *ATG16L1* SNPs evaluated in BU patients and healthy controls.

Gene	SNP rs# number	Chromosome position	Alleles	Gene location	aa change	HapMap MAF	MAF in our study	HWE
NOD2	rs9302752	50719103	C>T	Near gene 5'	-	0.412	0.378	0.55
	rs13339578	50739105	A>G	Intron	-	0.441	0.483	0.83
	rs2066842	50744624	C>T	Missense	P268S	0.009	0.008	1.00
	rs5743278	50745996	C>G	Missense	A725G	0.083	0.053	1.00
	rs4785225	50746546	G>C	Intron	-	0.455	0.484	0.96
PARK2	rs1514343	163213083	G>A	Intron	-	-	0.420	0.60
	rs1333955	163213454	C>T	Intron	-	0.228	0.242	1.00
	rs1040079	163214027	G>A	Intron	-	-	0.470	0.64
ATG16L1	rs2241880	233274722	T>C	Missense	T300A	0.270	0.297	0.10

SNP – single nucleotide polymorphism; aa – amino acid; MAF – minor allele frequency; HWE – Hardy-Weinberg equilibrium. Chromosome positions are from NCBI database, assembly GRCh37.p13. HapMap MAFs were identified in the HapMap-YRI population.

To assess the risk and progression of BU according to *NOD2*, *PARK2* and *ATG16L1* SNPs, we compared their genotype frequencies between BU patients and age- and gender-matched healthy controls. Whereas no significant variations in the distribution of genotypes among cases and controls were observed in the overall test of association, the rs1333955 SNP in the *PARK2* gene was significantly associated with increased susceptibility to BU upon modelling of a dominant mode of inheritance [OR, 1.43 (95% CI, 1.00 – 2.06); P=0.05] (Table 2).

Table 2. Genotype distributions and association test results of SNPs in the *PARK2* gene among BU patients and age- and gender-matched healthy controls.

Gene	SNP rs# Number	Alleles: status [a]	Genotype, n (%) [b]			P value [c]		
			A/A	A/a	a/a	Overall	Recessive model	Dominant model
PARK2	rs1333955	C>T						
		BU	107 (52.2)	83 (40.5)	15 (7.3)	0.113	0.24	0.05
		Controls	177 (61.0)	99 (34.2)	14 (4.8)			
	rs1040079	G>A						
		BU	90 (30.4)	148 (50.0)	58 (19.6)	0.161	0.22	0.07
		Controls	47 (23.2)	107 (52.7)	49 (24.1)			
	rs1514343	G>A						
		BU	70 (33.7)	109 (52.4)	29 (13.9)	0.314	0.13	0.78
		Controls	97 (32.4)	145 (48.5)	57 (19.1)			

[a] The first nucleotide represents the major allele.
[b] Genotypes were defined according to the major (A) and minor (a) alleles at each SNP.
[c] Association tests for the overall association (A/A vs. A/a vs. a/a), and the recessive (A/A + A/a vs. a/a) and dominant (A/A vs. A/a + a/a) genetic models were carried out using Fisher's exact t test.

Of interest, a similar though less significant association was also observed for patients carrying the rs1040079 SNP in the same gene [OR, 1.45 (95% CI, 0.96 – 2.18); P=0.07]. Although the rs1333955 SNP was found to be in strong LD with rs1514343 and rs1040079 (Figure 1), none of the four haplotypes determined was significantly associated with the development of BU (Table S2). No associations with the risk of BU were detected for SNPs in *NOD2* or *ATG16L1* (Table S3).

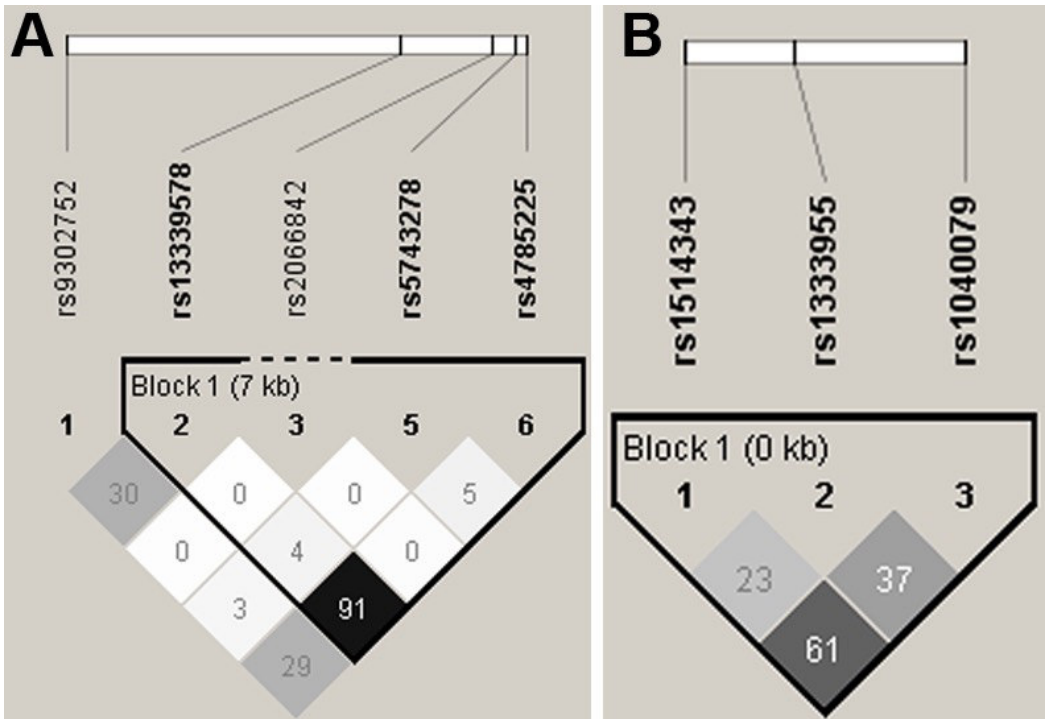

Figure 1. Haploview® pairwise analysis of Linkage Disequilibrium (LD) between NOD2 gene SNPs (A) and PARK2 gene SNPs (B). The r2 colour scheme was used. Measures of r2=0 are represented in white (not significant); 0<r2<1 are represented in shades of grey; r2=1 are represented in black (significant). The numbers inside the box indicate the r2 in a percentage version. An r2 of 0 indicates complete linkage equilibrium, whereas an r2 of 1 indicates complete LD.

Table S2. Haplotype frequencies and association test results in the *PARK2* gene among BU patients and age- and gender-matched healthy controls.

Haplotype[a]	Alleles[b]:status	Frequency (%)	P value[c]
H1	A-C-G		
	BU	0.396	0.29
	Controls	0.430	
H2	G-T-A		
	BU	0.273	0.11
	Controls	0.229	
H3	G-C-A		
	BU	0.224	0.74
	Controls	0.216	
H4	G-C-G		
	BU	0.099	0.22
	Controls	0.123	

[a] Haplotypes with frequencies >0.05 in either BU patients or controls are shown.

[b] Pairwise linkage disequilibrium (LD) blocks were defined using the confidence intervals method. Genotype frequencies of the selected SNPs were used to phase haplotype configuration.

[c] P values were calculated based on approximate chi-square distribution.

Table S3. Genotype distributions and association test results of SNPs in the *NOD2* and *ATG16L1* genes among BU patients and age- and gender-matched healthy controls.

Gene	SNP rs# number	Alleles: status [a]	Genotype, n (%) [b]			P value		
			A/A	A/a	a/a	Overall	Recessive model	Dominant model
NOD2	rs13339578	A>G						
		BU	61 (29.5)	93 (44.9)	53 (25.6)	0.263	0.39	0.30
		Controls	76 (25.3)	157 (52.4)	67 (22.3)			
	rs2066842	C>T						
		BU	204 (99.0)	2 (1.0)	0 (0.0)	0.476	1.00	0.31
		Controls	273 (97.8)	6 (2.0)	0 (0.0)			
	rs4785225	G>C						
		BU	60 (29.0)	93 (44.9)	54 (26.1)	0.128	0.21	0.28
		Controls	74 (24.7)	162 (54.0)	64 (21.3)			
	rs9302752	C>T						
		BU	81 (39.1)	94 (45.4)	32 (15.5)	0.984	0.82	0.94
		Controls	118 (39.5)	137 (45.8)	44 (14.7)			
	rs5743278	C>G						
		BU	188 (90.4)	19 (9.1)	1 (0.5)	0.403	0.23	0.62
		Controls	267 (89.0)	33 (11.0)	0 (0.0)			
ATG16L1	rs2241800	T>C						
		BU	103 (50.0)	84 (40.8)	19 (9.2)	0.144	0.13	0.41
		Controls	138 (46.3)	143 (48.0)	17 (5.7)			

[a] The first nucleotide represents the major allele.

[b] Genotypes were defined according to the major (A) and minor (a) alleles at each SNP.

[c] Association tests for the overall association (A/A vs. A/a vs. a/a), and the recessive (A/A + A/a vs. a/a) and dominant (A/A vs. A/a + a/a) genetic models were carried out using Fisher's exact t test.

In addition, and although the rs13339578 SNP in the *NOD2* gene was in strong LD with both rs5743278 and rs4785225 SNPs (Figure 1), no associations were found for the haplotypes formed by this block (Table S4).

Table S4. Haplotype frequencies and association test results in the *NOD2* gene among BU patients and age- and gender-matched healthy controls.

Haplotype[a]	Alleles[b]:status	Frequency (%)	P value[c]
H1	G-C-C		
	BU	0.481	0.70
	Controls	0.468	
H2	A-C-G		
	BU	0.464	0.61
	Controls	0.448	
H3	A-G-G		
	BU	0.051	0.91
	Controls	0.052	
H4	A-C-C		
	BU	0.005	0.13
	Controls	0.015	

[a] Haplotypes with frequencies >0.05 in either BU patients or controls are shown.

[b] Pairwise linkage disequilibrium (LD) blocks were defined using the confidence intervals method. Genotype frequencies of the selected SNPs were used to phase haplotype configuration.

[c] P values were calculated based on approximate chi-square distribution.

Since the clinical presentation of BU varies dramatically and epidemiological data has pointed out that host genetic factors may be involved in these phenotypes [1], we further evaluated the genetic susceptibility to the severe WHO category 3 or the ulcerative form of BU. We found that both the rs9302752 and rs2066842 (P268S) SNPs in the *NOD2* gene significantly increased the predisposition of patients to develop category 3 lesions following a dominant genetic model [OR, 2.23 (95% CI, 1.14 – 4.37); P=0.02; and OR, 12.7 (95% CI, 0.60 – 269); P=0.03), respectively] (Table 3).

Table 3. Genotype distributions and association test results of SNPs in the *NOD2* and *ATG16L1* genes with the severe WHO category 3 or the ulcerative form of BU disease.

Gene	SNP rs# number	Alleles[a]: status	Genotype, n (%)[b]			P value[c]		
			A/A	A/a	a/a	Over all	Recessive model	Dominant model
NOD2	rs9302752	C>T						
		Cat. 1 or 2	63 (43.8)	60 (41.7)	21 (15.6)	0.05	0.64	0.02
		Cat. 3	15 (25.9)	33 (56.9)	10 (17.2)			
		Non-ulcerative	24 (38.7)	28 (45.2)	10 (16.1)	1.00	0.86	0.94
		Ulcerative	57 (39.3)	66 (45.5)	22 (15.2)			
	rs13339578	A>G						
		Cat. 1 or 2	42 (29.2)	68 (47.2)	34 (23.6)	0.54	0.27	0.82
		Cat. 3	16 (27.6)	24 (41.4)	18 (31.0)			
		Non-ulcerative	17 (27.4)	27 (43.6)	18 (29.0)	0.76	0.46	0.67
		Ulcerative	44 (30.4)	66 (45.5)	35 (24.1)			
	rs2066842	C>T						
		Cat. 1 or 2	143 (100)	0 (0.0)	0 (0.0)	0.08	1.00	0.03
		Cat. 3	56 (96.6)	2 (3.4)	0 (0.0)			
		Non-ulcerative	62 (100)	0 (0.0)	0 (0.0)	1.00	1.00	0.35
		Ulcerative	142 (98.6)	2 (1.4)	0 (0.0)			
	rs5743278	C>G						
		Cat. 1 or 2	131 (90.3)	14 (9.7)	0 (0.0)	0.35	0.11	0.82
		Cat. 3	53 (91.4)	4 (9.1)	1 (1.7)			
		Non-ulcerative	54 (87.1)	7 (11.3)	1 (1.6)	0.21	0.12	0.29
		Ulcerative	134 (91.8)	12 (8.2)	0 (0.0)			
	rs47885225	G>C						
		Cat. 1 or 2	41 (28.5)	68 (47.2)	35 (24.3)	0.60	0.33	0.90
		Cat. 3	16 (27.6)	24 (41.4)	18 (31.0)			
		Non-ulcerative	16 (25.8)	28 (45.2)	18 (29.0)	0.73	0.53	0.51
		Ulcerative	44 (30.3)	65 (44.9)	36 (24.8)			
ATG16L1	rs2241880	T>C						
		Cat. 1 or 2	76 (52.8)	55 (38.2)	13 (9.0)	0.36	0.74	0.17
		Cat. 3	24 (42.1)	27 (47.4)	6 (10.5)			
		Non-ulcerative	27 (43.6)	25 (40.3)	10 (16.1)	0.07	0.02	0.22
		Ulcerative	76 (52.8)	59 (41.0)	9 (6.2)			

[a] The first nucleotide represents the major allele.

[b] Genotypes were defined according to the major (A) and minor (a) alleles at each SNP.

[c] Association tests for the overall association (A/A vs. A/a vs. a/a), and the recessive (A/A + A/a vs. a/a) and dominant (A/A vs. A/a + a/a) genetic models were carried out using Fisher's exact t test.

None of the other SNPs in *NOD2*, *PARK2* or *ATG16L1* revealed association with WHO category 3 (Table S5). In what regards susceptibility to the ulcerative form of BU disease, the rs2241880 (T300A) SNP in the *ATG16L1* gene was found to significantly protect patients from presenting the ulcer phenotype when a recessive genetic model was applied [OR, 0.35 (95% CI, 0.13 – 0.90); P=0.02] (Table 3). None of the other SNPs in *PARK2* or *NOD2* genes revealed associations with the degree of ulceration (Table S5).

Table S5. Genotype distributions and association test results of SNPs in the *PARK2* gene with the severe WHO category 3 or the ulcerative form of BU disease.

Gene	SNP rs# number	Alleles[a]: status	Genotype, n (%)[b]			P value[c]		
			A/A	A/a	a/a	Overall	Recessive model	Dominant model
PARK2	rs1514343	C>T						
		Cat. 1 or 2	47 (32.4)	78 (53.8)	20 (13.8)	0.56	1.00	0.33
		Cat. 3	23 (39.7)	27 (46.5)	8 (13.8)			
		Non-ulcerative	25 (40.3)	30 (48.4)	7 (12.3)	0.40	0.47	0.18
		Ulcerative	45 (30.8)	79 (54.1)	22 (15.1)			
	rs133955	G>A						
		Cat. 1 or 2	78 (54.9)	56 (39.4)	8 (5.6)	0.82	0.12	0.13
		Cat. 3	107 (52.2)	83 (40.5)	15 (7.3)			
		Non-ulcerative	29 (47.6)	26 (42.6)	6 (9.8)	0.54	0.37	0.39
		Ulcerative	78 (54.2)	57 (39.6)	9 (6.2)			
	rs1040079	G>A						
		Cat. 1 or 2	33 (23.6)	74 (52.8)	33 (23.6)	0.82	0.55	0.86
		Cat. 3	13 (22.4)	29 (50.0)	16 (27.6)			
		Non-ulcerative	15 (24.2)	29 (46.8)	18 (29.0)	0.50	0.32	0.80
		Ulcerative	32 (22.5)	78 (55.0)	32 (22.5)			

[a] The first nucleotide represents the major allele.

[b] Genotypes were defined according to the major (A) and minor (a) alleles at each SNP.

[c] Association tests for the overall association (A/A vs. A/a vs. a/a), and the recessive (A/A + A/a vs. a/a) and dominant (A/A vs. A/a + a/a) genetic models were carried out using Fisher's exact t test.

DISCUSSION

We compared the prevalence of SNPs in autophagy-related genes in confirmed cases of BU and in randomly selected community controls equally exposed to similar risk factors such as relationship and same behaviors (recreational or not) related to stagnant waters around villages.

We found that the rs1333955 SNP in the *PARK2* gene was significantly associated with development of BU. The PARK2 protein – known as parkin – is associated with the process of protein ubiquitination, acting as an E3 ligase and targeting proteins for proteasomal degradation [7]. The ubiquitin-mediated pathway is a complementary system for autophagy activation and that contributes to pathogen elimination, including *M. tuberculosis*, by surrounding bacteria with conjugated ubiquitin chains. Our findings support a role for the *PARK2/PACRG* gene cluster in susceptibility to *M. ulcerans* infection, suggesting that mechanisms linked to ubiquitination and proteasome-mediated protein degradation might unveil a common pathway in the intracellular fate of this pathogen. The fact that the same SNP has been associated with a higher risk of leprosy [8] points to a pertinent role for this gene in both infections. In addition, PACRG has been suggested to preferentially bind to hydrophobic molecules, such as lipids [9]. Mycolactone, a lipid mycotoxin, was recently shown to inhibit translocation of newly translated proteins into the endoplasmic reticulum [10], culminating in their degradation by the proteasome. Accordingly, we have recently reported that mycolactone induces an increased amount of ubiquitinated proteins in the cell by affecting cytoskeleton constituents and cytoskeleton-dependent intracellular trafficking [5]. Ultimately, this points to likely critical consequences of the rs1333955 SNP on the proteasomal degradation induced by mycolactone and might explain, at least in part, its association with risk of BU.

Because autophagy is a pivotal immunological mechanism mediating protection to infection by intracellular pathogens [4], mycolactone-induced impairment of autophagy might have implications for the progression of BU disease. Previous studies have revealed that the NOD2-ATG16L1 axis is important for maintaining intracellular immune homeostasis [11]. The rs9302752 and rs2066842 SNPs in the *NOD2* gene were found to be significantly associated with a severe phenotype of BU disease, reflected by the WHO Category 3, suggesting a crucial role of genetic variability of the *NOD2* locus in defining severity of BU disease. The rs9302752 SNP is located in the upstream region of the gene, and therefore it might deregulate promoter

activity and influence gene expression and susceptibility to infection. Indeed, silencing *NOD2* expression in human macrophages was reported to result in a local spread of *M. tuberculosis*, with an impairment in NOD2-mediated production of cytokines [12]. In addition, the rs2066842 SNP underlies the P268S amino acid substitution, and has been found to affect host recognition of bacterial muramyl dipeptide. As such, we hypothesize that a failure in the innate immune recognition of *M. ulcerans* via NOD2 might divert the proper activation of immunological autophagy, therefore permitting progression of infection and development of more severe phenotypes.

The non-ulcerative and ulcerative forms of BU can be observed as stable clinical phenotypes, and not all patients progress to the latter [1]. We found the rs2241880 SNP in the *ATG16L1* gene to be associated with protection of BU patients from an ulcerative clinical form. ATG16L1 is a master regulator of the core autophagy machinery, initially identified as a pivotal risk factor for Crohn's disease [13]. The rs2241880 variant is located in the coding region of *ATG16L1* and leads to the Thr300Ala (T300A) amino acid substitution, which has recently been found to enhance its self-degradation by caspase 3, thereby impairing autophagy activation [14]. Of interest, the T300A variant also decreased selective autophagy, resulting in increased interleukin (IL)-1β signaling and decreased antibacterial defense [14]. Increased levels of IL-1β are also associated with a more exuberant local inflammation. Indeed, non-ulcerative forms of BU such as edema and plaque are considered more inflammatory than ulcerative lesions [15].

Our study has some limitations. In particular, the study was conducted in a single population, and therefore it requires confirmation in larger groups and independent cohorts, as well as the assessment of the functional consequences of the associated variants and their influence to the immune response dynamics. It is however important to note that our case-control study has a robust sample size and the critical advantage that controls were carefully matched to cases regarding environmental exposure to mycobacteria.

Our findings indicate that specific genetic variants in autophagy-related genes influence susceptibility to the development of BU and its progression to severe phenotypes, highlighting the multiple additive effects of single genetic factors and their complex interactions towards the overall weight of the human immune response to *M. ulcerans*. Ultimately, this study reinforces the applicability of host genomics as an important factor to be considered in the stratification of

infection risk in endemic regions and, more importantly, for the definition of patient groups more likely to advance to more severe and debilitating phenotypes of BU disease.

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CHAPTER 5

GENERAL DISCUSSION

Buruli ulcer (BU) is an infectious disease mainly found in tropical regions of Africa, America, Asia, and Australia, with West Africa as the main endemic area. BU is caused by *Mycobacterium ulcerans* and presents itself as a necrotising skin and soft tissue disease, which can progress to bone damage. The causative agent of BU belongs to the same family as those that cause leprosy and tuberculosis (TB), but uniquely, *M. ulcerans* produces an exotoxin, mycolactone, which further contributes to tissue damage. BU can affect all age groups, although it is predominantly seen in children. Published studies on exposure and patient behaviour risk factors for BU development were subjected to an extensive review in the first chapter. Previous to the present work, there was emerging evidence in the literature for a major role of genetic factors in BU development:

(a) not all subjects develop clinically overt disease, even after sustained exposure to *M. ulcerans* in endemic wetlands. In fact, a previous study showed that titres of antibodies against *M. ulcerans* in samples of serum from affected and unaffected subjects were similar [1, 2];

(b) the disease presents a wide spectrum of classical clinical manifestations and their relative frequency in African populations tends to be similar [3];

(c) cases of spontaneous healing have been reported, although the rate at which this outcome occurs is still not known [4-6];

(d) cases tend to cluster within families. Sopoh *et al* (2010) demonstrated for the first time that BU history in the family is associated with occurrence of the disease (odds ratio= 2.89; p=0.004), since not all contemporaneous generations developed BU [7];

(e) molecular studies on genetic variability inducing susceptibility/resistance to the disease are scarce, although one study performed by Stienstra *et al* (2006) demonstrated an association between susceptibility to BU and the D543N polymorphism in the SLC11A1 gene, which encodes the natural resistance-associated macrophage protein (NRAMP1) [8].

These observations, associated to the low bacterial diversity reported among the *M. ulcerans* strains isolated in endemic regions [9, 10], suggest an important contribution of host genetic variability in susceptibility/resistance to BU. Based on this premise, we decided to uncover immune-related genetic variants that can influence the host immune response and therefore contribute to the individual pattern of resistance/susceptibility to BU.

Genetic studies of infectious diseases in African populations present specific challenges. Most reported large-scale genetic studies have been conducted in populations of European ancestry and are not applicable to African populations, besides being characterized by greater diversity and shorter regions of linkage disequilibrium (LD) than other major populations groups [11]. BU poses additional specific challenges in conducting association immune-susceptibility studies, when compared to other infectious diseases, including other mycobacterioses. Firstly, subject recruitment (cases and controls) is difficult mostly because of the need to control similar exposure patterns to a specific environment (e.g. water contact). Secondly, technical difficulties in blood/tissue storage and DNA isolation (in quality and quantity) in the low resource regions where BU is endemic has limited the analysis of candidate regions or genes [8]. Thirdly, detailed BU phenotype definition, as a requirement to focus on gene variability associated to phenotypes [12], has been lacking in previous clinical-epidemiological BU studies.

In recent years, strong evidence has emerged for a host genetic basis associated with the development of TB or leprosy, as well as its subtypes or phenotypes [13]. Regarding leprosy, most individuals develop efficient immunity against *Mycobacterium leprae* with no signs of clinical disease [14] but, in a small proportion of exposed individuals, leprosy can manifest in an array of clinical forms, ranging from the localized tuberculoid to the systemic lepromatous disease, associated with a typically Th1- or Th2-type immune response, respectively [15]. Studies on the immune response against *M. leprae* have been used as a research tool in assisting genetic characterization of leprosy patients, thus allowing the determination of possible associations between, mostly, innate immune-related genes and the development of leprosy and its different clinical forms. A similar concept has been explored for TB and its clinical variants, as described in the first chapter. The latest studies emphasize that severe childhood TB, adult TB and latency/infection TB phenotypes, should be considered subgroups of subjects, defined on the basis of individual (e.g. age at TB onset) or environmental (e.g. pathogen strain) factors, associated to specific human genetic variants controlling the various stages and forms of the disease [16].

Given the lack of precise phenotype definition for BU, based on clinical, epidemiological or pathologic data, we first decided to clarify the definitions of the different BU phenotypes, specifically in what concerns the more severe clinical forms, before proceeding to genetic inter-individual variability studies.

Previous research has classified the different classical presentations of BU, from nodules and oedemas to plaques and ulcers. In addition to the categorization in the classical types referred above, the World Health Organization (WHO) recognizes three levels of BU severity: category 1) lesion diameter <5cm; category 2) lesion diameter 5-15 cm and; category 3) lesion diameter >15cm associated with multi-focal lesions, bone damage and /or at a critical site on the body. However, a clear BU phenotype definition, tackling the possible associations between classical and severe forms and the impact of time delay to seek medical treatment on disease progression, had not been taken into account in the previously published clinical-epidemiological BU studies. In response to the need for a robust definition of BU phenotypes, we conducted the clinical-based study described in Chapter 2, examining records of 476 patients from Benin, West Africa, who had *M. ulcerans* infection confirmed through laboratorial testing. The cohort followed the standard demographics described in other studies, with 51.5% of male patients, 67% of patients under the age of 15 and a median age of disease diagnosis of 12.

In order to explore how BU disease progresses, we studied the relationship between lesion presentation and the time between the first signs/symptoms remembered by the patient and attendance for medical care. Of the 476 patients studied, 32% presented with a non-ulcerated form of skin lesion (nodule, oedema or plaque), with a median time delay between initial symptoms and seeking medical care of 32.5 days (IQR 30-67.5). On the other hand, 67% of BU patients presented ulcerated lesions and 1% showed bone damage, with a median time delay of 60 days (IQR 30-120). This difference was shown to be statistically significant, which supports previous studies suggesting that time is an important factor in disease progression from non-ulcerated to ulcerated BU skin lesions [17]. This finding highlights the importance of early BU diagnosis and treatment.

In this study, presented in Chapter 2, further analysis was carried out regarding the relationship between lesion severity and the time delay in seeking medical care. We observed that 33% of the patients presented WHO category 3 lesions while 66% presented WHO categories 1 and 2. Interestingly, in this case, no statistical difference was seen between the median time delay for WHO category 3 severe lesions (60 days) and category 1 and 2 lesions (60 days) (P = 0.92). The same behaviour was observed restricting the analysis to multi-focal lesions (median 90 days; IQR 56-217.5; P = 0.09) and larger lesions (diameter >15cm) (median 60 days; IQR 30-120; P = 0.92), when compared with unifocal (median 60 days; IQR

30-90) and small lesions (diameter ≤15cm) (median 60 days; IQR 30-90). These observations indicate that rather than lesions becoming progressively more severe, larger lesions or multi-focal lesions present a stable phenotype and therefore should represent a separate phenotype of BU disease.

In conclusion, we showed in this first experimental chapter that time-delay to seek medical attention is not related with more severe BU phenotypes, implying that severe clinical forms should be considered as distinct phenotypes of the same disease and subjected to specific risk factor investigation, including environmental and host-genetic association.

The results presented in Chapter 2 highlight that intrinsic regulatory mechanisms, namely associated with the host immune response and local biochemical/physical factors, likely have relevant roles in determining severe phenotypes, thus justifying more structural immune-related genetic studies. Indeed, the development of disease phenotypes in TB, leprosy and other infections, results from host gene and environmental factors interaction, requiring well-constructed genetic epidemiological studies. With this purpose, we built a population-based BU biological bank, composed of biological samples from clinically characterized healthy endemic controls and subjects with laboratorial confirmation of past or present BU, living in an endemic BU area in Benin (West Africa). HIV co-infection was an obligatory excluding criteria and all included patients completed anti-bacillary treatment according to the WHO recommendations, which was complemented with surgical procedures, when necessary [WHO, 2004]. As described in Chapter 3, the constitution of the cohort and the technical issues related to blood collection and DNA extraction protocols were carefully addressed, minimizing previously identified methodological errors [18].

The SNPs selected for the studies that were undertaken by our laboratory resulted from a comprehensive literature review and from dbSNP. The SNPs were chosen under the criteria of having been previously associated with other mycobacterioses and/or being located within innate and adaptive immune-related genes. A total of 42 SNPs from the genes *DECTIN1*, *TLR1*, *TLR2*, *TLR4*, *TLR6*, *IL4*, *IL10*, *PARK2*, *PACRG*, *NOD2*, *DCSIGN*, *VDR*, *TNFA*, *MBL2*, *CCL5*, *IFNG*, *IFNGR2*, *IL12RB1*, *IL12RB2* and *BCL2L11* were tested for association with risk/protection to BU development, as well as to the development of distinct disease phenotypes. In the context of this thesis, in Chapter 3 and 4, we focused on the most relevant statistical associations: *TNFA* and autophagy-related variant genes.

In Chapter 3, we focused on TNF-α, a relevant mediator involved in the innate branch of the host immune response, known to play a role in controlling *M. ulcerans* in animal models of infection [19] and found in BU lesions [20]. Of the 3 genotyped SNPs (rs1800629, rs1800630 and rs1799724), we only observed an association with the *TNFA* promoter gene polymorphism rs1800629 [-308 $G^{low} \rightarrow A^{high}$]. We observed that the -308 A allele was associated with increased risk susceptibility to BU (OR = 3.98; 95% CI, 1.04–15.2; P = 0.03), when considering a recessive model of transmission. None of the considered SNPs associated with severe phenotypes.

Our results showed that the *TNFA* promoter gene polymorphism rs1800629 plays a key role in susceptibility to BU development, which is line with previous studies in TB. Indeed, rs1800629 was associated with a Tunisian population with TB, since its mutant allele increased the risk of developing extrapulmonary TB [21]. Additionally, a recent meta-analysis reported that this mutant allele is associated with an increased risk of pulmonary TB, but only when analysis was performed according to ethnicity in a Chinese population [22]. The same SNP had a reversed effect (protective) in leprosy, according to two different studies [23, 24], although there is a major bias in including distinct phenotypes known to present distinct *TNFA* expression profiles [25].

The functional significance of the rs1800629 promoter polymorphism on *TNFA* gene expression is still unclear, since multiple functional effects related to the transcription of this polymorphism have been described. While some authors report that the AA genotype presents increased promoter activity than the GG genotype at the -308 locus [26, 27], others describe that there are no transcriptional differences between the two genotypes or even that the differences observed between genotypes are cell-type specific or stimulus-specific [28]. Additionally, chromatin conformational changes in association to the A allele could lead to altered availability of the promoter binding site to a repressor or an activator, altering the transcription of the *TNFA* gene [27-30]. Ultimately, we will need to progress to functional studies using animal or cellular models to substantiate this gene in its proper immunological function during *M. ulcerans* infection.

In Chapter 4, we focused on genetic variants in the autophagy-related genes *NOD2*, *PARK2* and *ATG16L1* and their association, not only with the risk of developing BU and progression to an ulcerative form, but also with severe phenotypes of the disease. Genetic variants were genotyped in our cohort, composed of 208 BU patients (70.2% with an ulcerative

form and 28% with the severe WHO category 3 phenotype) and 300 healthy endemic controls. We report the rs1333955 SNP in *PARK2* to be significantly associated with increased susceptibility to BU [OR 1.43; P = 0.05]. In addition, both the rs9302752 and the rs2066842 SNPs in *NOD2* genes significantly increased the predisposition of patients to develop category 3 (OR, 2.23; P = 0.02; and OR 12.7; P = 0.03, respectively), whereas the rs2241880 SNP in *ATG16L1* was found to significantly protect patients from presenting the ulcer phenotype (OR, 0.35; P = 0.02).

Our findings therefore indicate that specific genetic variants in autophagy-related genes influence susceptibility to the development of BU and its progression to severe phenotypes. It is important to note that, despite the fact that our individual *p* values are at the limit of significance, we are convinced of the strength of our study design. Using a robust sample size with carefully selected matched controls with a strictly controlled exposure to the mycobacteria, we believe that the limited significance of our associations for autophagy-related variants highlights the adding effects of single genetic factors with modest phenotypic consequences and their complex interactions towards the overall weight of the immune response. Actually, it is known that autophagy is a regulated process, contributing to the innate control of intracellular pathogens by triggering the autodigestion of cytoplasmic components and driving pathogen clearance, being dependent on microtubule cytoskeleton and dynein-driven transport, with dynein playing a role in the delivery of autophagosome contents to lysosomes during autophagosome-lysosome fusion [31]. Since microtubules and dynein are affected by mycolactone [32], cytoskeleton-related changes might potentially impair the autophagic process and impact the risk and progression of *M. ulcerans* infection, as we show with gene variants and consequent functional effects. Previously, the function of specific components of the autophagic machinery, namely NOD2, PARK2 and ATG16L1, has been associated with resistance to several intracellular pathogens, including *Mycobacterium tuberculosis* [31]. In Chapter 4, we show that the rs9302752 and rs2066842 SNPs in the *NOD2* gene were found to be significantly associated with a severe phenotype of BU disease (WHO category 3), suggesting a crucial role of genetic variability of the *NOD2* locus in defining severity of BU disease. Furthermore, considering non-ulcerative and ulcerative forms of BU as stable clinical phenotypes, as shown in Chapter 2, we found the rs2241880 SNP in the *ATG16L1* gene to be associated with protection of BU patients from ulcerative clinical forms. The rs2241880 variant is located in the coding region of *ATG16L1* and leads to the Thr300Ala (T300A) amino acid

substitution, which has recently been found to enhance its self-degradation by caspase 3, thereby impairing autophagy activation [33]. The T300A variant also decreased selective autophagy, resulting in increased interleukin (IL)-1β signaling and decreased antibacterial defense [33]. Increased levels of IL-1β are also associated with a more exuberant local inflammation. Indeed, as reviewed in Chapter 1, non-ulcerative forms of BU, such as oedema and plaque, are considered more inflammatory than ulcerative lesions [34].

As shown in this thesis, several genetic variants are responsible for phenotypic changes in the host response to *M. ulcerans* and can contribute for the individual pattern of resistance/susceptibility to BU. These genomic differences can be passed to offspring along with specific marker genes, being inherited as a single unit due to close proximity within a locus. The advantages of family studies for genetic epidemiology have long been established [35], since they not only enrich for genetic loci containing rare variants and provide control for heterogeneity and population stratification, but also allow to better follow the transmission of variants with phenotypes, as well as reveal the effects of parental origin of alleles [36].

To increase the power to express and/or detect rare recessive alleles, early methods in genetic epidemiology used twins, sibling pairs, and other relative pairs to establish the relative recurrence risk of a disease. Additionally, consanguineous marriages and multi-marriages of a single man can enrich for genetic loci containing rare variants, such as those related with primary immunodeficiencies [37]. These marriages are common in Central and West Africa and during our epidemiological studies, we were able to discover three multiplex families resident in Lalo at Couffo Department in Benin, showing high values of recurrence risk ratio, thus reflecting family aggregation of BU and the most probable involvement of host genetic factors. In all families, there is a male element that shows several conjugal unions (Figure 1). The disease only develops in descendants of one of the conjugal unions, revealing a mendelian mode of transmission that can be associated to the presence of homozygotes in the offspring that are not present as such in either parent. As such, the study of these families in the near future can be a powerful tool to identify monogenic susceptibility genetic variants.

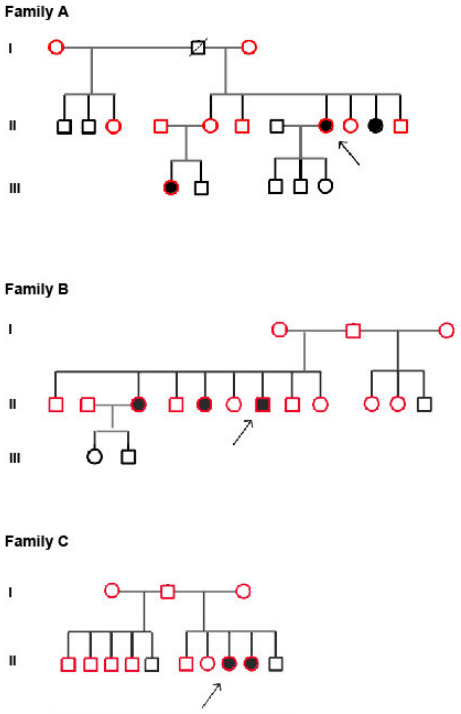

Figure 1. Pedigree of three multiplex families with BU cases (black symbols). Squares represent male family members and circles female family members. Symbols with a cross denote deceased family members. Each generation is designated by a Roman numeral (I, II, III). Individuals selected for blood extraction are in red color. All elements are HIV negative.

Overall, the results presented in this thesis are of primary interest to those working in the field of BU and may have a relevant impact on biomedical and clinical research. Due to the high genetic similarity between *M. ulcerans* and other pathogenic mycobacteria, such as *M. tuberculosis, M. leprae* and *Mycobacterium marinum*, these results could also be explored in other mycobacterial diseases.

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