Epidemiology of *Mycobacterium ulcerans* disease in the Bankim Health District of Cameroon and monitoring of the healing process of Buruli Ulcer lesions

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PD Dr. Maja Weisser und Prof. Dr. Gerd Pluschke

Basel, 8. Dezember 2015

Prof. Dr. Jörg Schibler

Dekan

Dedicated to my parents

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Summary

Buruli ulcer (BU) is a necrotizing skin disease caused by *Mycobacterium ulcerans* which, if untreated, can lead to extensive tissue destruction and ulceration. The disease has been reported from over 30 countries with the highest prevalence in West Africa. Generally it is assumed that *M. ulcerans* is acquired from environmental sources, but BU is considered a "mysterious disease" because the natural reservoir and the mode of transmission are still not identified. Clinically BU presents with a spectrum of forms ranging from non-ulcerative lesions to large ulcers. The gold standard for diagnosing BU is IS2404 qPCR, which is a sophisticated technology not applicable in the field, where BU is often diagnosed on the basis of clinical signs and symptoms only. Direct microscopic smear examination after Ziehl-Neelsen staining, which has a low sensitivity, is the only point-of-care laboratory diagnostic method currently available. Since 2004, the WHO recommends to treat BU with a combination of streptomycin and rifampicin daily for 8 weeks. While this specific treatment is highly effective in killing the bacteria, healing of large ulcers may require long periods of time.

The Bankim Health District (HD) in the Mapé dam basin of Cameroon has been recently identified as BU endemic area and a new BU field research site was established by us in 2010. Within the framework of this thesis, we have contributed to strengthening of the local BU treatment and research site by the implementation of a surveillance and documentation system to promote a continuous case detection and follow up of patients, to investigate the pathway of transmission and to perform a comprehensive spatio-temporal distribution analysis of BU in the area.

Local clinical and microscopic diagnosis was re-confirmed by qPCR, bacterial culture and histopathology performed in Basel. The in-depth analysis on 148 qPCR confirmed cases underlined that BU is a pediatric disease in Africa and that the lesions occur mainly at the limbs with no differences amongst males and females. We obtained information on the exact geographical origin of 136 qPCR positive BU patients through mapping of their houses and farms. Results revealed for the majority of patients residence or agricultural activities close to the Mbam river. Sites of environmental contact of BU patients were screened to search for potential reservoirs of *M. ulcerans.* At one village water site, DNA of *M. ulcerans,* was persistently found over more than 2 years, indicating that the pathogen may persist in detritus.

Because some of the BU lesions healed very fast, while others showed an impaired healing process, we analyzed tissue samples in detail for the presence of wound healing and scarring

biomarkers. Using the histopathological approach, we evaluated the use of markers of cell activation, myofibroblast formation and matrix deposition for the monitoring of the healing of BU lesions. While α-smooth muscle actin-positive myofibroblasts were not found in untreated lesions, they emerged during the healing process. These cells produced abundant extracellular matrix proteins, such as procollagen 1 and tenascin and were found in fibronectin rich areas. After antibiotic treatment many cells, including myofibroblasts, revealed an activated phenotype. Healing wounds showed dermal tissue remodelling by apoptosis, and increased cytokeratin 16 expression in the epidermis.

Taken together, the results described in this thesis were obtained by a multidisciplinary approach. They contribute to our understanding of BU epidemiology and transmission, as well as of pathogenesis, wound healing and may eventually help to improve diagnosis, treatment and prevention of BU.

Chapter 1

Introduction

Buruli ulcer (BU) is a neglected tropical disease (NTD) caused by *Mycobacterium ulcerans,* the third most common mycobacterial infection after tuberculosis and leprosy. It is worldwide present but largely a problem of poor tropical West African countries causing a lot of human suffering. Major efforts and measures to control the disease were initiated by the World Health Organization (WHO) in 1998 with the "Yamoussoukro Declaration on Buruli Ulcer". Over the last 20 years these measures, together with interventions tackling other NTDs, were enhanced and implemented with the objective to intensify research, diagnosis, treatment, management and prevention to control these diseases and to reduce their burden [1].

1.1. History and Epidemiology

BU disease was first discovered by Sir Albert Cook in 1987 in Uganda but the definitive characterization and the isolation of the agent responsible for the disease, occurred only in 1948 by MacCallum, an Australian bacteriologist. He published an article titled "A new mycobacterial infection in man" in which he described 6 Australian cases reporting an unusual skin ulceration with undermined edges and presence of acid fast bacilli in the lesion, (AFBs) [2]. Despite this first report, the name BU originated later as a consequence of the discovery of a large numbers of cases in the Buruli County (Nakasongola Disctirc) in Uganda, during the 1960s [3]. In the same period and in the following years, a significant number of cases were reported from other West and Central African countries including the Democratic Republic of Congo, Nigeria, Gabon, Cameroon and later also from Benin and Ghana [4–8]. Since new cases and foci of BU were progressively found not only in Africa and Australia but also in countries in South America and in South East Asia, in 1998 the WHO initiated the global BU initiative with the aim to disclose this emerging topic and to control and coordinate research activities [9]. Currently BU is reported from over 30 countries all around the world (Figure 1), being a concrete and relevant public health problem [10]. In 2014, 12 out of the 15 countries reporting regularly to WHO, stated around 2200 new cases, more than half of cases reported in 2009 [11]: a part for few countries, the trend of the number of cases seems to progressively decline but the reasons behind are not yet elucidated [12]. Considering the fact that BU is typically a disease of people living in remote areas with limited access to health infrastructures, insufficient data collection and reporting systems and also strong traditional beliefs, the real number of the cases reported is still an issue and it could be anunderestimat [13]. Buruli ulcer disease is characterized by a focal distribution, generally associated with rural wetlands or areas in which stagnant water is present so it seems to be directly related to

environmental factors [14,15]. It can affect individuals of all ages but in African countries it occurs mostly in children and young adults between the age of 5 and 15 and in individuals over 50 years while it is under represented in children below the age of 5 [16–18]. The typical localization of the lesions is on the limbs and particularly the lower ones and no differences between genders are observed in terms of BU incidence [14,18,19].

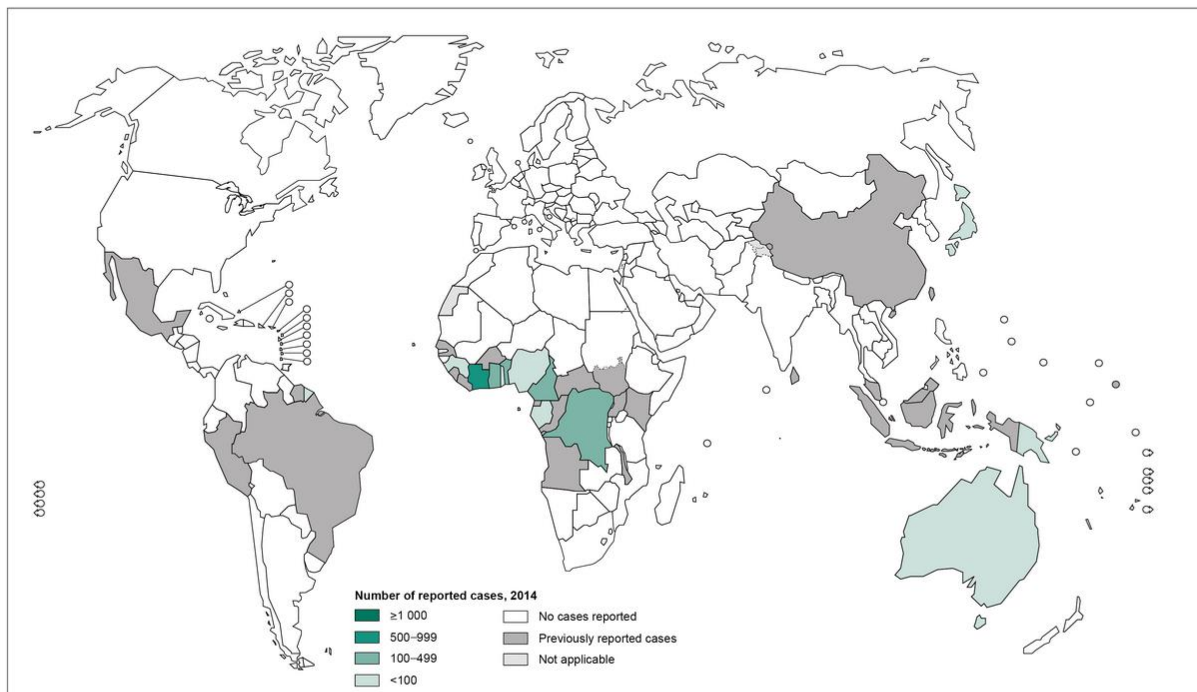

Figure 1: A global map representing the countries which reported case in 2014. Data source, World Health Organization global Health Observatory Map Gallery

1.1.1 BU in Cameroon

In Cameroon BU was described for the first time in 1969, in cases coming from the Nyong River valley, in the Central region of Cameroon close to the villages of Akonolinga and Ayos [20]. After this first description, between the beginning of 2000 and 2009 suspected cases were also reported from the Bankim Health District (HD) in the Adamawa region [21]. Currently in Cameroon there are 3 major endemic foci for BU: Ayos/ Akonolinga/ Ngoantet in the central region of Cameroon, Bankim in the Adamawa region and Mbonge in the south West region (A. Um Book, personal communication). The district of Bankim, is believed to have become a BU hotspot after large alterations of the environment when in 1989 an artificial lake, the Mapé dam basin, was created by damming the Mbar River (A. Um Book, personal communication) [22]. The Bankim HD in the Mapé Dam region of Cameroon in which our activities were carried on from 2010 to 2014, consists of 7 health areas (Atta,

Songkolong, Somié, Nyamboya; Bamkin Rural and Urbain, Bandam) and is bordered by 4 other HDs (Nwa, Malantuen, Mayo Darlé and Yoko). Main geographical features of the area are the artificial Mapé Dam described above and the Mbam River which is creating a natural border between the Bankim HD and the Yoko HD (Figure 2) [18]. In Cameroon, the National BU Control Program (NBUCP) was initiated in 2002 sharing the aim of the GBUI declaration established by the WHO. It was built up in collaboration with the NGOs FAIRMED and MSF Switzerland with the objectives to detect cases early, confirm 70% of clinical BU suspected cases by PCR, treat all active BU cases and heal at least 95% of BU cases in order to reduce the suffering of the affected population (E.T. Njih, Plos NTD under review). The national BU program included the implementation of BU diagnostic and the opening of treatment centers (BU-DTCs) in the main cities and in the periphery to provide adequate infrastructure and trained health personnel in the BU endemic areas.

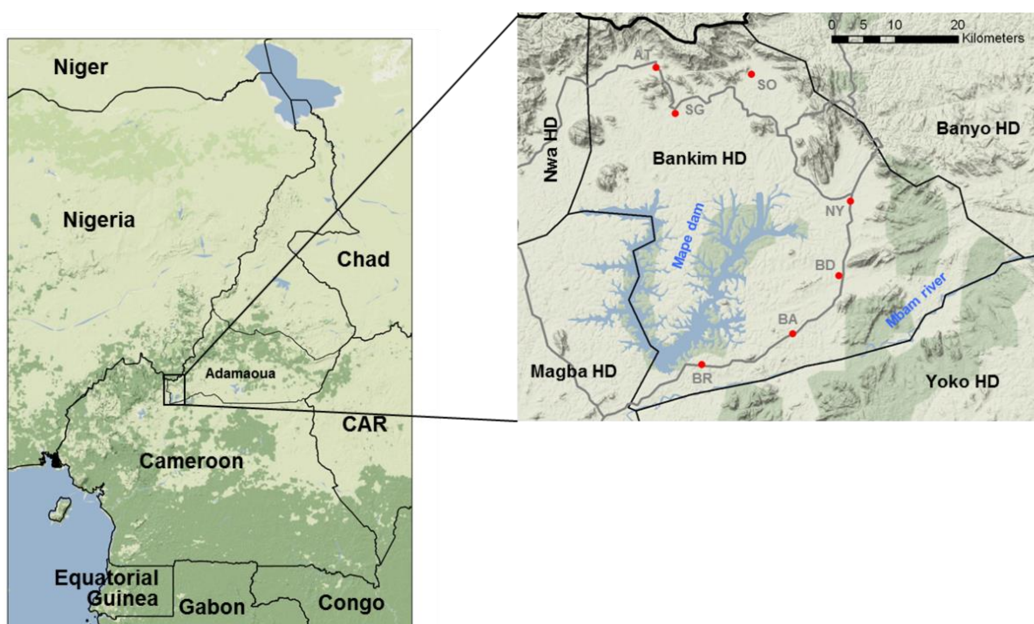

Figure 2: Bankim Health District in the Adamawa region of Cameroon.
Figure curtsy of Martin W. Bratschi.

In the Bankim HD the health infrastructure consists of a public district hospital in Bankim, the reference center for the treatment of BU, and 6 public and 4 private primary health centers with personnel trained to take care of BU patients.

The remote and poor nature of this district contributes to another important characteristic associated to the described area which is the strong traditional and belief component. To seek treatment at traditional healers close to the villages and the use of traditional remedies is

common. Additionally the skeptical approach to the hospital and current chemotherapy or surgery, makes the management of this disease in the described area, particularly challenging (Awah PK personal communicate).

1.2 Causative Agent

1.2.1 Genetic diversity of M. ulcerans

The pathogen *M. ulcerans* is believed to have evolved from the ancient progenitor *M. marinum,* an aquatic pathogen which causes granulomatous lesions in fish and occasionally also skin lesions in humans. Despite the two mycobacteria share more than 97% overall nucleotide identity, *M. ulcerans* is more virulent and causing the human disease Buruli ulcer [23].

The difference between the two species is mainly due to the major changes acquired by *M. ulcerans* during the evolution and the divergence from *M. marinum* [24]. Specifically the genetic differences characterizing *M. ulcerans* are the acquisition of the pMUM001 mega-plasmid of 147 kb encoding for the polyketide synthesis system responsible for the production of mycolactone, which is involved in the pathogenesis of BU disease, as well as acquisition of two insertion sequences (IS) [25,26]. The insertion sequences (IS) *IS2404* and *IS2606* are genetic sequences used to differentiate mycolactone-producing mycobacteria MPMs from *M. marinum* and they are used as targets for the *M. ulcerans* specific polymerase-chain reaction (PCR), the current most sensitive diagnostic tool for BU [27–29]. The acquisition and the expansion of these two sequences have led to extensive pseudogene formation and genome rearrangement [23]. In addition, during the evolution, *M. ulcerans* also has undergone genome reduction and rearrangement by gene deletion suggesting an adaptation to a new ecological niche in the environment, different from the one of its ancestor [19,30].

The discovery of other mycobacteria not associated to BU but also producing mycolactone isolated from fish (*M. solariae*, *M. pseudoshottsii*) and frog (*M. liflandii*), enhanced the hypothesis that they developed from *M. marinum*. Over time, *M. ulcerans* diverged into two different lineages namely “ancestral” and “classical”. *M. ulcerans* isolates from Australia and Africa are included in the “classical” linage while strains from Japan, China and South America represent the “ancestral” linage.

Genetic analyses of different *M. ulcerans* strains causing disease in humans revealed that the pathogen is highly clonal and high resolution typing methods have to be used to differentiate

strains circulating in the same endemic area in order to gain knowledge about spatial-temporal spread of genetic *M. ulcerans* variants [31–33].

1.2.2 Mycolactone

M. ulcerans produces a macrolide exotoxin called mycolactone which is considered to be the key virulence factor of *M. ulcerans* responsible for the majority of the pathology related to BU disease, including the characteristic necrosis of the subcutaneous tissues.

The secreted exotoxin is able to passively diffuse through the membrane of cell inducing necrosis and apoptosis [34,35] additionally it is responsible for the suppression of the host immune response by acting on the adapted cell mediated immunity and as well as the innate immune response. By doing so the progression of the disease is promoted [36,37]. Macrophages, monocytes, B-cells and T-cells are partially blocked via the suppression of interleukins, tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ) [34,38,39].

Different *M. ulcerans* strains produce different closely related forms of mycolactone which display differences in the general structure and have a defined geographical pattern. The chemical variability between the 5 forms (A/B, C, D, E and F) is mainly based on the heterogeneity of the lower side chain which can vary in length, number of double bonds or number and position of the hydroxyl groups, in contrast with the macrolide core and the upper side chain which maintained conserved structure [40,41]. According to these structural differences and the geographical clustering, five different variants of mycolactone have been identified among a worldwide collection of strains; mycolactone A/B is produced by isolates from Africa while isolates from Australia and South Asia respectively produce the C form and the D form of mycolactone. Different forms of mycolactone seem to be associated with different virulence and mycolactone A/B appears to be the most potent [40,42]. In addition to the mycolactones produced by the human *M. ulcerans* phatogen two other variants, E and F, are found to be produced by the fish pathogen (*M. pseudoshottsii*) and the frog pathogen (*M. liflandii*), also called mycolactone producing mycobacteria (MPM) [37]. Recently for research purposes, synthetic mycolactone forms have been produced with the aim to have more insight into the structure-activity relationship [43].

1.3 Natural Reservoir and Transmission

Both, natural reservoir and transmission of *M. ulcerans* are poorly understood and still under investigation. Despite this, it is commonly assumed that endemic foci are associated with wetlands, areas with slow flowing or stagnant water and more recently also with proximity to

rivers indicating human behavior associated with water as a risk factor. Behavioral studies disclosed that living, swimming and working close to water, the failure to wear protective cloths, poor wound care, and agriculture activities are risk factors [19,44–46]. Increased BU incidence has also been associated to human interventions like damming of rivers, deforestation, increased agriculture activities but also to natural events which created unprecedented wetland modifying the environment and causing relevant effects on the organization and function of preexisting ecosystems [19,47,48].

While it is known that other closely related human mycobacteria including *M. tuberculosis* and *M. leprae* are characterized by person to person transmission, for *M. ulcerans* this is still not clear and it is hypothesized that it is acquired through environmental contact [19]. Human to human transmission is exceptionally rare with just one case reported and occurred following a human bite [49]. In addition to the hypothesis of the environmental acquiring, others routes and mechanism of transmissions have been proposed and investigated over the years ranging from aerosol contaminations to vector transmission by amoebae and snails, water bugs or other insects biting human [50–53]. Aquatic networks-interface forming biofilms which create an optimal microhabitat for the bacterium, have also been investigated and it was speculated that they could confer selective advantages [54]. Although the potential of different African aquatic bugs to serve as environmental reservoir has been shown, direct transmission by biting has never been demonstrated. So far DNA of *M. ulcerans* was only detected in African invertebrates which are not hematophagous [19,52,55]. In contrast to Africa, in Australian people have less direct contact with the environment and it is also know that tourists can be at risk, so in these areas, alternative routes of transmission have been investigated. Examples of strong correlation between incidence of BU cases and incidence of vector borne infections transmitted by mosquitos (Ross River Virus / Barmah Forest Virus) have been reported and effectively *M. ulcerans* DNA was identified in mosquitos captured during a BU outbreak in southern Australia [56]. In addition to this, more recently, Fyfe et al. found *M. ulcerans* positive skin lesions and/or feces in possums, small terrestrial marsupial, captured at Point Lonesdale, one of the endemic focus in Australia which might serve as the reservoir [57,58].

Despite the presence of sensitive PCR techniques which are able to detect very small amounts of *M. ulcerans* DNA in the environmental samples, it is so far not possible to determine if viable bacteria are present. Considering these limitations and the few positive samples found in highly endemic BU countries, results of environmental studies are difficult to interpret and they have therefore to be taken with caution [19,29].

In recent times a number of studies in highly endemic communities have been realized, but more studies targeting local populations and their behavior could be an approach to get more insight in the transmission issue. To identify the mode of transmission and the natural reservoir, could lead to the development or implementation of preventive measures to protect and reduce the impact of this disease among the populations at risk.

Figure 3: A typical swampy area in Cameroon where BU is present.

1.4 Pathogenesis

1.4.1 Clinical presentation

BU presents with a spectrum of clinical forms ranging from non-ulcerative stages to the presence of large open ulcers. It is generally assumed that early lesion are represented by the nodular or edematous form which can eventually progress to ulcers characterized by undermined edges and cotton wool like appearance [13,59]. Lesions are usually classified according to the WHO criteria on the basis of the lesion size: Category I (small lesion <5 cm), Category II (5-15 cm of diameter) and Category III (single lesion > 15 cm of diameter, multiple lesions or lesions at critical sites such as eye, breast and genitalia, osteomyelitis) [60]. Unless secondary infection due to other bacteria which can infect the lesion, most ulcers are painless probably as a consequences of the nerve damage caused by the toxin at the site of the lesion [61,62]. Despite the fact that the majority of the lesion are reported to occur at the extremities, any part of the body can be affected [63]. The incubation time can vary between few weeks up to years; patients, especially in Africa, usually present quite late at the hospital or at the health center showing large ulcerations which can lead to serious consequences including a very long stay at the hospital and lifelong disabilities like contractures, scarring and in the most severe cases even amputation [64,65]. Approximately 10% of the reported BU

cases, manifest severe conditions consisting of multiple lesions or the development of osteomyelitis with bone involvement [66,67].

A peculiar phenotypic characteristic of *M. ulcerans* is the very low optimal growth temperature ranging from 28ºC to 30ºC [15]. This restricted growth temperature plays a role in the pathogenesis of the BU disease by limiting the involvement of the tissues to cooler areas [15].

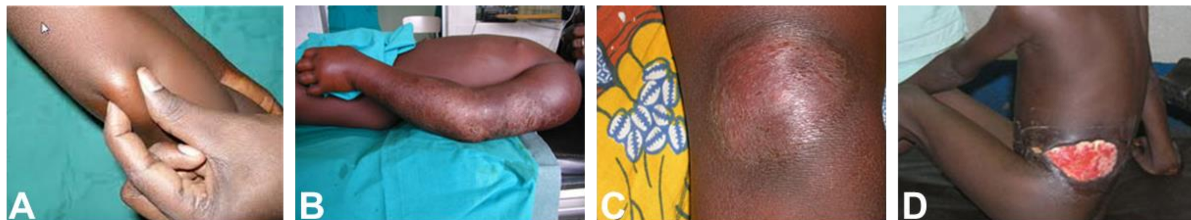

Figure 4: Clinical forms of BU. Typical presentation of a nodule (A), Oedema (B), plaque (C) and ulcer (D). Images are adapted from the WHO web site.

1.4.2 Histopathology and Immunity

Typically, the histopathological features present in the tissue sections from untreated BU patients include an extensive necrotic and edematous subcutaneous area, in which fat cell ghosts and limited infiltration surrounding the remaining blood vessels are present. Epidermal hyperplasia is also characteristic while the dermis appears with relatively intact collagen and reduced infiltration around glands and vessels as shown in Figure 5 [68,69].

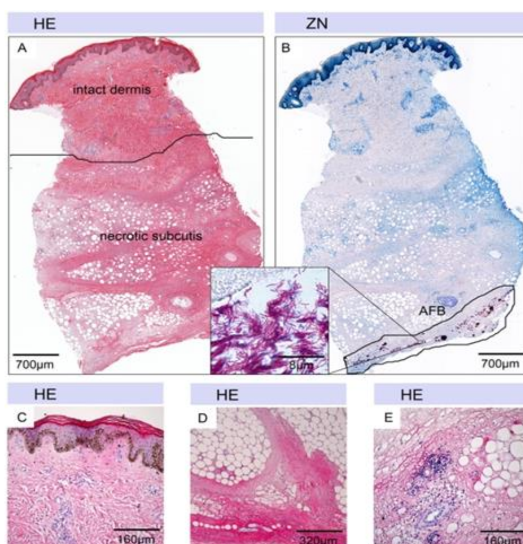

Figure 5: Histopathological feature of untreated BU lesion (Source: Ruf MT et al, 2011, Plos NTD)

The clinical and histopathological features of BU suggest an immunological spectrum of host responses over time. In early stages mycolactone induces death of the tissue and strongly suppresses the immunity of the host. In contrast, in latest stages and during the administration of antibiotic treatment, leukocyte infiltration and granuloma formation can be observed and the host immune system might overcome the action of the toxin allowing the commencement of the healing process (Figure 6).

Bacteria are generally visible in the necrotic subcutaneous area as extracellular clusters, typically focally distributed, but according to this immunological spectrum, as the lesion starts to heal, lymphoid aggregates and granulomatous inflammation develop and they start to destroy bacteria which then can be found intra-cellular also as debris [70–72].

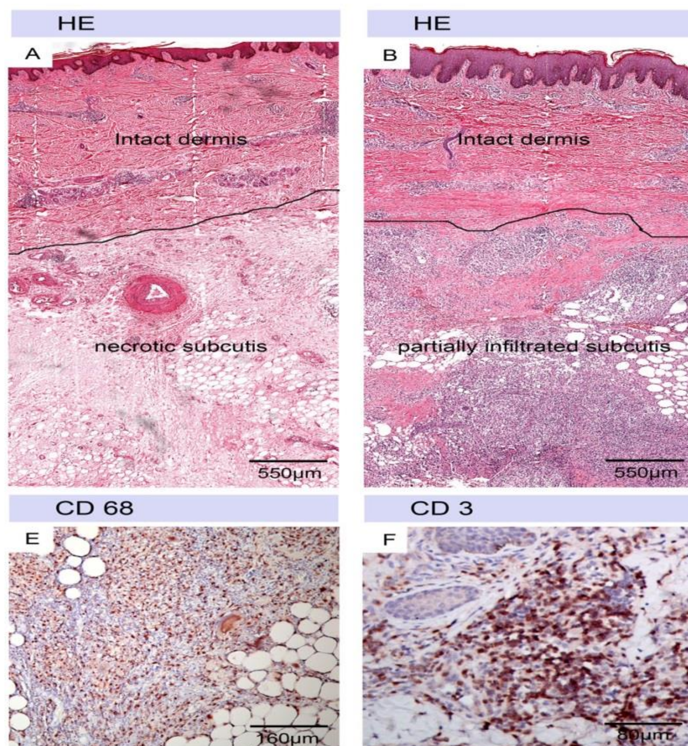

Figure 6: Histopathology of a BU lesion after antibiotic treatment (Source: Ruf MT et al, 2011, Plos NTD)

1.5 Diagnosis

Diagnosis of BU in rural areas is often only based on clinical signs, in order to confirm the clinical suspicion, although WHO strongly recommends to reconfirm the diagnosis by laboratory testing [27,73]. The clinical diagnosis of BU, even if done by trained and experienced health staff, can be complex not only for the ulcerative forms, but even more for the pre-ulcerative forms. Differential diagnosis for BU includes tropical ulcers, necrotizing

fasciitis, diabetic or venous ulcers, yaws and also cutaneous tuberculosis for ulcerative forms, while pyogenic abscess and cellulitis can be confused with nodule and edematous forms [27,74]. BU ulcers display certain specific signs like undermined edges, presence of cotton wool and the absence of fever and pain which can help with the differential diagnosis [75]. According to WHO, cases require two positive laboratory results even if in in presence of high clinical suspicion, one positive test result is enough [76].

In order to achieve reliable laboratory results, specimen sampling is a very important step and different methods are used for different BU forms. Pre-ulcerative lesions are typically sampled by fine-needle aspiration (FNA) while ulcers can be sampled by using a cotton wool swab to swab the undermined edges of the whole lesion. Specimens from both methods can be used for culturing, direct AFB staining and IS2404 PCR. For histopathology punch biopsies or surgical excision are needed [77].

Currently the four laboratory diagnostic methods available for BU are direct smear examination, PCR, histopathology and culture of *M. ulcerans (*Figure 4) [59].

Direct smear examination of swab specimens taken from the undermined edges of the ulcers, is a rapid method where acid fast bacilli (AFB) are detected after staining with Carbol fuchsin (Ziehl-Neelsen Method). Analysis is done with a simple light microscope. It is a simple and cheap method which doesn't require sophisticated equipment making it particularly appropriate to use locally in limited resources countries. However, the sensitivity is quite low (< 60%) hence, the presence of few bacteria can remain undetected and lead to the generation of false negative results. Also specificity is low, due to the fact that there are other mycobacteria which might cause skin lesions and are detected by Ziehl-Neelsen staining [77]. Further, the reliability of the results is strictly dependent to the quality of the sampling performed and the capacity of the laboratory personal to appropriately stain and read the slides.

On the other side, histopathology, culture and PCR require a good laboratory infrastructure, specific and expensive equipment and highly qualified personnel making those three additional methods not applicable in the field but only in reference laboratories.

Histopathology can be performed either on small punch biopsies or on surgical debridement. There are typical histopathological features associated with *M. ulcerans* infection: necrosis of the subcutaneous tissue with appearance of fat ghost cells, edema, epidermal hyperplasia, reduced infiltration and also clusters of extracellular bacteria [69,70]. Because typically bacteria are located deep and very focal in the subcutaneous layer, AFBs may not be present in all histopathological specimens. To obtain an adequate histopathological sample, tissue

needs to consist of all the three skin layers (epidermis, dermis and subcutis), which is one of the major challenges associated with this method. In addition, histopathology is useful to gain deeper insight into the general state of the tissue and also to monitor the course of the treatment studying the local immune response [78].

Similarly, the culture of *M. ulcerans*, is difficult and time-consuming and has a sensitivity between 20-60% according to the laboratory performing the assay [59]. However it is the only method which can distinguish between viable and dead bacteria and can therefore be used to monitor the treatment response and the development of drug resistance [59]. Currently, quantitative real time PCR (qPCR) of *M. ulcerans* DNA based on the amplification of the insertion sequences (IS) IS2404 and IS2606, is considered to be the gold standard method and is largely used in national and international reference laboratories. qPCR is highly sensitive and specific but requires strict and very frequent quality control to ensure the accuracy of the results [79].

Due to limited access to treatment centers and laboratory services, the development of novel point of care diagnostic tests which are easy and cheap to use in remote rural areas to reconfirm the clinical diagnosis, is of high priority. Different strategies to face this need are currently being developed, including the detection of *M. ulcerans* DNA by the loop-mediated isothermal amplification (LAMP) technique, and the detection of *M. ulcerans* antigens by an antigen capture assay [80–82]. The cell wall-associated protein MUL_3720 has been identified as a promising target for an antigen capture assay [83]. A panel of high affinity antibodies against this antigen was generated and a sandwich-ELISA format with a sensitivity rate comparable to that of microscopic detection of acid fast bacilli in smears from clinical specimens was developed. Preliminary results show that this approach has great potential to be developed into a field-compatible point of care diagnostic test for *M. ulcerans* infection (personal communication G. Pluschke).

1.6 Treatment

Until 2004, BU was traditionally treated by wide surgical excision of the lesion including a large margin of healthy skin surrounding the necrotic area to try to reduce the rate of relapses. Generally skin grafting to facilitate the closure of the lesion had been necessary afterwards [13,84,85]. Despite these measures, relapses occurred often (in around 17% of the patients) leading to multiple interventions and long periods of hospitalization and rehabilitation [13]. In response to successful pilot studies evaluating the efficacy of a combined antibiotic regimen

with rifampicin and streptomycin (R/S), in 2004 WHO published a provisional guidance on the role of specific antibiotics in the management of *Mycobacterium ulcerans* disease [86]. Although initial doubts, R/S therapy appears to be effective in the treatment of all forms of BU lesions, achieving almost recurrence-free healing (0-2%) and acceptable levels of side effects [87,88]. Currently the standard antibiotic treatment recommended by WHO, includes the daily administration of oral rifampicin at 10 mg/kg and intramuscular streptomycin at 15 mg/kg for 8 weeks. Despite the efficacy of the antibiotic treatment to kill the pathogen, debridement and skin grafting are often also required particularly for large lesion to speed up and facilitate the healing process achieving the best functional result possible [89]. Because streptomycin is administrated by injection and due to its side effects which are infrequent but indeed present especially in children, efforts are ongoing to establish a fully oral therapy by replacing streptomycin with other molecules. Clarithromycin seems to be an effective alternative and it is for instance successfully used in combination with rifampicin to treat pregnant women [90,91]. In addition, in Australia and French Guyana the antibiotic treatment regime currently used is comprised of oral rifampicin at 10 mg/kg daily and oral clarithromycin at 7.5 mg/kg twice a day for 8 weeks [60,92]. Identification of new compounds active against *M. ulcerans* remain a priority and research activities are strongly encouraged also because rifampicin, the most effective drug against BU, is also one of the first line drugs for the treatment of tuberculosis which might lead to the development of resistances. In all instances, even if the antibiotic treatment is highly effective, wound care which is being recognized to play an important role in BU care, together with physiotherapy is fundamental to prevent disabilities and life-long sequela especially when lesions are located at joints [93,94]. Despite the effectiveness of the treatment, it has been reported that in particular in very remote area, some patients initially prefer to consult traditional healers and use traditional herbal remedies before seeking modern medical treatments. Such practices may lead to worsening of the lesion conditions which can lead to prolonged wound care treatment, delay in healing and increase the possibility of long-term disabilities [95]. Alternative treatments using local application of heat exploiting the temperature sensitivity of *M. ulcerans*, were suggested already during the seventies when water filled heat jackets were used by Meyers *et al*. to treat 8 patients successfully [96]. More recently, in 2009, a proof of concept trial with six BU patients with ulcerative lesions was realized and lesions were treated with bags filled with a phase change material (PCM) for up to 56 days. These bags can reach a temperature of around 40ºC, for several hours [97]and can be applied without the need of electricity onto the lesion. All patients were cured and lesions healed by the application of

heat packs alone. Based on these promising results a Phase II, single center, open label, non-comparative clinical trial with 53 patients was initiated. Results showed the efficacy of the local hyperthermia with a cure rate of around 92% at 6 months after completion of treatment [98].

Although it is generally held that early or uncomplicated BU lesions can spontaneously clear, rare cases of self-healing are reported [89,92].

During or after the antibiotic treatment a temporary worsening of the lesion or/and the appearance of secondary lesions might be experienced. This phenomenon called "paradoxical reaction", is an inflammatory reaction against *M. ulcerans* antigens. Often this worsening is misinterpreted as a recurrence or relapse [99,100].

To date, no specific vaccine against *M. ulcerans* is available and the only vaccine that could potentially have an effect against mycobacterial diseases is the BCG (Bacillus Calmette-Guérin) containing the attenuate bovine tuberculosis bacillus. While it is recognized that BCG causes a cross-protection against leprosy, no clear evidence has been reported about its efficacy to prevent *M. ulcerans* disease [101]. Despite the difficulties due to the unclear nature of the bacterium, the development of a new vaccine seems possible and so active research is ongoing in this area [102].

1.7 Wound healing

One of the major problems associated with BU is the evolution of the lesions which can be very different from one patient to another. Some of the lesions are healing very fast following a normal wound healing process while others require a lot of time leading to several problems included high cost for care and hospitalization and also lifelong sequaela [61,103].

Wound healing *per se* is a complex process, consisting of integrated phases: homeostasis and inflammation, tissue replacement and tissue remodeling which are characterized by inter and intra-cellular level variations [104,105]. Once the clotting cascade occurred, inflammatory cells migrate into the wound bed and promote the inflammatory phase, consisting of infiltrating macrophages, lymphocytes and neutrophils which clean the wound area, release cytokines to induce inflammation and to stimulate fibroblasts, keratinocytes and other components involved in the following phases of the wound healing process [106]. The tissue replacement phase is characterized by epithelial proliferation and migration through a ''temporal'' extracellular matrix (ECM) composed of different proteins including fibronectin, tenascin and pro-collagen which act as a support for the fibroblasts migrating into the wound

bed. Fibroblasts together with endothelial cells support capillary growth and formation of granulation tissue. Myofibroblasts, which are specialized fibroblasts, are the main producers of collagen in healing wounds [107]; by contracting ,using α-smooth muscle actin (αSMA), they promote the shrinkage and the closure of wounds [108]. After the healing is completed, the myofibroblasts are normally eliminated and their persistence in wound granulation tissue is considered pathological [109]. The final remodeling phase of wound healing consists of few modifications including the reduction in the number of newly formed vessels to slowly return to conditions similar to healthy skin tissue [110].

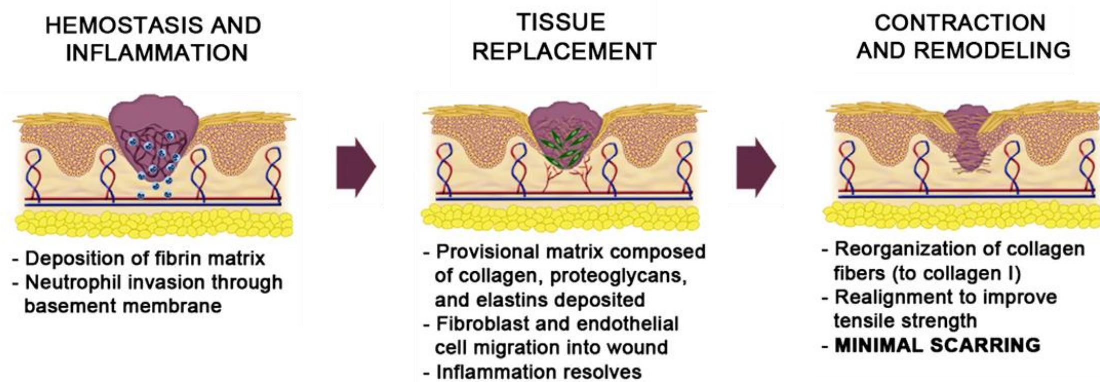

Figure 7: Phase transition in a normal wound healing process (Source: Wells A.et al, 2015, MATBIO)

1.7.1 Wound Healing Markers

In order to investigate the wound healing process in BU and in particular the causes interfering the normal course of the healing process, it is possible to use biological markers recognized to be important in this process which are the following ones.

Cytokeratin 16 is expressed in the epidermis and is a marker for keratinocyte hyper-proliferation which is one of the characteristic histopathological features in BU lesions; it is not present in healthy epidermal skin [111].

Pro-Collagen I is the main component of connective tissue; In normal skin it is found in limited quantities in the dermis and subcutis but during the wound healing process the amount can strongly increase especially in the subcutaneous tissue [112].

Tenascin together with *Fibronectin*, are extracellular matrix proteins which show a patchy distribution in the dermis, in the epidermal adnexa and mesenchymal structures in healthy skin. During the wound healing process Tenascin is upregulated in the vessels and also in the subcutis where increased amounts of Fibronectin are also present [106].

α-*SMA* is a differentiation marker of smooth muscle cells and in healthy normal skin is generally present in the walls of blood vessels. It is commonly used as a marker for the detection of myofibroblasts which show, in healing wound tissues, a strong expression [108,113].

Phosphorylated S6 Ribosomal protein (PS6) is a component of the mTor pathway which is the mammalian target of rapamycin involved in the regulation of many major cellular process including growth, proliferation and death of cells and it is also implicated in pathological conditions. PS6 it is a marker for the activation of the mTor pathway which can be used to detect activated cells in a tissue [114–116].

Cleaved Caspase-3 is a critical executor of apoptosis and it is responsible for the proteolytic cleavage of many key proteins. It can be used to monitor the process of elimination of cells which are no longer required from one to another phase of the wound healing process promoting the tissue regeneration and the wound healing [117,118].

1.8 Skin diseases related to Buruli ulcer

The ulcerated BU lesions, especially the ones which are very big or located at the joints, often present complicated and long wound healing and often require a grafting intervention to heal. During the lesion closure process, it may happen that scar formation results in an uncontrolled development of the tissue which leads to fibroproliferative diseases such as keloids or hypertrophic scars (HTS). These kinds of scarring problems have been documented in the BU disease and they may lead to severe contractures, deformities or thickened skin associated with lifelong disturbances [119].

1.8.1 Keloids and Hypertrophic scars

Keloids and Hypertrophic scars are a fibrous tissue overgrowth that results from an abnormal wound healing process and they generally develop in response to a trauma even if a spontaneous appearance cannot be completely excluded [120]. HTSs are described as elevated scars that do not spread beyond the wound margins and they can often regress naturally over time. They mainly contain type III collagen orientated parallel to the epidermis and organized in filaments. In contrast keloids continue to evolve and they are characterized by a thinned epithelium which can even brake and create focal ulcerations [121]. Keloids are characterized by an excess deposition of ECM proteins, primarily disorganized type I and III collagen and α-SMA in the form of myofibroblasts; in addition increased angiogenesis, overexpression of

cytokines and inflammatory cell infiltration has been observed [120,122]. The mTor pathway is known to be involved in the overproduction of ECM proteins and therefore might be up regulated in other inflammatory fibroproliferative diseases as HTS.

Other diseases not caused by an altered wound healing process but indeed characterized by abnormal growth and presentation of the epidermis are inflammatory skin diseases which include psoriasis and atopic dermatitis (AD).

1.8.2 Psoriasis and Atopic Dermatitis

Psoriasis and atopic dermatitis (AD) are chronic inflammatory skin diseases associated with immunological alterations. Although AD and psoriasis are clinically and pathologically different they share common features like scaly skin and altered epidermal differentiation and both are associated with a high burden of morbidity and stigmatization [123]. Psoriasis is characterized by infiltration of inflammatory cells into the dermis and epidermis with hyperproliferation and abnormal keratinonocyte differentiation accompanied with hyper-activation of the innate immunological cells which lead to a thickened epidermis [124,125]. AD is typically found in children and is also characterized by an inflammatory status of the upper skin layers resulting in incomplete differentiation of the epidermis [126]. While it is known that the mTOR pathway plays a role in the pathogenesis of psoriasis and also that clinical data suggest that mTOR inhibitors provide therapeutic benefit for this disease, less is known about the potential involvement of this important factor in other more general epidermal inflammations like AD [125,127].

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Chapter 2

Goal and Objectives

2.1 Goal

The aim of this thesis was to contribute to the understanding of *M. ulcerans* transmission and its clinical epidemiology through a five-year longitudinal study in the Mapé Basin of Cameroon. We further aimed to monitor the antibiotic treatment outcome for BU lesions using immunohistochemical approaches.

2.2 Objectives

1. To study the presence and distribution of *M. ulcerans* DNA in the environment at sites of contact of laboratory reconfirmed patients
2. To conduct a comprehensive epidemiological and spatial distribution analysis of the evolution of BU over time in the Mapé Basin of Cameroon
3. To develop a protocol for a set of antibodies targeting wound healing markers and to investigate the healing process of BU lesions and in particular the factors affecting the normal evolution of the healing process
4. To histopathologically examine scarring conditions within the wound healing process of confirmed BU lesions

Chapter 3

Mycobacterium ulcerans persistence at a village water source of Buruli ulcer patients

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Abstract

Buruli ulcer (BU), a neglected tropical disease of the skin and subcutaneous tissue, is caused by *Mycobacterium ulcerans* and is the third most common mycobacterial disease after tuberculosis and leprosy. While there is a strong association of the occurrence of the disease with stagnant or slow flowing water bodies, the exact mode of transmission of BU is not clear. *M. ulcerans* has emerged from the environmental fish pathogen *M. marinum* by acquisition of a virulence plasmid encoding the enzymes required for the production of the cytotoxic macrolide toxin mycolactone, which is a key factor in the pathogenesis of BU. Comparative genomic studies have further shown extensive pseudogene formation and downsizing of the *M. ulcerans* genome, indicative for an adaptation to a more stable ecological niche. This has raised the question whether this pathogen is still present in water-associated environmental reservoirs. Here we show persistence of *M. ulcerans* specific DNA sequences over a period of more than two years at a water contact location of BU patients in an endemic village of Cameroon. At defined positions in a shallow water hole used by the villagers for washing and bathing, detritus remained consistently positive for *M. ulcerans* DNA. The observed mean real-time PCR Ct difference of 1.45 between the insertion sequences IS2606 and IS2404 indicated that lineage 3 *M. ulcerans*, which cause human disease, persisted in this environment after successful treatment of all local patients. Underwater decaying organic matter may therefore represent a reservoir of *M. ulcerans* for direct infection of skin lesions or vector-associated transmission.

Summary

Buruli ulcer (BU) is a neglected tropical disease caused by *Mycobacterium ulcerans* which affects mainly children in West Africa. Although it is commonly believed that the infection originates from an environmental source, both the reservoir of *M. ulcerans* and the mode of transmission to human patients remain to be elucidated. Previous investigations indicated that transmission likely takes place away from the homes of patients. We therefore screened the farms as well as village and farm water locations of 46 laboratory confirmed BU patients of the Mapé Basin of Cameroon for the presence of *M. ulcerans* DNA by real-time PCR. In this analysis three positive village water locations were identified. By studying one of these locations in great detail we found that *M. ulcerans* DNA persists in underwater detritus in one section of the village water location even after all local cases had been treated. The detritus may represent a reservoir of *M. ulcerans* from where infection could take place through either direct contamination of skin lesions or through contamination or colonization of insect vectors.

Introduction

Buruli ulcer (BU) is a neglected tropical disease of the skin and subcutaneous tissue caused by the environmental pathogen *Mycobacterium ulcerans*. The disease, which can affect all age groups and both sexes, has been reported in over 30 countries but is most frequent in West Africa. Typically, BU presents with ulcers with undermined edges but clinical manifestations also include nodules, oedema and plaques. Lesions can encompass entire limbs if patients report late for treatment [1]. The WHO recommends that all cases should be laboratory confirmed by microscopy, polymerase chain reaction (PCR), primary culturing or histology [2]. However, because of the limited access to laboratory facilities in BU endemic areas, cases are often diagnosed based only on clinical signs and there is a pressing need for a simple, sensitive and specific point-of-care diagnostic test [3]. Historically, BU was treated using wide scale excision of the lesions. Since 2004, the WHO recommends a combination therapy of daily streptomycin and rifampicin for 8 weeks as the standard treatment for BU [1].

In Africa, the major risk factor for BU is proximity to stagnant or slow flowing water, but other factors such as poor wound care, and failure to wear protective clothing have also been identified in case-control studies [4]. It has further been reported, that man-made modifications of the environment may increase the incidence of BU [4]. Despite relentless efforts, both the reservoir and the exact mode of transmission of BU remain a mystery. Numerous investigations of the environment have attempted to identify the source of the pathogen with so far only limited success. Studies in Ghana and Benin, have examined environmental samples for the presence of the *M. ulcerans* insertion sequence (IS) 2404. Some of these studies have identified many IS2404 positive sites and found positive samples in both BU endemic and non-endemic areas [5]. On the other hand, a study from Ghana has reported that only very few samples were real-time PCR positive [6]. These difficulties to conclusively identify the environmental reservoir of *M. ulcerans* and the fact that investigations on its genome have revealed that the pathogen has undergone substantial niche adaptation [7,8], have led investigators to look for invertebrate or vertebrate animal reservoirs [4,7]. Specifically the role of aquatic insects as potential reservoirs has been evaluated [9,10] and a recent study analyzing transmission networks has found that a specific taxa of aquatic invertebrates may be involved in the transmission of BU [11]. While to date no mammalian reservoir has been detected in Africa, possums have been identified as an animal reservoir of *M. ulcerans* in the southern Australian BU endemic area [12]. The mode of transmission from an animal or environmental reservoir to human patients also remains to be elucidated. Both insect bites, from mosquitos or water bugs, and direct inoculation of bacteria into the skin

from an environmental reservoir after skin trauma have been suspected to be relevant for transmission [4] and several parallel modes of transmission may need to be considered [13].

The objective of the current study was to longitudinally monitor environmental contact water sources of laboratory confirmed BU patients for the persistence of *M. ulcerans* DNA.

Material and Methods

Ethical statement

Approval for this study was obtained from the Cameroon National Ethics Committee (N°041/CNE/DNM/09 and N°172/CNE/SE/2011) and the Ethics Committee of Basel (EKBB, reference no. 53/11). Participation was voluntary and all patients, independent of their study participation, were treated according to national treatment guidelines. All cases who participated in the study or their legal guardian provided written informed consent.

Study area, patient inclusion and patient confirmation

All real-time PCR confirmed cases identified in the Mapé Basin of Cameroon [13] between the beginning of December 2009 and the end of November 2011, were eligible for inclusion in this study. For definitive BU diagnosis, clinical samples were collected, DNA extracted and IS2404 real-time PCR performed as previously described [13–15]. Environmental sampling was performed between February 2011 and June 2013.

The main water bodies of the study area are the Mapé Dam and the Mbam River [13]. The region experiences two rainy seasons, a short one from mid-March to mid-May and a long one from mid-June to the end of September, with the rest of the year being dry.

Selection of environmental sampling locations and sampling procedures

Patients selected for in-depth investigation were interviewed to determine where they lived for the year before the onset of BU. Homes of as many non-participating real-time PCR confirmed cases as possible were also identified and mapped. If participating patients had a home both in their village and at their farm, an interview was used to determine where they spent more time. After achieving an accuracy of less than 10m, a GPS receiver was used to map the patient's home. Together with the patient, a close friend or relative, locations of regular environmental contact of the patient were then visited. The investigated and mapped locations included the patient's farm(s) and the location(s) where she/he obtained water while at home (VW: village water sources) or at the farm(s) (FW: farm water sources). Locations used to obtain water for drinking, cooking, bathing, clothes washing and dish washing were visited. At all locations, soil and plant material was collected. At the water contact locations, a water sample was also collected. Samples collected at the farms were dry soil and plants

growing on dry grounds. Plant and soil samples from the water contact locations, were collected from either in the water, at the water's edge or in the moist area around the water.
Additionally, animal fecal samples were collected in the highly BU endemic village of Mbandji 2. Samples were collected around the homes of laboratory confirmed BU patients and included the feces of chickens, ducks, pigs, goats and sheep.
At two water contact locations located in Mbandji 2 (VW12 and VW13) we performed repeated and in-depth sampling over a period of more than two years (Table S1). In addition to the samples collected at the initial time point (t=0) as described above, samples were collected from VW12 and VW13 at seven additional time points (t=2.1, 4.8, 7.7, 10.5, 15.3, 20.3 and 27.4 months). At the two initial time points, samples were collected from 3 sampling sites at each VW location. At the remaining time points, samples were collected at 21-22 sampling sites around VW12, with 3-5 sample replicates at each sampling site. At the same time points, VW13 was sampled at 14-16 sampling sites with 1-3 sample replicates collected at each sampling site. Details of the sampling sites and the number of replicates collected at each sampling site and at each time point are given in Table S1. All samples, with the exception of those collected at sampling sites 7 and 13, which were plants on dry soil, were collected from inside the water or at the water's edge.
At the last follow-up time point (t=27.4 months), further soil samples from inside the water were collected around the log at location VW12. At each sampling site 3-5 replicates of the same type of sample were collected. At several sampling sites on either side of the log, samples were repeatedly collected in the course of a few days. At sampling site 55, additional samples of various natures were collected.
All environmental samples were stored at 4ºC until analysis.

Environmental DNA extraction and real-time PCR

From the environmental samples, DNA was extracted and real-time PCR performed as previously described [14,15]. Briefly, approximately 200µL of each soil, plant and fecal sample was transferred to a lysing tube and DNA extracted using the Fast DNA Spin Kit for Soil (MP Biomedicals, product number 116560-200) and a Precellys24 homogenizer (Bervet Bertin Technologies). From the plant samples, DNA was extracted from a mixture of leaves and stems and if possible roots. For water samples, 1mL was transferred to a lysing tube, the tubes centrifuged for 10 min (14'000 rpm), the supernatant removed and the samples then processed like the other samples. All samples were at least once extracted by the above

method. Some samples were also processed once by homogenizing them in lysing matrix E tubes in the presence of MT Buffer (MP Biomedicals) and Phosphate Buffered Saline (MP Biomedicals), pelleting debris (10 min at 14'000 rpm) and then extracting DNA from the supernatant with the QIASymphony (Qiagen) and the QIAsymphony DSP Virus/Pathogen Midi Kit (Qiagen, product number 937055). The two different extraction methods yielded comparable DNA quantities, as assessed by real-time PCR, when applied in parallel to positive environmental samples (data not shown). For each extraction, a reagent control was included.

Extracted DNA (1µL of 100µL) was run twice in the IS2404 real-time PCR assays as previously described [14,15]. In the IS2404 real-time PCR, an internal positive control (IPC, Applied Biosystems) was included to detect PCR inhibition. Inhibited samples were diluted 1/5 and 1/10 and analyzed again. In each real-time PCR run both negative and positive controls were included. If a sample was positive in at least one of the IS2404 real-time PCR assays, DNA was extracted from a second aliquot of the same environmental sample. If again at least one of two parallel IS2404 real-time PCR assays was positive, the corresponding environmental sample was considered positive for IS2404. DNA extracted from these samples (1 µL and 5 µL) was then subjected to IS2606 and keto reductase (KR) real-time PCR as previously described [14,15]. If the extracts of a particular sample, were at least once positive for these two additional targets, the sample was considered positive for *M. ulcerans* DNA. All IS2404 positive samples that were not positive for both of the other targets were not considered to contain *M. ulcerans* DNA and were not included in the analysis. Most of the real-time PCR assays were performed in a StepOne Plus Real-Time PCR System (Applied Biosystems) and analyzed using the StepOne Software (v2.2.2; Applied Biosystems). Only samples extracted using the QIASymphony as well as samples analyzed in Cameroon, were real-time PCR tested for the presence of IS2404 by a Mastercycler Realplex 4 ep Gardient S (Eppendorf) and the data analyzed by Mastercycler ep Realplex (version 2.2).

Statistical data analysis

Descriptive statistics were computed using R (The R Foundation for Statistical Computing; version 2.15.1) and RStudio (RStudio, Boston, USA; version 0.95.262). Maps were drawn in ArcGIS ArcMap (Economic and Social Research Institute, Redlands, USA; version 10.0).

Results

Screening of environmental contact locations of BU patients for the presence of *M. ulcerans* DNA

From December 2009 to November 2011, 67 real-time PCR confirmed cases of BU were identified in the Mapé Basin of Cameroon. Of these patients, 46 were selected for in-depth environmental contact analysis based on their origin in the southern part of the Mapé Basin and their availability to participate in the study. The homes and farms as well as the VW and FW locations of the patients were mapped (Figures 1A and 1B). The median direct distance between the homes and farms was 1.5 km (interquartile range = 0.6 km to 5.3 km). While some patients lived permanently at their farm, others travelled more than 15 km to get from their home to their farm (Table 1). As shown in Figures 1A and 1B, many of the BU patients in the southern Mapé Basin moved south and east towards the Mbam River for their farming activities.

Environmental samples (171 soil, 153 plant and 109 water samples) were collected at the farms (n=49), FW (n=43) and VW (n=48) locations shown in Figure 1A. Of the soil and plant samples, 108/171 and 109/153 respectively, were collected in or around water. The remaining samples were collected from dry grounds. All environmental contact locations are numbered in Table 1; locations used by several patients are indicated by the same number. Additionally, pig, goat, sheep, chicken and duck fecal samples (n=24) were collected at 14 sampling sites in the BU endemic village of Mbandji 2 (Figure 1B).

All environmental and fecal samples were tested by real-time PCR for the presence of the *M. ulcerans* specific IS2404 DNA sequence. Three VW locations (VW12, VW31 and VW54) and one duck fecal sample (F07) tested positive (Figures 1A and 1B). At locations VW31 (Figure 1C) and VW54 (Figure 1D), soil samples collected in the moist area around the water wells were positive. Both of these water locations were used by one BU patient each (Table 1). Water from VW31 was reported to be used for bathing and washing of clothing and water from VW54 was used for all purposes including drinking. At location VW12, used by three of the patients living in the village of Mbandji 2, both a soil and a plant sample collected at the water's edge, tested positive. Further details on the results of a longitudinal study at VW12 are provided below.

Persisting real-time PCR positivity of detritus after successful treatment of the identified local BU patients

As shown in Figures 1B and 2A, six BU patients were notified during the study period in Mbandji 2, which is situated between the Mapé Dam and the Mbam River (Figure 1E). The locations of the homes of these patients are shown in Figure 2B and characteristics of the patients, which are not related to each other, are listed in Table 2. Patients 06, 13, and 34, aged 9, 5 and 57, respectively, all used the real-time PCR positive VW12 location (Figure 2B and Table 1). Furthermore, the only positive faecal sample (F07; from a duck) was collected in close proximity of the home of patient 13 (Figure 2B). The other three patients from Mbandji 2 used primarily four other VW locations (Table 2, Figure 2B).

To better characterize IS2404 real-time PCR positivity in Mbandji 2, we performed detailed longitudinal analyses of VW12 and the close-by IS2404 negative location VW13 (Figure 1B and 2B). VW12 was a permanent small water body with a wooden log lying in it (Figure 3A). The water was shallow and flowed slowly from the left to the right when approaching the log from the village of Mbandji 2. For most of the log, the left and right side of the water were not connected under the log; however at some points water could pass underneath the log. On the right side of the log, the vegetation was denser and a layer of detritus was accumulating. In contrast, the compacted ground on the left was not covered with detritus. Location VW12 was used by the local population – including patients 06, 13 and 34 (Table 1 and 2) – to wash clothing and for bathing. For these activities, locals stood in the water on the left side of the log. The father of patient 13 also reported that his daughter went to this location to play. In contrast, VW13 was used by the local population – including again patients 06, 13 and 34 – to obtain drinking water as well as water for cooking and bathing. In the front section of VW13, where there were planks of wood (Figure 3B), water emerged from several springs.

We collected and analysed environmental samples at eight time points over a period of 27.4 months at both VW12 (n=635) and VW13 (n=217) (Table S1). Particularly at location VW12, substantial seasonal alterations of the environment over the study period were observed (Figure 3A and 3B). None of the 217 samples collected at VW13 tested positive in the IS2404 real-time PCR and only one of 108 samples taken from the sand pits, which are located immediately to the north-west of VW12 and are part of the larger VW12 location, tested positive (Figure 4B and 4C, Figure S1 and Table S1). In contrast, at 7/8 time points, positive samples were obtained from at least one of the six positive sampling sites identified at VW12 (Fig. 4). In particular underwater detritus samples collected at sampling site 37 were positive at 5/6 time points tested (Figures 4A and 4B). The average IS2404 real-time PCR Ct values of

the positive samples varied between 34.0 and 38.4 (Figure 4B). As shown in Figure 4C, at the initial sampling time point, there was still one active case of BU (patient 34) using VW12 and there were still a total of three active BU cases in the entire village of Mbandji 2. However, from the third sampling time point on, no active BU case was using VW12 and from the fourth time point on, no active BU case was present in the entire village of Mbandji 2 (Figure 4C). Taken together environmental IS2404 real-time PCR positivity at the VW12 location thus persisted for more than one year after successful treatment of all BU patients identified in the village of Mbandji 2 (Figure 4).

Having identified the deposit on the right hand side of the log lying in VW12 as an IS2404 hotspot (Figure 4C), we analysed the soil all around the log in more detail. While compacted and sandy ground was found on the left, the ground was covered with decaying organic matter on the right hand side of the log (Figure 5A). At the eighth sampling time point we collected three replicates of soil samples every 1.14 m at a total of 14 sampling sites all around the log (Figure 5B). Using on site real-time PCR, we identified sampling site 55 (Figure 5C) as being positive (data not shown) and then sampled this location as well as other sampling sites around the log repeatedly over the next 12 days (Figure 5C). While all 59 samples collected on the left hand side and at the back of the log were negative, 9/62 samples from the right side of the log were positive. Positive samples were identified at sampling site 55 and the neighbouring sampling site 56 (Figure 5C) with an average IS2404 real-time PCR Ct value of 35.8 for all nine positive samples (Figure 5C) Additional sample types, including plants, roots or samples from the surface of the log, collected at sampling site 55 all tested negative (data not shown).

All the above mentioned IS2404 positive samples also tested positive in the IS2606 and the KR real-time PCR and the mean Ct difference (ΔCt) between IS2606 and IS2404 (IS2606-IS2404) of the samples was 1.45 (95% confidence interval from 1.10 to 1.80). This ΔCt indicated that the obtained PCR signal is not related to lineage 1 *M. ulcerans*, which are fish and frog pathogens, or lineage 2 *M. ulcerans*, both of which harbor only few copies of IS2606, but that the PCR signal we observed likely originates from lineage 3 *M. ulcerans*, which are found in human lesions and contain 63-98 copies of IS2606 per genome [7].

Discussion

In African BU endemic areas, both the nature of the environmental reservoirs of *M. ulcerans* and the mode of transmission to humans have so far remained unclear. The physical environment, e.g. biofilms, and organisms such as amoeba, insects, fish and frogs have all been proposed as possible reservoirs for the pathogen [4]. Investigations in Southern Australia have identified mammals, specifically possums, as a local reservoir of *M. ulcerans* [12]. However, no such mammalian source of the pathogen has been detected in Africa thus far [16]. As for the transmission to humans, hypotheses include insect vectors and direct inoculation from the environment via small skin lesions. Parallel modes of transmission may, depending on the environmental and epidemiological setting, be relevant [4,13]. A recent review on BU transmission, found that more evidence is needed to conclude that insects are involved in *M. ulcerans* transmission [4]. Interestingly, *M. marinum*, the closest relative and ancestor of *M. ulcerans*, occasionally causes human infection by inoculation through small skin lesions which are often not remembered by the patient because of the long incubation period [17].

Although BU may occur at all ages, the relative risk for children below the age of five to develop the disease is lower than for older children [13,18]. This appears to apply across different endemic areas in Africa and may indicate that exposure to *M. ulcerans* is increasing, once children are taking up new activities away from their homes [13,19]. Such activities could include going to the farm to work or to water sources for household activities, to collect water or to play. With this in mind and since proximity to water bodies undoubtedly is a risk factor for BU [4], we set out to systematically test environmental, and in particular water, contact locations of laboratory confirmed BU patients. Specifically we collected plant, soil and water samples at the farms as well as village and farm water locations of patients and tested them for the presence of *M. ulcerans* DNA.

Due to the abundance of other and faster growing microorganisms in the environment, routine cultivation of *M. ulcerans* from environmental samples has mostly failed [20] and to date only a single *M. ulcerans* isolate from a water-strider, has been reported [21]. Attempts to culture from our samples were not successful also because of the overgrowth by other mycobacteria. By PCR using pan-mycobacterial and hsp65 primers [22,23] and DNA sequencing, we detected species such as *M. shimoidei*, *M. psychrotolerans* and *M. chubuense* in our preparations (data not shown). Because of these difficulties, real-time PCR for IS2404 is commonly used to detect *M. ulcerans* in the environment. We applied most stringent quality control procedures with internal positive controls in each sample as well as negative and

positive controls in each real-time PCR run. Further, we only considered an environmental sample positive if it was positive in two separate DNA extractions. With this approach we are confident that the positive samples truly contain *M. ulcerans* DNA. We can however not exclude, particularly given the heterogeneity of the environmental samples, that some positive samples may be missed. Although IS2404 is considered a specific marker for *M. ulcerans* [24], the existence of IS2404 positive *M. ulcerans* ecotypes (lineage 1) that are largely avirulent for humans complicates interpretation of real-time PCR data and requires that samples are also tested for the presence of IS2606 and that the difference between the IS2606 and the IS2404 Ct value is analyzed [7,14]. Because *M. ulcerans* ecotypes that cause human disease in Africa and Australia (lineage 3) harbor a higher number of IS2606 sequences then those of linage 1, the ecovars can be separated based on the IS2606 to IS2404 ΔCT [7]. All 41 IS2404 positive environmental samples collected in the course of this study also tested positive for IS2606 with a mean IS2606 to IS2404 ΔCt of 1.45. This ΔCt is well below the ΔCt of 7 to 8 of the for humans less virulent lineage 1 *M. ulcerans* strains [14]. Two IS2404 and IS2606 positive samples tested negative in the real-time PCR for the lower copy number virulence plasmid associated KR sequence. Both of these samples, which had relatively high Ct-values for the IS2404 and IS2606 real-time PCR, were not included in the list of *M. ulcerans* DNA positive samples discussed in this paper.

Our screening of environmental contact locations of laboratory confirmed BU patients revealed that they travel considerable distances to get to their farms and some of the patients further reported to spend several months there. Molecular typing studies of disease isolates may help to identify if the patients were infected close to their homes or farms [25].

By testing environmental samples, we identified two *M. ulcerans* DNA positive water wells (VW31 and VW54) in two different villages. In a third village we identified an *M. ulcerans* positive duck fecal sample (F07) and a positive open permanent water location (VW12). While this rate of environmental positivity is similar to what has been found in a study from Ghana [6], positivity was much higher in another study also conducted in Ghana [26]. It is interesting to note that all three positive locations were permanent as opposed to seasonal water sources. Obtaining water from such water sources has previously been shown to increase the risk for BU [27]. The positive duck fecal sample merits further investigation to determine how waterfowl may contribute to the reservoir of *M. ulcerans*.

At VW31 and VW54 we did not investigate the local scenario any further and cannot exclude the possibility that these locations were contaminated with *M. ulcerans* DNA from the lesions of patients living close to the wells. However at VW12, a water source used by laboratory

confirmed BU patients from Mbandji 2, we observed longitudinal persistence of *M. ulcerans* DNA in underwater detritus for more than one year after successful treatment of the last BU patient. Continuous presence and case search in the village allowed us to detect all local cases and it is therefore unlikely that the source of the environmental positivity was from bacteria recently spread from a human lesion. Interestingly, the more sandy ground on the left of the log at WV12 was never real-time PCR positive for *M. ulcerans* DNA and even on the right side of the log the distribution of *M. ulcerans* DNA was highly focalized (Figures 4 and 5), with samples taken from sampling sites just a few meters apart giving different results. The persistent of real-time PCR positivity in the detritus is a strong indication that this micro-environment may represent a niche environment to which *M. ulcerans* has adapted in the course of evolution from the more generalist *M. marinum* [7,28]. How these findings are related to the recently identified potential role of aquatic worms in BU transmission [11] should be investigated further.

The previously described age distribution of BU patients in the Mapé Basin [13] and the here described findings of *M. ulcerans* DNA at village water sources, lead to the hypothesis that around the age of four both exposure to *M. ulcerans* and the risk of contracting BU increases. At this age, children are beginning to be sent to fetch water and may get in direct contact with the environmental source of the pathogen. Our data further suggest that, underwater detritus could represent a reservoir of *M. ulcerans*, from where infection could take place through either direct contamination of skin lesions or through contamination or colonization of insect vectors.

Acknowledgments

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Tables

Table 1: Environmental contact locations of laboratory confirmed BU patients tested for *M. ulcerans* DNA**.**

Patient ID	Village Water(VW) ***	Farm (F) ***	Farm Water (FW) ***	Distance Home–F (km)
01	VW01, VW02, VW03	F not visited	FW01, FW02	NA
02	VW04, VW05	F01	NA *	0.63
03	VW06, VW07	F02	FW03	12.14
04	VW08, VW09	F03	FW04, FW05	14.31
05	VW10, VW11	F04	FW06	5.62
06	VW12[#], VW13	F05, F06	FW07	7.54; 6.19
07	VW14, VW15, VW16	F07, F08	NA *	0.34; 0.65
08	VW17	F09	FW08	0.69
09	VW18, VW19	F10	FW09, FW10	3.03
10	VW20, VW21	F11	FW11	2.27
11	VW22	F12	FW12	1.08
12	VW23, VW24	F13, F14, F15	FW13	5.37; 5.26; 1.40
13	VW12[#], VW13	F16	FW not visited	2.87
14	VW25	F17	FW14	1.57
15	VW26	F18	FW15, FW16	3.20
16	VW27, VW28	F19	FW17, FW18	11.96
17	VW29	F20	FW19, FW20	3.51
18	VW30	F21	FW21	0.55
19	VW31[#]	F22	FW22	12.62
20	VW32	F23	NA *	0
21	VW not visited	F24	FW23	0
22	VW33	F25, F26	FW24	0.89;1.20
23	VW34, VW35	F27	FW25	2.00
24	VW36, VW37	F not visited	FW not visited	NA
25	VW38	F28	FW not visited	5.66
26	VW39	F29	FW26, FW27	1.48
27	VW40, VW41	F30	FW28, FW29	7.32
28	VW42, VW43	F31	FW30,FW31,FW32,FW33	4.40
29	VW44, VW45, VW46	F32 **	FW34	3.60
30	VW47	F33	FW35	15.35
31	VW48	F34	NA *	0.30
32	VW49	F35	NA *	0.99
33	VW50	F36	FW36	1.83
34	VW12[#], VW13	F16	FW not visited	2.98
35	VW51	F37, F38	NA *	0.08; 0.21
36	VW52, VW53	F39	NA *	0.38
37	VW54[#]	F40	NA *	0.24
38	VW55	F41	FW37	1.31
39	VW56, VW57	F42	NA *	0.59
40	VW58, VW59	F43, F44	FW38, FW39	1.13; 0.84
41	VW52, VW53	F45	NA *	0.79
42	VW60	F46	NA *	0
43	VW61	F47	NA *	0.02
44	VW not visited	F48	FW40, FW41, FW42	5.91
45	VW62	F49	FW43	13.52
46	VW48	F50	NA *	0.17

NA: not applicable
* Water carried to farm from home
** Location not tested by real-time PCR
*** VW, F and FW locations are individually numbered; locations which are shared between patients are identified by the same number
[#] Positive for *M. ulcerans* DNA

Table 2: Laboratory confirmed BU patients in Mbandji 2 during the course of the study**.**

Patient ID	Age	Gender	Clinical Form	Category	Disease Start Date*	Discovery Date	Treatment Start	VW used by the patient
06	9	M	nodule	1	unknown	13.04.2010	24.07.2010	VW12 and VW13
13	5	F	plaque	2	11.09.2010	06.11.2010	10.11.2010	VW12 and VW13
15	2	M	ulcer	3	26.10.2010	30.11.2010	03.12.2010	VW 26
33	11	M	ulcer	2	10.03.2011	05.05.2011	10.05.2011	VW 50
34	57	M	ulcer	1	01.03.2011	10.05.2011	12.05.2011	VW12 and VW13
40	42	M	ulcer	3	06.09.2009	07.08.2011	07.08.2011	VW 58 and 59

* Calculated based on information provided by the patient

Supplementary Table S.1: Number of environmental samples collected at each sampling sites of VW12 and VW13 at all sampling time points.

Sampling Site*	Sample Type	t = 0	t = 2.1 months	t = 4.8 months	t = 7.7 months	t = 10.5 months	t = 15.3 months	t = 20.3 months	t = 27.4 months
1	water	NA	NA	1	3	3	3	3	3
2	soil	NA	NA	1	3	3	3	3	3
3	plant	NA	NA	1	3	3	2	3	3
4	plant	NA	NA	1	3	3	3	3	3
5	water	1	NA	1	3	3	3	3	2
6	plant	1	NA	1	3	3	3	3	3
7	plant	NA	NA	1	3	3	3	3	3
8	soil	NA	NA	1	3	3	3	3	3
9	soil	NA	NA	NA	NA	3	3	3	3
10	plant	NA	NA	1	3	NA	NA	NA	NA
11	water	NA	NA	1	3	3	3	3	3
12	soil	NA	NA	1	3	3	3	3	3
13	plant	NA	NA	1	3	3	3	3	3
14	water	NA	NA	1	3	NA	NA	3	3
15	soil	1	NA	NA	3	NA	NA	NA	NA
16	plant	NA	NA	NA	NA	3	2	3	3
17	soil	NA	NA	5	3	3	3	3	3
18	water	NA	NA	4	3	NA	3	3	3
19	plant	NA	NA	5	3	3	3	3	3
20	plant	NA	NA	5	3	3	3	3	3
21	water	NA	NA	4	3	3	3	NA	2
22	soil	NA	NA	5	NA	3	3	3	3
23	water	1	1	NA	5	NA	NA	5	NA
24	soil	NA	NA	5	NA	5	5	NA	5
25	soil	2	1	4	5	5	5	5	5
26	water	NA	NA	NA	5	NA	NA	NA	NA
27	plant	2	3	5	NA	5	5	5	5
28	soil	NA	NA	5	NA	5	5	5	5
29	plant	NA	NA	NA	5	NA	NA	NA	NA
30	water	NA	NA	5	5	5	5	5	3
31	soil	NA	NA	5	5	5	5	5	5
32	plant	NA	NA	5	5	5	5	5	5
33	plant	NA	NA	5	5	5	5	4	5
34	water	NA	NA	4	5	5	5	5	5
35	plant	NA	NA	5	5	5	4	5	5
36	plant	NA	NA	5	4	5	5	5	NA
37	soil	NA	NA	5	5	5	5	5	5
38	water	NA	NA	5	5	5	5	5	5
39	plant	NA	NA	5	5	5	5	5	NA
40	plant	NA	NA	5	5	5	5	5	5
41	plant	NA	NA	5	5	5	5	5	5
42	plant	NA	NA	5	5	5	5	NA	5
43	plant	NA	NA	5	5	5	5	4	5

* Sampling sites 1 to 16 are at VW13 and 17 to 43 are at VW12.

Figures

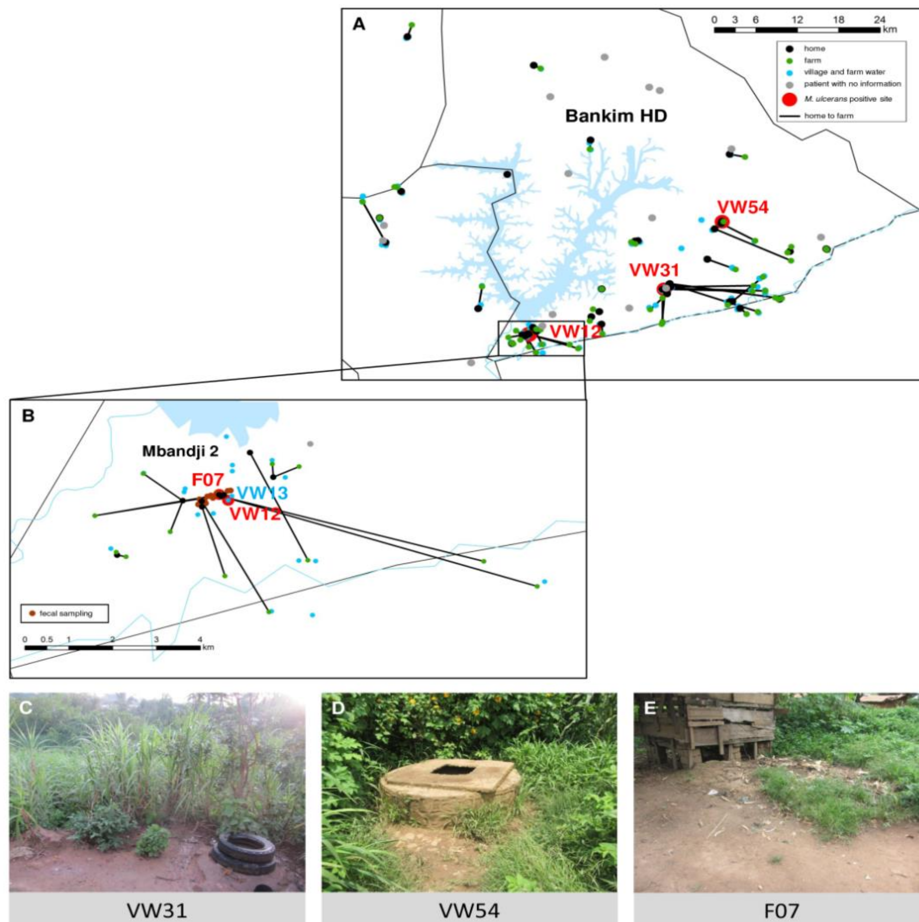


Figure 1: Environmental contact network of laboratory confirmed BU patients from the southern Mapé Basin.

Panel A and B (detailed view of the village of Mbandji 2) show the houses where the 46 laboratory confirmed BU patients in our study lived (black points), the farm(s) where they worked (green points) and the locations where they obtained their water (blue points) during the year before the onset of BU symptoms. The home of each patient is connected with their farm(s) as applicable. Homes of 17 of the 21 non-participating laboratory confirmed BU patients were also mapped and are shown in grey. At the farms and water contact locations, soil (n=171), plant (n=153) and water (n=109) samples were collected. Furthermore, in Mbandji 2 (B), animal faecal samples were collected around patients' houses (brown points). All samples were tested for the presence of *M. ulcerans* DNA and three village water locations were found to be positive (red points; VW12, VW31 and VW54). Further, at location F07 a positive duck faecal sample (red point) was collected. Photographs of locations VW31, VW54 and F07 are shown in C, D and E, respectively. Finally, Panel B also shows a negative water contact location (VW13) which was studied in detail.

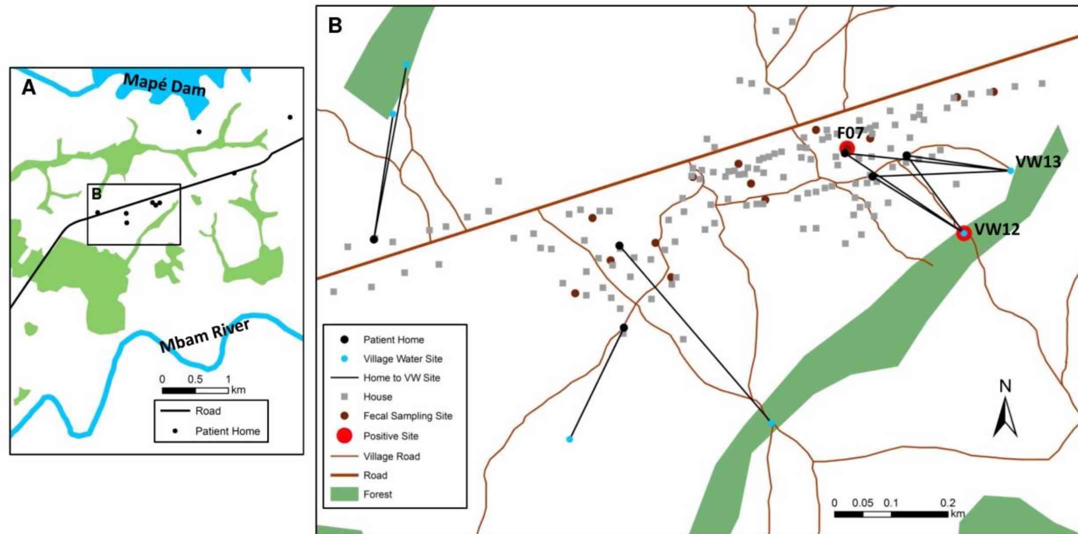


Figure 2: Water contact locations in Mbandji 2 which were investigated in detail.
Based on the high case number and the identification of two environmental locations which were positive for *M. ulcerans* DNA, water contact locations in Mbandji 2 were analysed in detail. The town is located between the Mapé Dam and the Mbam River (A). Panel B shows the locations of the homes of the 6 patients from Mbandji 2 in our study (black points) and each of the homes is connected with the village water location(s) used by the respective patient. Faecal sampling sites are also shown (brown points). Locations which tested positive for *M. ulcerans* DNA are highlighted in red (B). A positive (VW12) as well as a close by negative (VW13) village water locations were studied in more detail. Images are based on a 0.5m resolution WorldView-2 image take on March 12th 2011.

Figure 3: Alterations of the environment at locations VW12 and WV13 at the sampling time points.
Photographs of locations VW12 (A) and VW13 (B) are shown at selected environmental sampling time points.

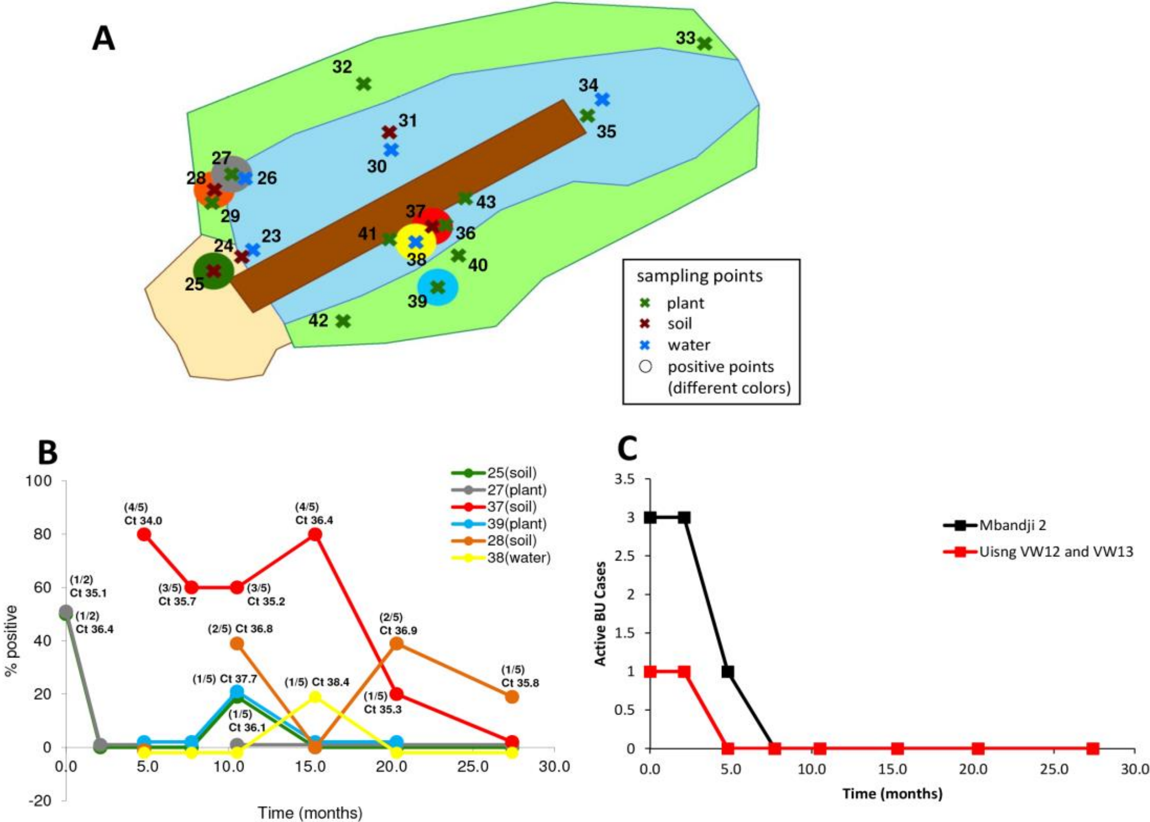


Figure 4: Persistence of *M. ulcerans* at a village water location of BU patients.
Panel A shows a diagram of the water hole at VW12A from where samples were collected at eight time points over a period of 27.4 months. Soil sampling sites are shown as brown crosses, water sampling sites as blue crosses and plant sampling sites as green crosses. Table S1 shows how many samples were collected at each sampling site and each time point. All samples were tested for the presence of *M. ulcerans* DNA by real-time PCR. At 7 sampling time points, *M. ulcerans* real-time PCR positive samples were identified at VW12 (B with positive sampling sites identified by the larger coloured circles and C). Panel C (line colours correspond to the circle colours in panel B) shows the rate of positivity of the collected sample replicates as well as the average Ct value for the IS2404 real-time PCR performed on the positive samples. Finally, panel C shows the number of active BU cases in the village of Mbandji 2 (black line) and the number of active BU cases using VW12 (red line) at the environmental sampling time points.

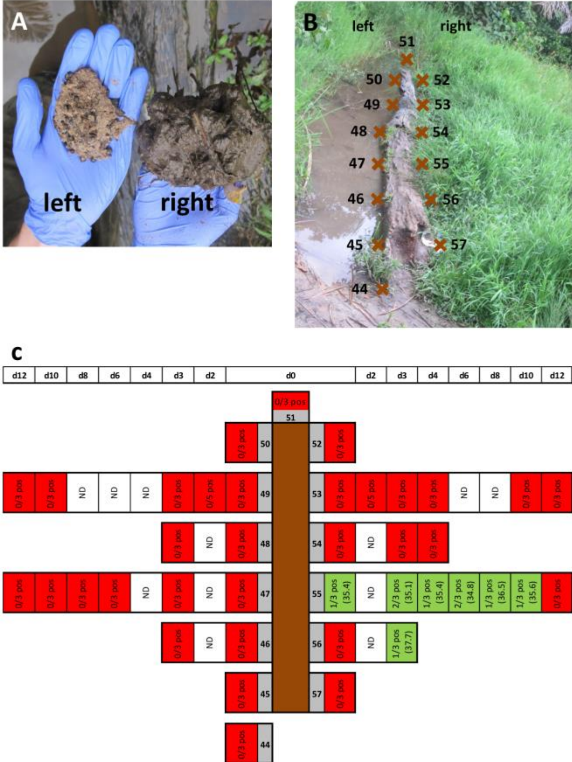
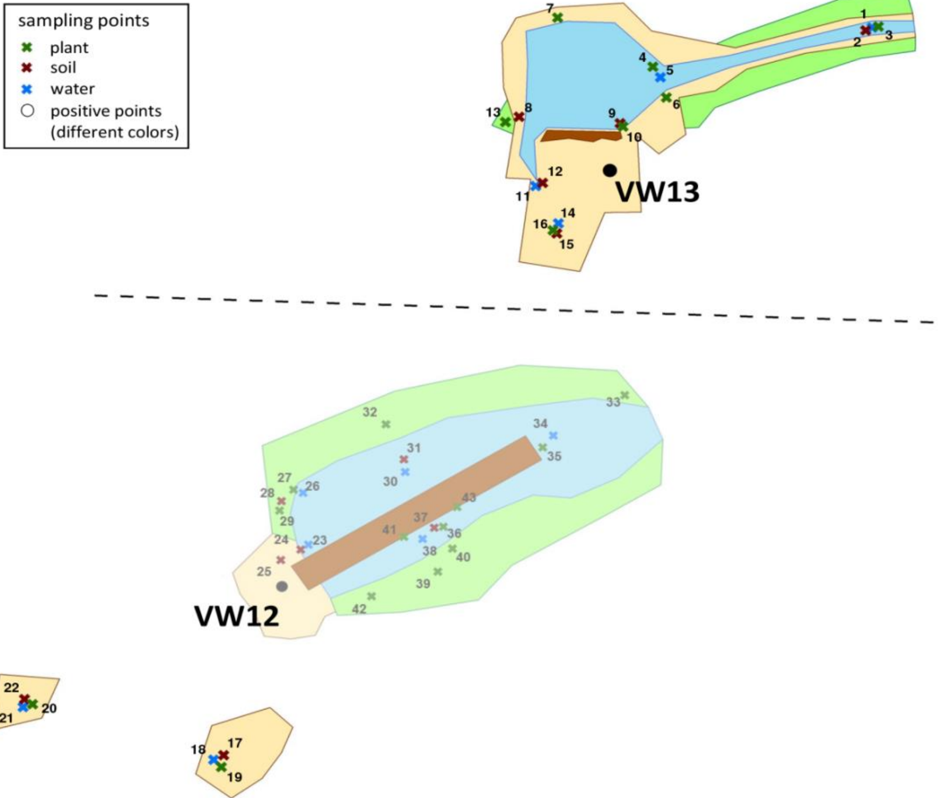


Figure 5: In-depth analysis of soil surrounding the log at VW12.
Panel A shows the nature of the soil on the right and the left hand side of the log. To better understand positivity of samples at location VW12, we performed sampling all along the log on either side (B; brown crosses indicate sampling sites). Selected sampling sites were re-sampled over the next 12 days as indicated in panel C. Panel C further shows the rate of positivity among the replicates collected at each sampling time point and the average Ct value of the IS2404 real-time PCR performed.



Supplementary Figure S1: Sampling sites at VW13 and the sand pits at VW12.

Diagram of VW13 and the sand pits close to VW12 with the sampling sites; soil sampling sites are shown as brown crosses, water sampling sites as blue crosses and plant sampling sites as green crosses. For details on the main water body of VW12 (transparent part) see Figure 4.

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Chapter 4

Epidemiology of Buruli ulcer in the Bamkin Health District of Cameroon: a longitudinal study

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Abstract

Buruli ulcer (BU) is a neglected tropical disease of skin and soft tissue caused by *Mycobacterium ulcerans.* While the disease has been reported from more than 30 countries worldwide, most cases occur in West Africa. Here we report results of a longitudinal study on BU cases reporting to the health facilities of the Bankim Health District (HD) of Cameroon. Following a house-by-house survey in the spring of 2010, a system for continuous registration of new BU cases was established. Of the 274 clinically diagnosed cases between 2010 and 2014, 148 (54%) were re-confirmed by IS2404 qPCR as BU. Locally performed microscopic examination detected acid-fast bacilli in 76.2% of the analyzed qPCR positive lesions. However, there was also evidence for false positive microscopy results. 63.5 % of the laboratory reconfirmed BU patients originated from the Bankim Health District, the others came primarily from the surrounding districts. A substantial proportion of patients lived close to the Mbam River or had their farms close to it. The population age adjusted prevalence showed a biphasic pattern with the lowest prevalence in the <4 year olds, and peak prevalence's in the 12 to 14 and in the <50 year olds. 43.2% of the patients had WHO category 3, 28% category 2 and 28.8% category 1 lesions, respectively. The mean duration before medical consultation was 27 weeks for category 3, 11 weeks for category 2 and seven weeks for category 1 patients, respectively. Taken together, our data show that 1. a point-of care diagnostic test for BU is urgently required, 2. even well-established BU treatment centers may receive primarily patients with advanced lesions and 3. research on the mode of transmission of *M. ulcerans* should focus on agricultural activities.

Introduction

Buruli ulcer (BU) is a necrotizing disease of skin and soft tissue caused by *Mycobacterium ulcerans [1]*. While BU has been reported from more than 30 countries worldwide, the majority of cases are occurring in West and Central Africa. More than 80% of all BU cases are currently reported from Benin, Cameroon, Côte d'Ivoire, Democratic Republic of the Congo and Ghana [2,3]. Within the endemic countries, cases are focally distributed in rural areas [4,5].
BU presents with pre-ulcerative forms ranging from nodules to plaques and edema, which may develop into potentially large ulcers with characteristic undetermined edges [1,6]. The cytotoxic macrolide exotoxin mycolactone of *M. ulcerans* is the key factor in the pathogenesis of the disease [7–9]. While in African BU endemic settings, most patients are children [1], the disease can affect individuals of all ages [10–12]. Typically the lesions occur at the extremities, but all parts of the body can be affected [12]and in most settings no differences in BU incidence between women and men have been observed [13]. The severity of the disease is classified by the WHO into three categories: "Category 1" single lesions with ≤ 5 cm diameter, "Category 2" single lesion 5-15 cm diameter and "Category 3" lesions with diameter > 15 cm, multiple lesions, lesions at critical sites (eyes, breast or genitals) or osteomyelitis [14].
Despite the WHO recommendation to use laboratory diagnostic tests to reconfirm clinical diagnosis, especially in rural and remote areas, many cases of BU are currently diagnosed based only on clinical signs and symptoms, since no sensitive point-of care test is available.
The WHO recommended treatment for BU consists of daily administration of oral rifampin (10 mg/kg) combined with intramuscular streptomycin (15 mg/kg) for 8 week under supervision [14]. Clarithromycin (7.5 mg/kg) can be used to replace streptomycin [15]. Patients who are not promptly managed and treated, can develop very large lesions, requiring long wound healing times and leading to long term functional disabilities [1,16,17].

Although risk factors for developing BU, such as proximity to slow flowing or stagnant water bodies, not wearing protective clothing and poor wound care, have been identified, the mode of transmission and the natural reservoir of *M. ulcerans* are still unknown [18]. Several theories have been proposed, including transmission by an insect vector or direct inoculation from an environmental reservoir into skin lesions[18–23]. In Cameroon, BU was described for the first time in 1969, in patients coming from the Nyong River valley, in the Centre region close to the villages of Akonolinga and Ayos [24]. In 2001 a survey in that area identified numerous cases of active and inactive BU [24]. Between 2007 and 2009 first suspected BU cases were reported from the Bankim Health District (HD), which turned out to be one of the BU endemic foci of

Cameroon [25,26]. On the basis of these findings, in 2010 an exhaustive house-by-house survey was realized in the Bankim HD and a surveillance and recording and reporting system was established in the area [12] to continuously monitor all cases. In the present paper we report the results from the longitudinal observations covering all BU cases registered in the Bankim HD of Cameroon for the five year period from 2010 till 2014.

Material and Methods

Ethical statement

Ethical approval for the house-by-house survey and the subsequent surveillance system was obtained from the Cameroonian National Ethics committee (041/CNE/DNM/09 and 172/CSE/SE/2011) and the Ethics Committee of Basel (EKBB, reference no. 53/11). In addition, all patients or their guardians, if underage, signed an informed consent. All patients detected in the Bankim HD during the course of the study, were, regardless of their consent to participate in the study, treated according to the national BU treatment guidelines of Cameroon.

Study area

The study was conducted in the Mapé Dam region of Cameroon. The area consists mainly of the Bankim HD but also includes part of the neighboring HDs namely the Nwa HD, the Malantuen HD, the Mayo Darlé HD and the Yoko HD. Main environmental features of the area are the Mapé Dam, an artificial lake created by damming the Mapé River in 1988 and the Mbam River which delimits the natural border between the Bankim HD and the Yoko HD.

Surveillance method

After the first house-by-house survey realized in 2010 [12], a continuous surveillance system to monitor the occurrence of new cases of BU was established in the Mapé Basin of Cameroon. The surveillance approach was based on community and health personnel referrals, as well as on regular supervision to health centers carried out by trained and experienced health workers. During supervisory visits, suspected BU cases were observed and if necessary referred to the Bankim district hospital to be further evaluated. Prior to treatment initiation, swabs or a fine needle aspirate (FNA) were collected from lesions for laboratory reconfirmation. Several dry swabs were collected from ulcerative lesions or a FNA was drawn from non-ulcerative lesions and then transferred onto a dry swab to facilitate handling and storage. In addition, clinical and

demographic information was collected from the BU patients. Geographic coordinates of the house where the patients lived for the year before the onset of the disease and the farm where they worked in the same period were collected using a Global Positioning System (GPS) receiver. Further, photos documenting the state of the lesions at the moment of enrolment were collected. All clinically confirmed cases between 2010 and 2014 were included in the cohort and evaluated in the presented study.

Laboratory confirmation of BU cases

Specimens from suspected BU cases were examined in the laboratory of the Bankim district hospital using Ziehl–Neelsen (ZN) staining for the detection of acid-fast bacilli (AFB). ZN was performed according to the WHO guidelines [6] and the reading was done by an expert laboratory technician. The rest of the swab samples were locally stored at 4ºC before being shipped to the Swiss Tropical and Public Health Institute in Basel, Switzerland for IS2404 quantitative polymerase chain reaction (qPCR) analysis. The qPCR analysis was done according to the protocol developed by Fyfe et al. [27]. Briefly, swabs were broken into glass bottles containing glass beads mixed with 2 mL of PBS and vortexed for 1.5 minutes. The DNA was extracted from 1mL of liquid using the Roche Respiratory Specimen Preparation kit and QIAamp DNA Mini kit, and amplified by qPCR using the StepOnePlus system. Results were analyzed using the Applied Biosystem StepOne Software. qPCR-positive reconfirmed samples were also used to initiate a *M. ulcerans* primary culture on Löwensein-Jensen medium, as previously described [28].

Statistical analyses

The measured outcomes and patients' characteristics were summarized using counts and proportions if these were categorical and as means, standard deviations, medians and ranges if they were of continuous nature. The differences in categorical outcomes were determined using the Fisher's exact test, and if the outcomes were continuous, the Mann-Whitney U-test or Kruskal Wallis tests were used. The association between binary outcomes and factors of interest was calculated using logistic regression models, examining first the association with single factors and then adjusting for other factors of interest. Results are expressed as odds ratios. The level of significance was set at $p<0.05$. SAS release 9.4 (SAS Institute Inc., Cary, USA) was used for the statistical analysis. Geographic data were analyzed with ArcGIS ArcMap (Economic and Social Research Institute, Redlands, USA; version 10.2.1)

Results

From January 2010 to December 2014, 274 suspected BU cases were enrolled in our cohort in the Bankim HD in the Adamawa region of Cameroon, where the Bankim District Hospital is the referenced center for BU treatment in that area. The annual number of reported clinically confirmed cases of BU was variable with the largest number being registered in 2011 (n=68); after which a progressive decline to 41 in 2014 was observed (Figure 1B).

Of the 274 clinically diagnosed cases 148 (54%) were re-confirmed by IS2404 qPCR. 105 of these 148 qPCR reconfirmed cases had also been analyzed by ZN staining at the local laboratory in Bankim and AFB were detected in 80 (76.2%) of these samples (Table 1). With samples from 136 qPCR positive patients, attempts were also made to cultivate *M. ulcerans*. From 45 of the 136 qPCR positive cases (33%), *M. ulcerans* could be cultured. In an initial phase, attempts were also made to cultivate *M. ulcerans* from samples of qPCR negative patients; none of these samples yielded a positive culture. However, samples from 19 qPCR negative patients yielded positive ZN readings. Most likely these represented false positive results, since the number of ZN positive and qPCR negative results decreased after re-training of local laboratory staff and improvement of microscopy equipment.

The seasonal distribution of BU cases was irregular, with the highest numbers of laboratory reconfirmed BU cases being reported during March, April and October (Figure 1A). The ratio between qPCR positive and negative cases varied throughout the year with qPCR negative cases dominating in February, June and August (Figure 1A). When analyzed on an annual basis, the qPCR reconfirmation rate was lowest in 2010 (Figure 1B).

Geographic origin and movement radius of BU cases

Of the 148 laboratory reconfirmed BU patients enrolled, 94 (63.5%) were from the Bankim HD with most of the others coming from the surrounding districts. Specifically, 22 patients (14.9%) came from the Malantouen HD, 15 (10.1%) from the Yoko HD, 9 (6.1%) from the Nwa HD and 1 (0.7%) from the Mayo Darlé HD. The remaining 4 patients, for whom the area of origin could be determined, came from further away, with one patient traveling more than 400 km from Douala. To obtain information on the exact origin of the patients coming from the Bankim and surrounding HDs (n=143), we set out to map the location of their house. This was possible for 136 of the patients with the remaining ones having been lost to follow-up. The distribution of the homes of 131 of these patients is shown in Figure 2 with the remaining five coming from locations not included in the area covered by the map. While BU patients originated from throughout the area shown in Figure 2A, clusters of patients were observed in particular in the

area between the Mapé Dam and the Mbam river (n=42) and in the upstream area of the Mbam river (n=26). Areas with these clusters are demarcated in Figures 2A. For the patients coming from these two clusters, we also mapped their farms to gain a better understanding of their movement radius. As shown in Fig 2B, all but one of the patients living in the upstream area of the Mbam, had their farms close to their homes and close to the Mbam river. The one patient, whose farm is not shown, reported to engage in farming 34 km south-west of the home. While some of the patients originating from the area between the Mapé Dam and the Mbam River had their farms close to their homes, most of them, particularly those coming from the town of Bakim also had their farms close to the Mbam river (Figure 2C). Satellite images (Fig. 3) depict that the area along the river used for agricultural activities is regularly flooded in September during the major rainy season. Farming takes place in these temporally flooded areas (Fig. 4).

Clinical characteristic of laboratory reconfirmed BU patients

More than half of the 148 laboratory reconfirmed patients (54.7%; n=81) were ≤ 15 years old. However, the population age adjusted prevalence of BU relative to the age group 0 to <2 year olds, showed a biphasic pattern with the lowest prevalence in the 2 to <4 year olds, and peak prevalences in the 12 to 14 and in the <50 year olds (Figure 5). The median age of the patients was 13 years (max 73 and min 0.5 years) and the mean was 22 years. Among females the median age was 16 years and the mean 23, while among males the median was 13 years and the mean 20 years. The majority of the patients (96.6%) presented with a single lesion. Ulcerative lesions were most common (77.6 %) followed by plaques (11%), edema (7.6%) and nodules (3%). 43.2% of the patients had WHO category 3 lesions, while WHO categories 2 (28%) and category 1 lesions (28.8%) were less common. The mean duration before consultation was 18 weeks, 27 weeks for category 3, 11 weeks for category 2 and seven weeks for category 1 patients, respectively.

Among the 148 qPCR positive patients, 36 (27.7%) were treated with antibiotics and received wound care and rehabilitation assistance as in-patients, while 94 (72.3%) received treatment and wound care as out-patients. Among the 36 patients treated as in-patients, 31 (86.1%) presented with a category 3 lesion, 1 (2.8%) patient had a category 2 lesion and 4 (11.1%) patients had a category 1 lesion. 25/148 (16.9%) patients received split skin grafting.

Among the 148 laboratory reconfirmed patients the gender ratio was balanced with 73 (49.3%) females and 75 (50.7%) males. However, the gender ratio varied significantly with age with males dominating among younger (< 15 years) and females among the older (> 15 years)

patients (OR 2.6, 95% CI 1.12-6.19, p=0.025 and OR 2.49, 95% CI 0.19-0.82, p=0.012 respectively) (Table 2).

Discussion

In 2013, we reported the results of a house-by-house survey for BU, leprosy and yaws realized in 2010 in the Bankim HD of Cameroon [12]. The present study complements these data presenting results of a five-year longitudinal analysis realized in this BU endemic area of Cameroon. As already reported from other BU endemic areas [2], we observed a progressive decline of the number of cases from 2011 to 2014. The reasons for this general phenomenon are not yet clear. In our setting a change in the strategy for case finding over time may have contributed. Initially a system of active case-finding with trained teams from the Bankim hospital carrying out screening and sensitization campaigns all around the health district addressing in particular communities suspected to be at risk was established. In addition, community volunteers were trained to increase community participation and developing an interface to refer suspected BU patients to the treatment centers. With an increase in community awareness about BU, the active surveillance approach was gradually converted to a passive approach with cases coming on their own to the treatment centers. This reduction in active case finding activities could partially explain the observed decline in cases. However, in a recent small scale survey for the main skin diseases, including BU, in rationally selected communities in the Bankim area, very few cases of BU were discovered; putting into question the hypothesis that the changing case finding strategy was the reason for the decline in the number of BU cases observed. Therefore, also other factors should also be considered. For example, the efficient case detection and treatment of cases has led to a significant reduction of the presence of patients with poorly attended ulcerative lesions in the communities. This is likely to correspond to a reduction in the spread of the bacteria from active ulcers into the environment which may play a role in transmission in the endemic settings. If this is indeed the case, early diagnosis and treatment may lead to a decrease in incidence.

Our geographical analysis of houses and farms of BU patients identified the banks of the Mbam river as a hotspot of BU prevalence in the district. The river is of fundamental importance for the daily activities and the life of the communities living or working in the area. It is surrounded by plantations, forest and also wet lands, most of them used as farms and it is suspected that in particular the river associated wetlands may be relevant for transmission of *M. ulcerans.* Also other studies have identified residence or activities related or close to river systems as a risk factor for BU [18]. It is remarkable in this context that the artificial lake created by the damming of the Mape river did not emerge as BU hotspot in the geographical analysis.

The observed seasonal variation in case numbers has similarities with other studies from Africa [29,30]. It has been hypothesized that the seasonal fluctuation is related to the transition from the dry to the rainy season and farming activities which appear to be a risk factor for BU disease [18]. In a BU endemic area of Northern Australia, a large spike in cases has been observed seven to eight months after an exceptionally long and wet rainy season [31]. Flooding may have resulted in increased transmission of *M. ulcerans* infection. Taking an average delay to presentation of 2.7 months into account, this suggests that the incubation period is about 4.5 months. This is in good agreement with a study on BU patients from southern Australia with a residential address outside a known BU endemic area [32]. Here a mean incubation period of 4.5 months has been estimated with a large variation (between 32 days and 264 days). Not only the incubation period for BU seems to be highly variable, but also other factors, such as farming activities may influence the time when patients with wounds report to the health system[33]. Nevertheless, it is tempting to speculate that in Bankim increased transmission during September's rainy season and flooding of the river banks may be responsible for the peak in BU cases reporting to the Bankim hospital in March and April of the following year.

Our analysis of the age spectrum of patients showed, that the BU prevalence in children below the age of four is very low. At this age, children have a smaller movement radius when compared with older children. Sero-epidemiological studies indicate, that younger children are less exposed to *M. ulcerans* than older children [34]. This suggests that the source of infection may be located at some distance from the movement radius of children below the age of four.

43.2% of the BU patients had WHO category 3 lesions, indicating substantial treatment delays [35,36]. The reasons leading to this delay are ranging from socio-culture reasons to economic reasons. Local beliefs which are in particular deeply rooted in rural communities can lead to first seek help from traditional healers [37]. A substantial number of patients came from villages far away from the Bankim hospital or other health centers and this was found to be a major concern for patients and accompanying relatives.

Patients in the cohort described here, were enrolled over 5 years giving the opportunity to show the evolution and the characteristics of the BU disease over time in the area in and around the Bankim. Analyzing the cohort, we highlight the importance of a combined approach in which active sensitization, a good surveillance and reporting system, reliable diagnosis, good clinical practice and a detailed understanding of the needs of the patients, are all important for BU control.

Tables

Table 1: Laboratory tests results; cross tables of qPCR results versus ZN results and qPCR results versus Culture results

Test	**ZN (+)**		**ZN (-)**	
	n	(%)	n	(%)
qPCR (+)	80	(54.05)	25	(16.89)
qPCR (-)	19	(20.43)	45	(48.39)
	Culture (+)		**Culture** (-)	
	n	(%)	n	(%)
qPCR (+)	45	(30.41)	91	(61.49)
qPCR (-)	0	(0.00)	26	(27.96)

Table 2: IS2404 qPCR results related to sex and age group

Age	**Gender**	**qPCR positive**		**qPCR negative**		**OR**	**95% CI**	**p-value**
		n	**%**	**n**	**%**			
≤15 years	Male	47	81.03	11	18.97	2.639	1.125-6.190	0.0257
	Female	34	61.82	21	81.03	1.0		
>15 years	Male	27	41.5	38	63.9	0.401	0.195-0.822	0.0127
	Female	39	58.5	22	36.1	1.0		

Table 3: clinical presentation of patients related to age and sex

		patients, n	%	Univariable			Bivariable		
				OR	95% CI	p-value	OR	(95% CI)	p-value
Lesion position									
head	≤15 years	5	6.2	4.211	0.479 - 36.97	0.1947	4.373	0.489 - 39.10	0.1868
	>15 years	1	1.5	1			1		
	Male	3	4	0.958	0.187 - 4.910	0.9593	0.796	0.151 - 4.186	0.788
	Female	3	4.2	1			1		
trunk	≤15 years	10	12.3	1.167	0.418 - 3.256	0.768	1.052	0.370 - 2.990	0.9237
	>15 years	7	10.7	1			1		
	Male	11	14.6	1.891	0.659 - 5.416	0.2356	1.905	0.655 - 5.532	0.2362
	Female	6	8.3	1					
upper limbs	≤15 years	28	34.5	1.279	0.632 - 2.585	0.4931	1.371	0.668 - 2.811	0.3886
	>15 years	19	29.2	1			1		
	Male	22	29.3	0.734	0.367 - 1.466	0.3817	0.665	0.326 - 1.352	0.26
	Female	26	36.1	1			1		
Clinic									
ulcer	≤15 years	59	72.8	0.536	0.238 - 1.207	0.1325	0.486	0.211 - 1.115	0.0886
	>15 years	55	83.3	1			1		
	Male	61	81.3	1.533	0.701 - 3.349	0.2839	1.714	0.766 - 3.833	0.1892
	Female	54	73.9	1			1		
oedema	≤15 years	8	9.8	3.506	0.718 - 17.10	0.1209	3.633	0.731 - 18.03	0.1145
	>15 years	2	3	1			1		
	Male	5	6.6	0.971	0.269 - 3.506	0.9647	0.814	0.219 - 3.016	0.758
	Female	5	6.8	1			1		

Figures

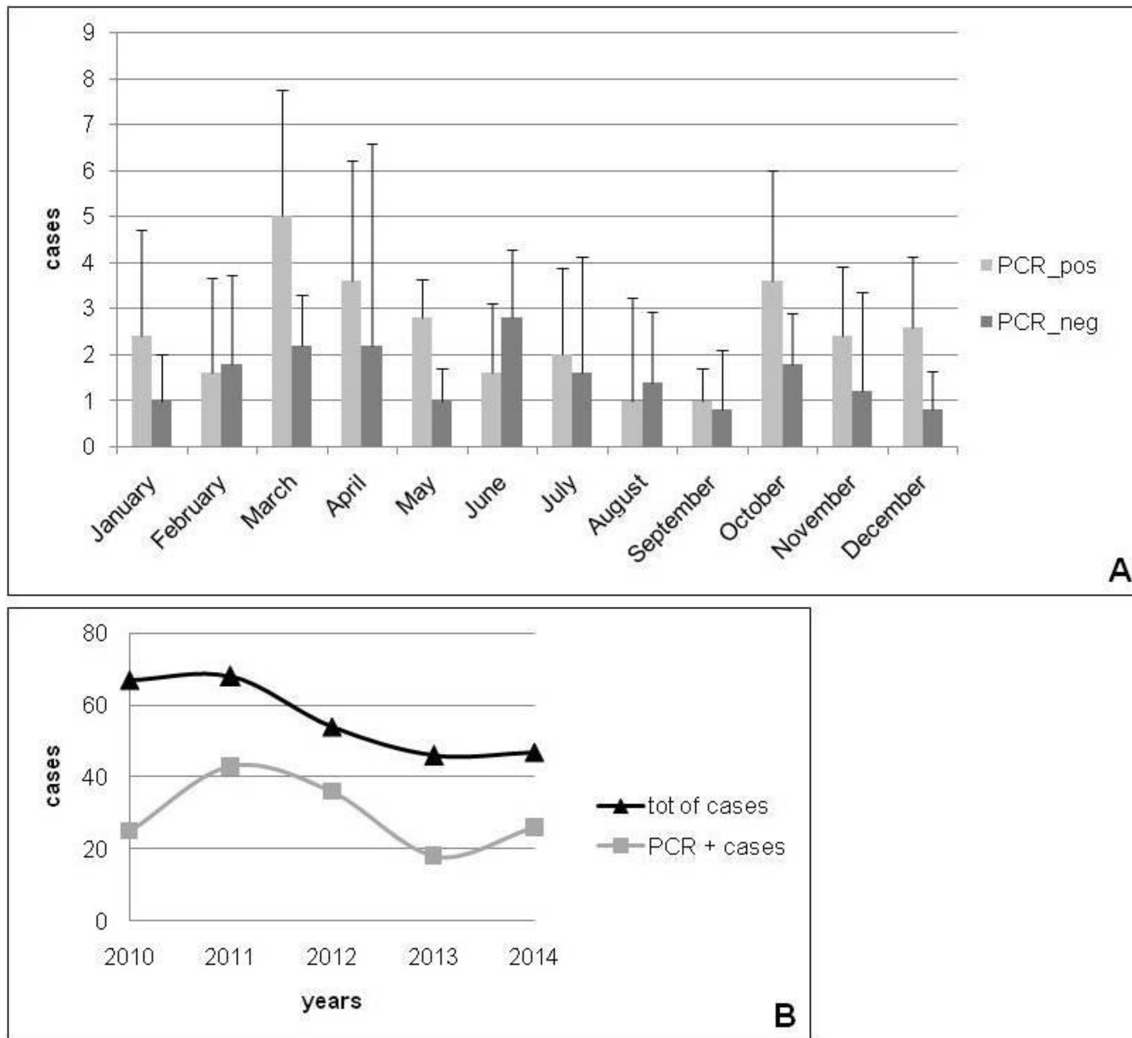

Figure 1: Average Monthly number of qPCR positive and negative suspected BU cases over 5 years. Panel A shows the average monthly incidence of qPCR positive and negative suspected BU cases identified between 2010 and 2014. Panel B shows the total number of suspected BU cases and the number of qPCR plosive cases detected per year over the same period.

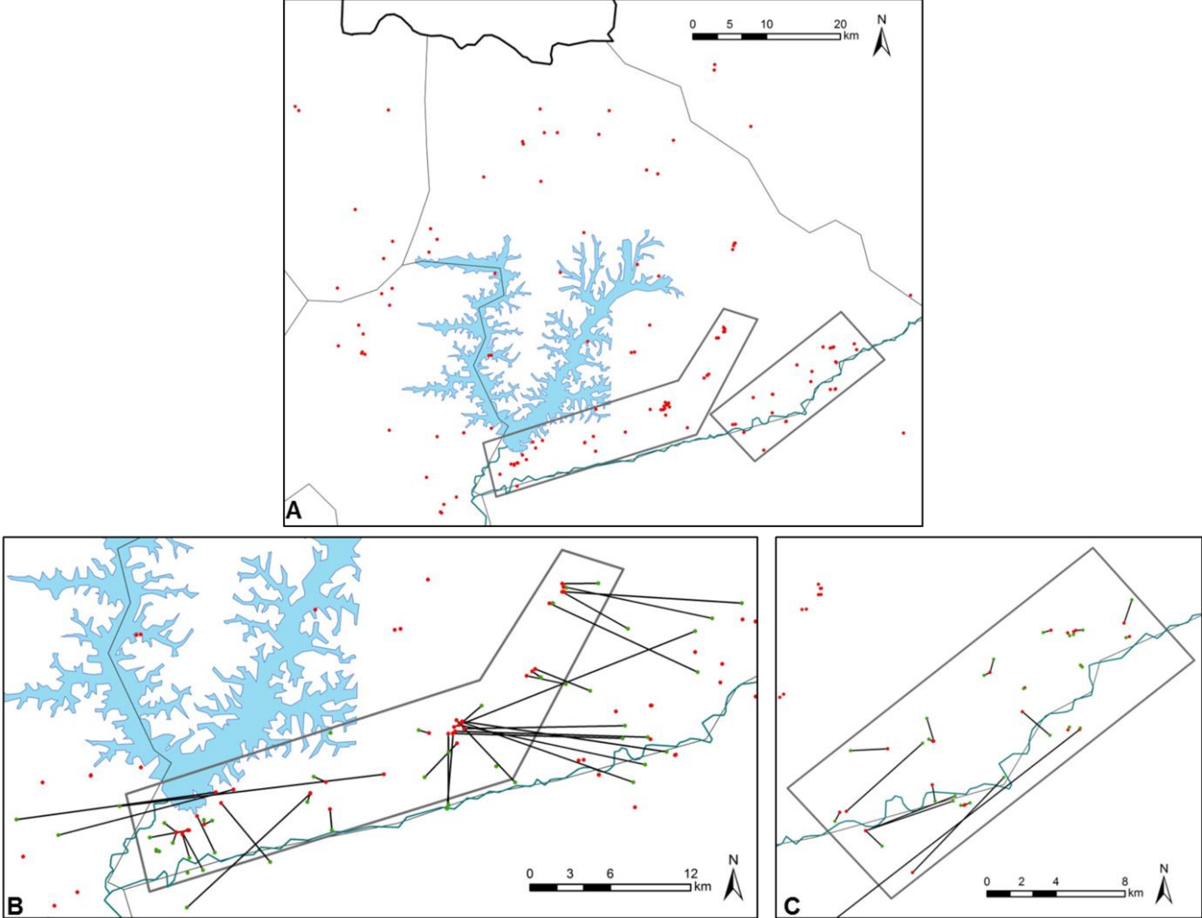

Figure 2: Houses and farms of qPCR positive BU cases from 2010 to 2014. Houses (in red) of 148 qPCR reconfirmed BU patients were mapped (A) and two main clusters of patients were identified in the Bankim HD; one between the Mapé Dam and the Mbam river (B) and the other in the upstream area of the Mbam river (C). For the patients living in the two clusters, farms (in green) were also mapped and are connected to the residence of the corresponding patient (B, C).

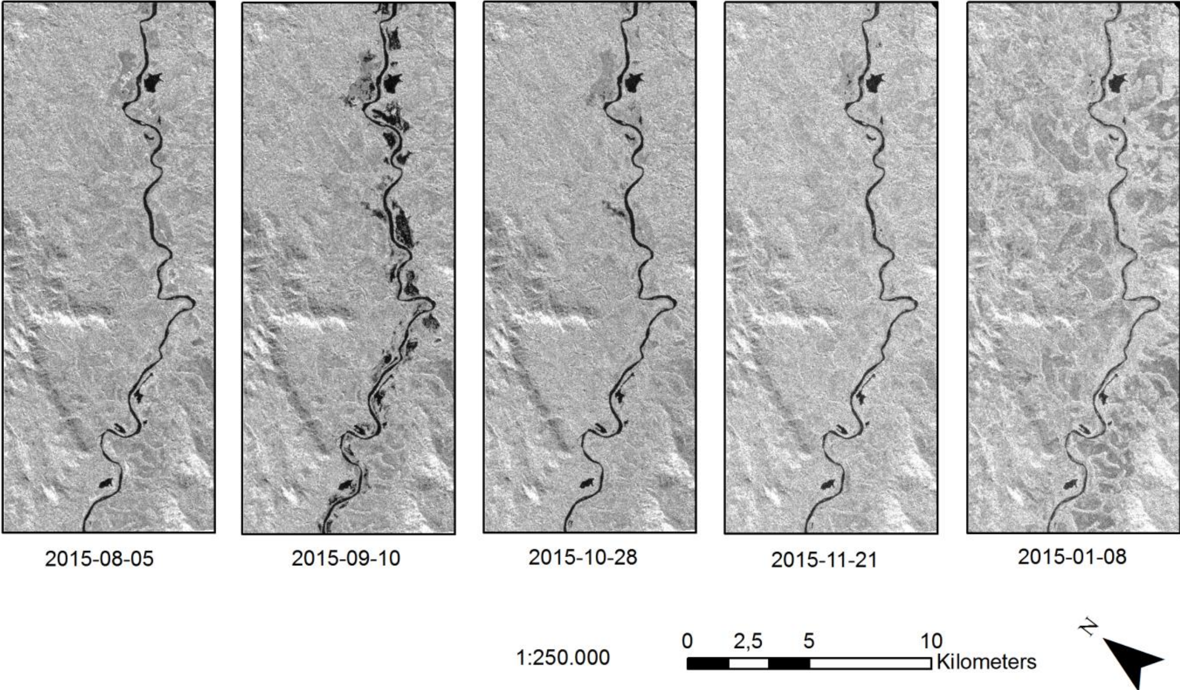

Figure 3: Flooding of the banks of the Mbam river during the rainy season in September.

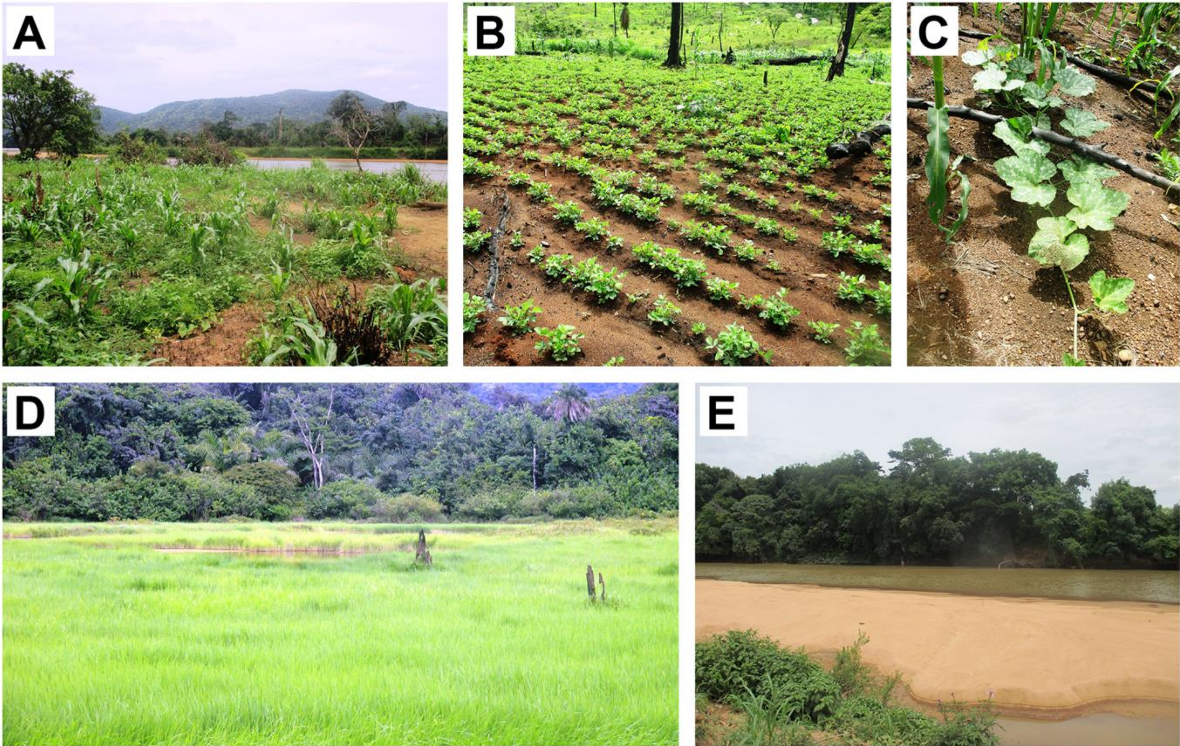

Figure 4: Land cover at the banks of the Mbam river. Areas used for agricultural activities (A-C) are interrupted by swamps (D). Sand banks emerge when the river waters are low (E).

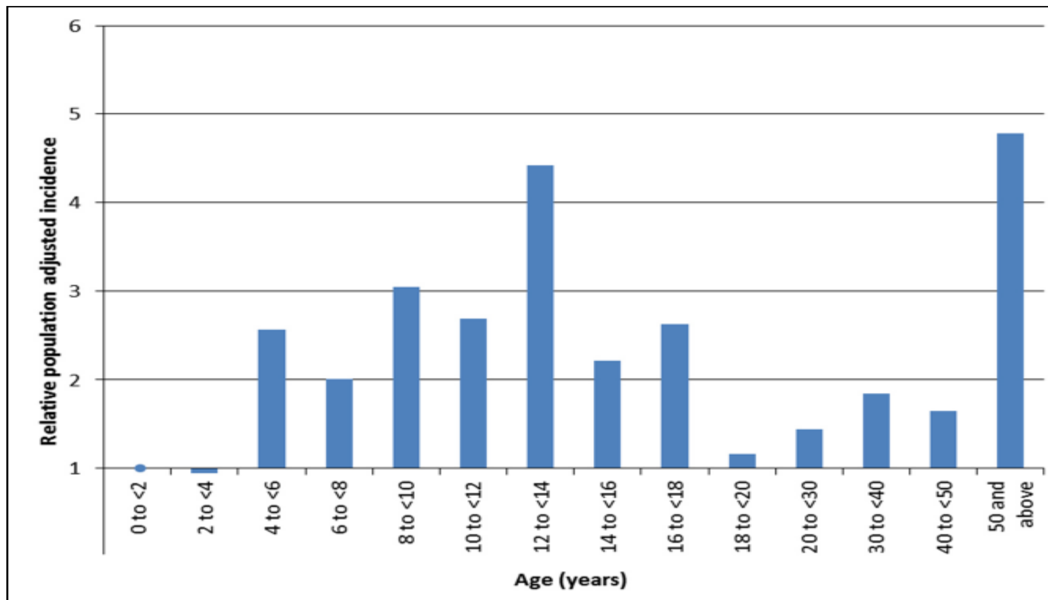

Figure 5: Relative population age adjusted incidence of PCR positive Buruli ulcer in the Bankim HD. On the basis of the population age distribution of the general population living in the study area and the age distribution of the qPCR positive BU patients, the population adjusted prevalence was computed. The prevalence shown is relative to the prevalence of patients aged between zero and less than two years.

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Chapter 5

Complete Healing of a Laboratory-Confirmed Buruli Ulcer Lesion after Receiving only Herbal Household Remedies

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Case Presentation

On March 7, 2011, an 11-year-old boy from the town of Bankim in the Adamaoua Region of Cameroon—a known endemic focus of Buruli ulcer (BU) [1]—was accompanied by his father to the district hospital in Bankim. The patient presented with a BU lesion classified as Category II, according to the classifications of the World Health Organization (WHO). The partially ulcerated plaque lesion, which was approximately 14 x 6 cm in size, had undermined edges characteristic of BU (Fig 1A) [2,3]. Following clinical examination and sample collection for diagnosis, the patient's family refused the standard WHO-recommended treatment for BU, which consists of daily rifampicin (10 mg/kg orally) and streptomycin (15mg/kg intramuscularly) for eight weeks [4], and the patient left the hospital. Wound exudates collected from the patient tested positive in the *Mycobacterium ulcerans*-specific IS*2404* quantitative polymerase chain reaction (qPCR) assay [5] with threshold cycle (Ct) values ranging from 20.0 to 28.6, indicating a high mycobacterial load. Swab exudates were also used for the initiation of a *M. ulcerans* primary culture on Löwenstein-Jensen medium, as previously described [6]. After 8.5 weeks of incubation at 30 °C, mycobacterial growth was observed, and the cultured mycobacteria were reconfirmed as *M. ulcerans* by IS*2404* colony PCR [6]. Whole genome sequencing of the isolate reconfirmed that it belongs to the local clonal complex of *M. ulcerans* [7].

One week after reporting to the hospital, the patient was visited at the family farm in proximity to the Mbam River, south of Bankim. Between the initial consultation and this encounter, the patient did not consult with any other health centre or traditional healer. However, the father of the patient applied herbal household remedies, derived from the barks of two trees, onto the lesion (Fig 2). Using standard tools in botany, the trees from which the herbal remedies were obtained could be identified as *Erythrophleum suaveolens* [(Guill. & Perr.), Brenan] and *Stemonocoleus micranthus* [8]. The application of the household remedies involved the washing of the lesion (at least once per day) with a decoction obtained by boiling the bark of *E. suaveolens.* In addition, a mixture of salt, powdered bark of *S. micranthus*, and the *E. suaveolens* decoction was applied onto the open lesion daily, over a period of three months (Fig 1B).

In May 2013, more than two years after the first encounter, the patient was examined again. At this point, the lesion had completely healed and no reduction in movement of the joint was observed (Fig 1C). At an additional follow-up visit in January 2014, the scar was found in

good condition and no signs of a relapse of the lesion, or the emergence of satellite lesions, were observed (Fig 1D, 1E, and 1F).

Ethical approval to investigate specimens from this patient was obtained from the Cameroonian National Ethics Committee (041/CNE/DNM/09 and 172/CNE/SE/2011) and the Ethics Committee of Basel (EKBB, reference no. 53/11), and written informed consent was obtained from the father of the patient for the publication of the details.

Case Discussion

The clinical presentation of *M. ulcerans* disease ranges from non-ulcerative nodules, plaques, or oedema to ulcers. The disease often starts as a non-painful nodule or indurated area, which may then ulcerate and develop BU-characteristic features, including undermined edges [9]. Large ulcerative lesions at joints, like the one described here, often take a particularly long time to heal completely and are often associated with long-term complications, such as disabilities in the form of contractures or limitations in movement. These long-term sequelae of BU may occur even if the patient receives the recommended antibiotic treatment and regular wound care [10]. Furthermore, patients with large lesions require physiotherapy and rehabilitation in addition to the antibiotic treatment and wound care [11,12].

It has been reported from various BU-endemic areas that some patients initially consult traditional healers before seeking modern medical treatments [13]. Such consultations with traditional healers may lead to a worsening of the lesions, which would, in turn, require prolonged wound-care treatment, lead to a delay in healing, and increase the possibility of long-term disabilities [14–16].

The BU patient presented here did not receive any therapeutic intervention, such as antibiotic treatment, surgery, or thermotherapy; instead, his father placed household remedies from the barks of two trees, *E. suaveolens* and *S. micranthus*, onto the lesion. Medicinal properties of the products of these two plants have been previously described, and it is known that they are used in traditional medicine in West and Central Africa to treat different conditions, ranging from fertility problems to stomach disorders. Interestingly, the bark of *E. suaveolens* has been reported to be used in traditional medicine to treat BU in Côte d'Ivoire and Benin [17–20]. In in vitro analyses, extracts from these plants have been shown to have anti-inflammatory and antibacterial properties; an ethanol extract from the bark of *E. suaveolens* has been specifically tested for activity against *M. ulcerans* [21–25]. Since spontaneous healing of BU cases has been reported [26], it is not firmly established that the healing of the lesion of the patient presented here was supported by herbal remedies. It is possible that the immune

responses of the patient could have cleared the infection, even without the application of household remedies. In addition, the daily washing and dressing of the wound described here, which is similar to the wound care performed in biomedical health care facilities, could have facilitated the healing of the wound. The effect of other aspects of care provided to this patient by the family, such as feeding, psychosocial support, and the encouragement of the patient to continuously move the arm to prevent any disability, may also have contributed to the complete healing of the lesion without any long-term sequelae.

However, the fact that this laboratory-confirmed BU lesion healed completely—after only receiving household remedies obtained from the trees *E. suaveolens* and *S. micranthus*—may merit further research on potential anti-mycobacterial activities of compounds contained in the bark of these two trees.

The combination of rifampicin with streptomycin or clarithromycin, given for eight weeks, is a well-established first-line therapy for all forms of active BU disease. While the search for next-generation antibiotics against mycobacteria is ongoing, efforts in the endemic areas must ensure that cases of BU are discovered early and that patients receive the WHO-recommended antibiotic treatment and professional wound management as early as possible.

Learning Points

- The combination of rifampicin with streptomycin or clarithromycin, given for eight weeks, is a well-established first-line therapy for all forms of active *M. ulcerans* disease.
- In African Buruli ulcer endemic areas, herbal remedies nevertheless continue to be applied to lesions of Buruli ulcer patients.
- Details of possible anti-mycobacterial activities of herbal remedies—obtained from plants that are repeatedly reported to be applied to Buruli ulcer lesions in several endemic countries in West Africa—may be worth further investigation.
- Given the possibility of spontaneous remission, the potential contribution of herbal remedies to the healing of Buruli ulcer lesions remains to be critically assessed.

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We would like to thank all the Bankim health care staff for their great support in the field. Thank you, also, to the Naturalis Biodiversity Center in Leiden, the Netherlands, for the identification of the plant specimens described in this case report.

Figures

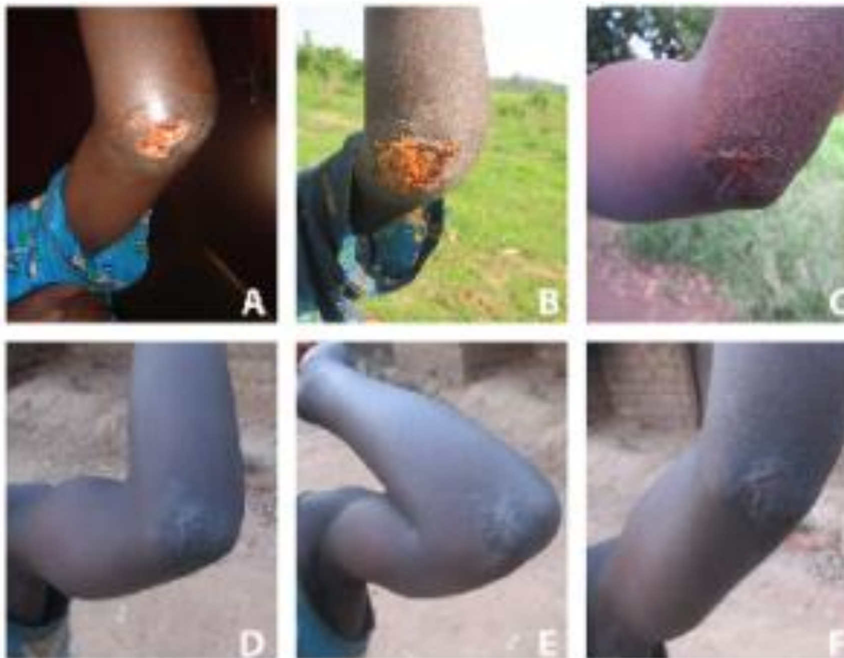

Figure 1: Clinical evolution of the Buruli ulcer lesion to which household remedies were applied. (A) Untreated, laboratory-reconfirmed Buruli ulcer lesion of a patient who presented to the district hospital in Bankim, Cameroon, in early March, 2011. (B) Appearance of the lesion one week later, with household remedies applied. (C) Completely closed lesion, two years later (September 2013). (D, E, and F) Follow-up pictures of the patient taken in January 2014, at which time no reduction in movement of the joint was observed and the patient could completely flex (E) and extend (F) the elbow.

Figure 2: Sources of the herbal products that were used as household remedies and applied to the laboratory-confirmed Buruli ulcer lesion. *Erythrophleum suaveolens* tree (A) and sampling of its bark (B). *Stemonocoleus micranthus* tree (C) and powdered bark (D).

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Chapter 6

Immunohistochemical monitoring of wound healing in antibiotic treated Buruli ulcer patients

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Abstract

Background: While traditionally surgery has dominated the clinical management of Buruli ulcer (BU), the introduction of the combination chemotherapy with oral rifampicin and intramuscular streptomycin greatly improved treatment and reduced recurrence rates. However management of the often extensive lesions after successful specific therapy has remained a challenge, in particular in rural areas of the African countries which carry the highest burden of disease. For reasons not fully understood, wound healing is delayed in a proportion of antibiotic treated BU patients. Therefore, we have performed immunohistochemical investigations to identify markers which may be suitable to monitor wound healing progression.

Methodology/Principal findings: Tissue specimens from eight BU patients with plaque lesions collected before, during and after chemotherapy were analyzed by immunohistochemistry for the presence of a set of markers associated with connective tissue neo-formation, tissue remodeling and epidermal activation. Several target proteins turned out to be suitable to monitor wound healing. While α-smooth muscle actin positive myofibroblasts were not found in untreated lesions, they emerged during the healing process. These cells produced abundant extracellular matrix proteins, such as pro-collagen 1 and tenascin and were found in fibronectin rich areas. After antibiotic treatment many cells, including myofibroblasts, revealed an activated phenotype as they showed ribosomal protein S6 phosphorylation, a marker for translation initiation. In addition, healing wounds revealed dermal tissue remodeling by apoptosis, and showed increased cytokeratin 16 expression in the epidermis.

Conclusion/Significance: We have identified a set of markers that allow monitoring wound healing in antibiotic treated BU lesions by immunohistochemistry. Studies with this marker panel may help to better understand disturbances responsible for *wound healing delays* observed in some BU patients.

Author summary

Coagulative tissue necrosis and local immunosuppression caused by the *M. ulcerans* macrolide toxin mycolactone are typical features of Buruli ulcer disease (BU). In particular in BU endemic remote rural areas of West Africa, patients often report with large ulcerated lesions. Despite the availability of an effective dual antimycobacterial antibiotic therapy, some ulcerative lesions may take long time to healing and represent a major burden for the patients as well as for the health system. Proper wound healing is a well-orchestrated process involving numerous cellular and acellular components. Here we have performed immunohistochemical studies with tissue from BU lesions collected before, during and after antibiotic treatment.

We identified a set of markers which are appropriate to evaluate formation of granulation tissue (alpha-smooth muscle positive fibroblasts), matrix deposition (pro-collagen 1, fibronectin and tenascin C), cell activation (phosphorylated S6), hyper proliferation of the epidermis (cytokeratin 16) and apoptosis (cleaved caspase 3) during wound healing. These markers may become suitable for assessing progression of tissue repair and for investigating the functional basis of impaired wound healing.

Introduction

Buruli ulcer (BU) is a necrotizing skin disease caused by *M. ulcerans.* It is primarily affecting the subcutaneous tissue and can, if untreated, lead to extensive tissue destruction and ulceration. The disease has been reported from more than 30 mainly tropical countries [1] around the world with the highest incidence in West Africa. The distribution of the disease is very focal and typically associated with rural wetlands in close proximity to stagnant or slow flowing water bodies [1,2]. The mode of transmission and the environmental reservoir of *M. ulcerans* are still not fully characterized.

The disease can affect all age groups, but the highest incidence is in children aged between 5 and 15 years and in the elderly [3,4]. Most of the lesions occur on the limbs, but all parts of the body can be affected. The currently recommended treatment consists of daily administration of oral rifampicin (10 mg/kg) and intramuscular streptomycin (15 mg/kg) for 8 weeks under regular supervision.

BU presents with a variety of clinical forms including nodules, plaques, edema and ulcers and in more severe cases multiple lesions as well as osteomyelitis have been observed. The disease often starts as a painless swelling or an area of induration which eventually may develop the characteristic features of BU such as large ulcers with undermined edges [5]. In particular in remote areas of Africa, patients tend to report late to the treatment centers and therefore often with very extensive and severe lesions. Long recovery periods are common and in the case of large lesions skin transplantation is required and permanent morbidities including functional limitations may be observed [6]. While mycolactone causes massive local immune suppression in active BU lesions, vigorous local immune responses are observed during anti-mycobacterial chemotherapy [7]. Paradoxical reactions including the enlargement of ulcers, progression of non-ulcerated plaques and edemas to ulcerative lesions, and the emergence of new lesions are frequently observed during chemotherapy [8,9]. However, neither ulceration of plaques [10] nor the appearance of new lesions [11,12] is necessarily an indicator of a failure of chemotherapy. While many BU lesions tend to heal fast after completion of anti-mycobacterial treatment in some patients, the healing process is severely delayed in others. Massive infiltration of lesions and the development of atopic lymphoid tissue are usually not interfering with wound healing. Vigorous local immune responses are thus a good marker of the success of anti-mycobacterial treatment [7], but not necessarily associated with complications. In patients being treated for BU, it is therefore difficult to differentiate between deterioration resulting from a local immune reconstitution

inflammatory syndrome [13] and deterioration resulting from other causes, such as disturbed orchestration of wound healing processes or secondary infections.

Wound healing is a complex process [14], consisting of a sequence of four overlapping and integrated phases: rapid homeostasis, inflammation, proliferation and tissue remodeling [15,16] which are characterized by inter- and intra-cellular level changes. Once the vascular constriction and the fibrin clot are in place, inflammatory cells migrate into the wound bed and promote the inflammatory phase characterized by the infiltration of macrophages, lymphocytes and neutrophils which clean the wound area, release cytokines to induce inflammation and to stimulate fibroblasts, keratinocytes and other elements involved in the subsequent phase of the wound healing process [17]. The inflammatory phase is in general followed, but partly also overlapping, with the proliferative phase. The proliferative phase is characterized by epithelial proliferation and migration through a “temporal” extracellular matrix (ECM) composed of several proteins including fibronectin, tenascin and pro-collagen I. This matrix acts as support for the fibroblast migration into the wound bed. Fibroblasts and endothelial cells are abundant at this time and they support capillary growth and formation of granulation tissue. Myofibroblasts, which are specialized fibroblasts, are the main producers of collagen in healing wounds. Initially type III collagen is produced and then replaced by type I collagen [18–20]. Myofibroblasts can contract by using a smooth muscle type actin-myosin complex, rich in α-smooth muscle actin (αSMA) and are involved in the contraction and closure of wounds [21,22]. αSMA is commonly used as a marker for the detection of myofibroblasts, but it is also present in pericytes located at the wall of blood vessels. After healing is complete, myofibroblasts are normally eliminated by apoptosis and in healthy tissue they are present only sub-epithelially in mucosal surfaces [15]. However, myofibroblasts seem to persist in wound granulation tissue that fails to resolve after healing, which is considered to be the cause of excessive matrix deposition in hypertrophic scars [15,23]. The final remodeling phase of wound healing is also characterized by a reduction in the number of newly formed vessels and a slow return to conditions similar to healthy skin tissue [15]. Here we have analyzed markers of cell activation, myofibroblast formation and matrix deposition in tissue biopsies from BU lesions before, during and after treatment.

Materials and Methods

Ethics statement

Ethical approval (clearance N^0 011, 12/10/2010) for the analysis of the clinical specimens was obtained from the provisional national ethical review board of the Ministry of Health Benin, registered under the N^0 IRB00006860. Tissue samples were taken for detailed immunohistological analysis, after written informed consent has been given by the patients or their guardians.

Study participants

Eight patients from a highly endemic region of Benin (Ze commune in the Atlantique department) with laboratory confirmed BU plaque lesions which reported to the Centre de Depistage et de Traitement de l'ulcere de Buruli d'Allada, between April and August 2009 were included in the study. For all eight patients, biopsies and material obtained during wound debridement or excisions were available. Samples were taken at 3 different time points: T1 before the start of antibiotic treatment (day-2 to 0), T2 during antibiotic treatment (day 26-34) and T3 after the completion of antibiotic treatment (day 56-72). The age of patients ranged from five to 70 years and lesions were mostly (5/8) present at the lower extremities (Table 1). Clinical diagnosis of BU was reconfirmed at least with 2 of 3 laboratory tests applied (ZN staining, IS 2404 PCR and histopathology). All patients tested negative for HIV, completed the eight weeks of antibiotic treatment as recommended by the WHO and lesions were closed and healed for all patients by day 127 after completion of therapy [10].

Tissue processing and staining

Punch biopsies and tissue samples removed during surgical procedures were transferred to a 10% neutral buffered formalin solution for 24 hours. Afterwards samples were stored and transported in 70% ethanol, embedded into paraffin and cut into 5 µm sections with a microtome. Sections were recovered on glass slides and after deparaffinization stained with Haematoxylin/Eosin (HE) to obtain an overview of the tissue structure and with Ziehl-Neelsen (ZN) to detect acid-fast bacilli.

For immunohistochemical and immunofluorescence analysis tissue samples were stained with the antibodies and protocols listed in Table 2. For immunohistochemical staining the ABC and the NovaRED Kits from Vector laboratories were used and sections were counterstained with Haematoxylin. Immunofluorescence staining was performed by using secondary

antibodies coupled to Alexa fluor 488 or Alexa fluor 594 and sections were counterstained with DAPI.

Histopathological features of tissue biopsies

The tissue biopsies analyzed here for wound healing markers have been analyzed previously for the development of inflammatory infiltrates [10]. Shortly, before start of treatment (T1), the plaque lesions presented with an intact epidermis and dermis with relatively intact collagen and minor infiltration around glands and vessels. The subcutis appeared necrotic and edematous with fat cell ghosts. Samples taken four to five weeks after start of antibiotic treatment (T2), showed some infiltration with $CD20^+$ B-cells, $CD3^+$ T-cells and macrophages and early granuloma formation. Large necrotic areas were still present at this stage. After completion of antibiotic treatment (T3) large surgically excised tissue specimens comprised areas with largely healthy appearance, strongly infiltrated areas and completely necrotic areas without infiltration [10].

Results

Emergence of αSMA-positive, phospoS6-activated myofibroblasts in antibiotic treated BU lesions

Immunohistochemical staining for αSMA revealed staining of blood vessel walls in the dermal tissue in all eight tissue samples from BU plaque lesions collected prior to antibiotic treatment (T1) (Fig. 1 A2, B2, C2). In contrast, no staining of blood vessel walls was observed in the necrotic subcutaneous areas of 7/8 lesions (Fig. 1: A3, B3). Only 1/8 lesions (Fig. 1 C3) showed also blood vessel staining in the subcutaneous tissue before commencement of therapy. During treatment (T2) no change of the αSMA staining pattern was observed in 6/8 lesions, compared to T1. However, in 2/8 lesions, small numbers of myofibroblasts were already present at this time point in the dermis and subcutis (data not shown). After completion of antibiotic treatment (T3) an increase in αSMA staining was observed in 7/8 patients. Extensive blood vessel staining was now also found in the subcutaneous tissue, indicative for the development of new blood vessels in the previously damaged tissue areas. In addition, in 7/8 lesions αSMA-positive myofibroblasts were found in the subcutaneous tissue in association with other infiltrating cells (Fig. 1A6, B6, C6).

Notably, myofibroblast-rich subcutaneous areas contained numerous cells, which showed phosphorylation of the S6 ribosomal protein (Fig. 2A, 2B), a well-established marker for downstream effects of mTor signaling. This prompted us to investigate whether S6 is activated in myofibroblasts of healing BU lesions. Double staining with antibodies specific for αSMA and the Serine$^{235/236}$ phosphorylated version of the S6 protein were performed on the seven myofibroblast-containing BU tissue samples collected after completion of antibiotic treatment (T3). αSMA-positive fibroblasts revealed Phospho-S6$^{235/236}$ staining, which indicates that the mTor pathway is activated in the myofibroblasts emerging in antibiotic treated BU lesions (Fig. 2).

The ECM proteins tenascin, fibronectin and pro-collagen 1 are expressed in healing BU lesions

Besides αSMA also the expression and distribution of the ECM proteins tenascin, fibronectin and pro-collagen 1 turned out to reflect progression to wound healing (Fig. 3). In tissue samples from untreated BU patients (T1), only weak cellular and/or subcellular staining of fibronectin and tenascin was observed primarily in the dermal region (Fig. 3 A2, A3). The amount of these proteins increased after completion of therapy (T3) in all eight patients, as

shown in Fig. 3 for two typical patient samples (B and C). The increase in these two proteins was most evident in subcutaneous areas, where also the αSMA-positive myofibroblasts emerged.

Before treatment (T1) only few cells, primarily located in the dermal tissue layer, expressed pro-collagen 1 (Fig 3 A4). After treatment (T3) substantial numbers of pro-collagen 1-positive cells were detected all over the tissue specimen (Fig. 3 B4, C4). These were particularly abundant in areas where αSMA-positive myofibroblasts were present (Fig. 3 B1, B2), suggesting that myofibroblasts are a major source of newly synthesized pro-collagen 1 in healing BU lesions.

Enhancement of cytokeratin 16 expression in keratinocytes early after commencement of BU treatment

Cytokeratin (CK16) expression in the epidermis is a marker for keratinocyte hyper-proliferation [24], as found associated with wound healing. It is not present in healthy epidermal skin (Fig. 4 A1). Epidermal hyper proliferation is a characteristic feature in BU lesions, and in 4/8 lesions collected prior to treatment some CK16 staining of keratinocytes was observed (Fig. 4 A2). CK16 staining increased in intensity and extension during (Fig. 4 A3) and in particular after completion of antibiotic treatment (Fig 4 A4). Along with this, epidermal hyperplasia was also more pronounced after therapy in 7/8 patients. Another characteristic observed for CK16 was the heterogeneity of the intensity of staining within individual specimen (Fig. 4B, Region1, Region 2), which may reflect diversity in keratinocyte activation in different areas of BU lesions.

Emergence of apoptotic fat cells after therapy may reflect tissue remodeling

Caspase 3 is a main effector caspase of the apoptotic cascade and antibodies specific for neoantigens of cleaved caspase 3 (CC3) are a useful tool to identify apoptotic cells in paraffin embedded tissue. For all BU lesions we observed a decrease of CC3-positive cells during treatment (T2) and an increase after therapy (T3) (Fig. 5). Before commencement of antibiotic therapy CC3-positive cells were observed in very small numbers in infiltrated areas of intact dermal tissue and around remaining blood vessels (Fig. 5A). The necrotic areas were devoid of CC3-positive cells, but contained abundant numbers of fat cell ghosts, which may have already gone through apoptosis (Fig. 5B). During treatment CC3-positive cells were only very rarely observed in the dermal and subcutaneous layer (Fig. 5C, D). After completion of

treatment some of the infiltrating immune cells were CC3-positive (Fig. 5E), which may reflect physiological elimination of inflammatory cells. Notably, 6/8 patients revealed strong CC3 staining of fat cells far away from the necrotic core (Fig. 5F), which suggests that apoptosis of fat cells may be an element of tissue remodeling in healing BU lesions.

Discussion

Specific treatment of BU is highly effective since the introduction of the R/S antibiotic combination therapy in 2004 and recurrence rates could be reduced [8]. Beside this promising development wound healing and wound management is still a major problem in the remote rural BU endemic areas of Africa where patients tend to present late to hospitals [25]. While the mycobacteria may be efficiently eliminated by the specific treatment, large open wounds may persist and often prolonged wound care and skin grafting are necessary [26,27]. Why some BU lesions heal very fast while others require a long time till complete healing, is unclear and not related to the size or lesion type. Paradoxical reactions may be caused by secondary bacterial infections [28], immune reconstitution inflammatory syndrome like mechanisms [11,13,29,30] or inappropriate cell activation and disturbed transition from the inflammatory to the healing phase. For future characterization of mechanisms causing wound healing delays, we have studied here the emergence and spatial distribution of wound healing markers in healing BU lesions.

Important key players during wound healing are αSMA-positive myofibroblasts. While in healthy skin αSMA is typically present only in cells located at the walls of blood vessels and in skin adnexa, it is also produced in healing wounds by myofibroblasts, a highly specialized cell type involved in granulation tissue formation, production of ECM proteins, and wound contraction [23]. After completion of antibiotic treatment we observed these specialized fibroblasts in substantial numbers in regenerating BU lesions. Minimal presence of myofibroblasts during the treatment phase (T2) is probably related to the massive mycolactone mediated tissue necrosis which delays granulation tissue formation.

Further analysis revealed that these myofibroblasts were activated via the intracellular regulatory mTor pathway, which mediates cellular events critical in cell proliferation, movement and metabolism [31,32]. In mice PI3K-Akt activation promotes cutaneous wound repair and an elevated mTor activity strongly accelerates wound healing [33].

Therefore it is speculated that activation of this pathway in humans might help to treat large, chronic and life threatening wounds and accelerate wound healing [34]. Antibodies binding to the phosphorylated S6 protein can be used to determine whether cells show an activation of the mTor pathway [32,35]. Here we observed that, after treatment of BU lesions, many cells, most notably αSMA-positive myofibroblasts, exhibited increased S6 phosphorylation, indicating enhanced protein synthesis and/or proliferation. In contrast, phosphorylated S6 protein positive cells are barely detectable in healthy skin [36].

Malnutrition or starvation is known to repress mTor activation (most likely via Insulin/IGF deficiency) [35] and may contribute to impaired wound healing in some BU patients. Based on these results, we will investigate in a next step whether mTor pathway activation can help to discriminate between healing and non-healing wounds in BU.

Activated myofibroblasts are not only involved in wound contraction but they also produce the ECM proteins fibronectin, tenascin and different pro-collagens. Together with newly formed blood vessels and inflammatory cells they built the granulation tissue, which is a prerequisite for successful healing of dermal injuries [15]. In healthy skin, fibronectin is found in blood vessels, in dermal/epidermal junctions and in hair follicles [17,20]. In contrast tenascin-C and pro-collagen 1, which is the zymogen of collagen 1, are almost absent in the healthy skin but are abundant in healing skin lesions [37–39]. The presence of activated myofibroblasts and deposition of ECM proteins in response to BU specific therapy is a strong indication for a successful ongoing wound healing process. We observed the formation of granulation tissue in all 8 patients after completion of therapy and in 2/8 patients already at time point T2 during chemotherapy. Indeed all BU lesions analyzed in this study, showed a good clinical outcome, no recurrences were observed and patients could be discharged from the hospital 42 to 127 days after completion of therapy [10].

Although, the epidermis and dermis of plaque lesions may stay closed and intact for a long time [10], the expression of CK16 is a clear indication that also the epidermis is affected in BU lesion. CK16 is a marker of epidermal hyper-proliferation and is expressed by activated keratinocytes in wounded tissue [24]. In antibiotic treated BU lesions we observed an increase of CK16 expression over time. Absence or only faint CK16 staining in tissue taken before therapy, support the idea that the cytotoxic and immunosuppressive effects of mycolactone, which arrests the lesions in a chronic wound healing state and also suppresses keratinocyte activation. Heterogeneous staining of the epidermal layer in larger surgical excisions excised at time point T3 may reflect disease activity in the underlying tissue. Augmented epidermal CK16 expression is also characteristic for inflammatory skin diseases with a hyper-proliferative epidermis, such as psoriasis, and CK16 is used as marker to evaluate the efficacy of anti-psoriatic treatments [40]. In addition, intra-dermal injection of the pro-inflammatory cytokine interferon-gamma has been shown to increase epidermal CK16 expression [41]. Therefore, enhanced CK16 expression in BU lesions after treatment is likely the consequence of increased dermal inflammation in response to successful antibiotic treatment and mycolactone clearing.

Each phase of the complex wound healing process is characterized by the presence of a specific population of cells producing specific proteins and fulfilling specific tasks. Tissue remodeling by controlled cell death (apoptosis) is as important as proliferation. If cell death is wrongly regulated and certain cells persist after their task is accomplished this may lead to wound healing complications, like the development of hypertrophic scars or keloids [23,42]. Additionally, infiltrating inflammatory cells need to be removed after the wounded area has been cleaned.

Here we used CC3 as an established marker which is detectable in a small time window in end-stage apoptotic cells. Its short duration of expression explains the small number of positive cells in our BU lesions. It is known that mycolactone induces apoptosis in vitro and in vivo leaving behind only necrotic tissue devoid of any surviving cells [43,44]. Accordingly, at time point T1, subcutaneous tissue presented nearly devoid of any intact cell, except for some remaining cells around blood vessels and CC3 positive cells were very rare. While a further decrease of CC3 staining was observed during antibiotic treatment (T2), after treatment (T3) CC3 staining became prominent in the granulation tissue and was even more pronounced in nearby fat tissue. At this stage the wound healing process is in a state between the inflammatory and the remodeling phase. The inflammatory phase sets in shortly after the start of treatment and is characterized by a strong mixed infiltration, presence of granulomas and giant cells as well as B-cell cluster [30]. Apoptosis of inflammatory cells observed in the temporary granulation tissue at time point T3 is a necessary step in order to form healthy new tissue [15]. No apoptotic myofibroblasts were detected, since they are still needed at this stage of the wound healing process. The observed emergence of apoptotic fat cells may also be a result of the ongoing tissue remodeling process and reflect the removal of superfluous or impaired fat tissue. In conclusion this study shows that markers like αSMA, fibronectin, pro-collagen 1, tenascin-C and CK16, are suitable to monitor healing of BU lesions. The present study also suggests that the mTor pathway might play an important role during wound healing in BU. Further investigations using the presented marker set to compare healing and non-healing BU lesions may help to clarify steps and aspects of this complex process. Whether wound healing deficiencies are associated with insufficient activation of the mTor pathway needs to be examined in a larger cohort.

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Figure

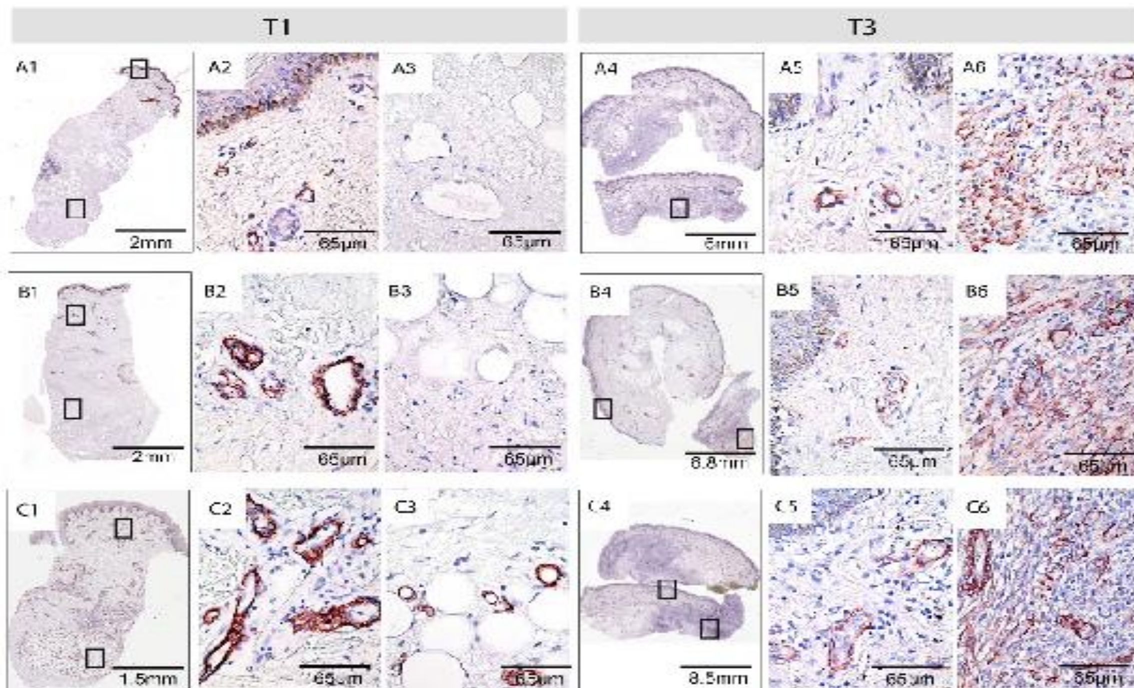

Figure 1: Emergence of αSMA-positive myofibroblasts in antibiotic treated BU lesions

Histological sections were stained with an anti-αSMA antibody and counterstained with Haematoxylin. Typical results with tissue specimens of three BU patients (A, B and C) are shown. Specimens were taken before or after completion of antibiotic therapy. A1, B1, C1: scans of the stained punch biopsies taken before commencement of antibiotic therapy. A2, B2, C2: higher magnification showing that αSMA staining in the dermis before treatment is restricted to blood vessels. A3, B3, C3: Higher magnification of the subcutaneous tissue showing blood vessel staining in only 1/8 patients (C3) and no specific staining in the other patients (A3, B3). A4, B4, C4: scans of the tissue excised after completion of antibiotic treatment. A5, B5, C5: αSMA-positive blood vessels were found in the dermis of all patients. A6, B6, C6: large numbers of αSMA positive myofibroblasts were found in strongly vascularized subcutaneous areas after completion of therapy.

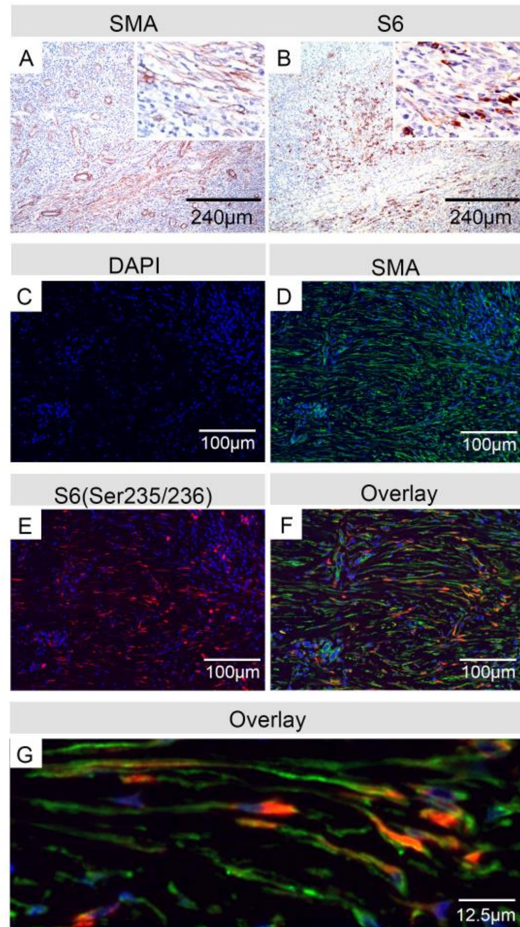

Figure 2: αSMA-positive myofibroblasts in antibiotic treated BU lesions show phosphorylation of the S6 ribosomal protein

Histological sections were stained either by immunohistochemistry and counterstained with Haematoxylin (A, B) or by immunofluorescence double staining (C-G) and DAPI counterstaining of nuclei (C). αSMA staining (A) and Phospho-$S6^{235/236}$ staining (B) were found in the same tissue areas. Immunofluorescence staining for αSMA (D) and Phospho-$S6^{235/236}$ (E) revealed co-staining of cells with fibroblast morphology (F, G), demonstrating that the mTor pathway is activated in the myofibroblasts emerging in antibiotic treated BU lesions.

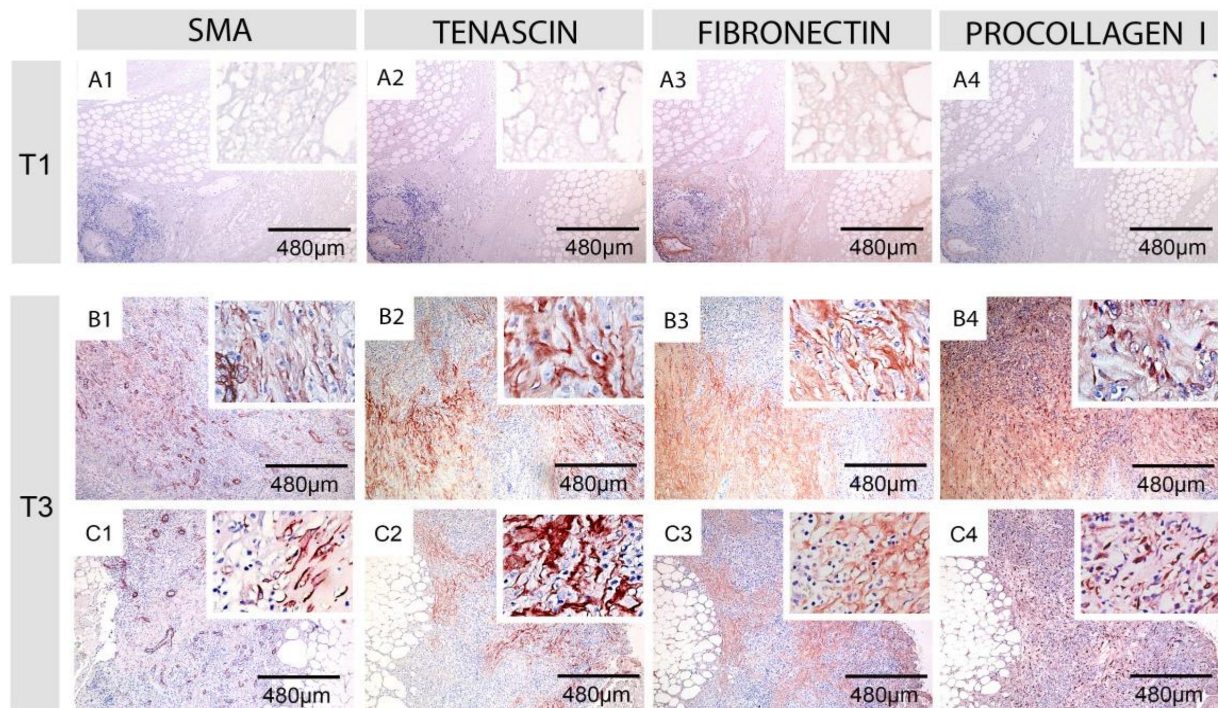

Figure 3: Increased expression of the ECM proteins tenascin, fibronectin and pro-collagen 1 in healing BU lesions

Serial histological sections were stained with antibodies against αSMA and the ECM proteins tenascin, fibronectin and pro-collagen 1 and counterstained with Haematoxylin. Panel A represents a typical lesion before commencement of antibiotic therapy (T1) and Panel B and C typical tissue specimens from two patients after completion of therapy (T3). Whereas no or only weak staining for αSMA, tenascin, fibronectin and pro-collagen 1 was observed before therapy (A1-A4), tissues turned strongly positive for all four markers after completion of treatment (B1-B4 and C1-C4). Staining of ECM proteins was most prominent in areas containing many αSMA positive myofibroblasts.

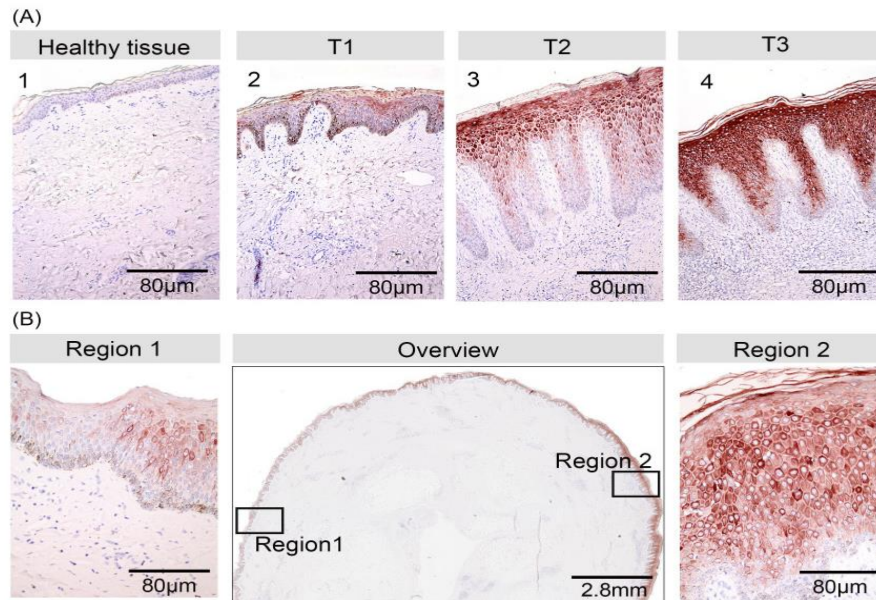

Figure 4: Increase of Cytokeratin 16 expression by keratinocytes during antibiotic therapy

Histological sections were stained by immunohistochemistry with an anti-Cytokeratin 16 antibodies and were counterstained with Haematoxylin. While healthy skin was completely devoid of Cytokeratin 16 staining (A1), some staining was observed (A2) in the epidermal layer of untreated BU lesions (T1). Staining intensity and epidermal thickness increased in samples collected during (T2) and after completion (T3) of antibiotic therapy (A3, A4). After completion of therapy (T3) heterogeneous staining (B, Overview), with some areas of the epidermal layer showing much weaker Cytokeratin 16 staining (Region 1) than others (Region 2) was observed.

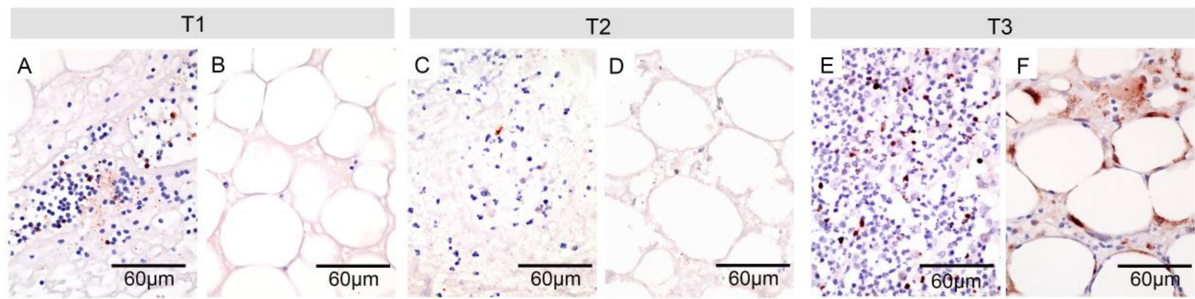

Figure 5: Emergence of apoptotic fat cells after completion of antibiotic therapy
Histological sections were stained by immunohistochemistry with anti-CC3 antibodies and were counterstained with Haematoxylin. Infiltrated necrotic areas (A, C, E) and fat cell layers (B, D, F) of the subcutaneous tissues are displayed. Before treatment some of the infiltrating cells showed CC3 staining (A). No staining was observed in the subcutaneous layer (B). During treatment (C, D) only very few cells showed CC3 staining. After treatment substantial numbers of infiltrating cells were CC3-positive (E) and in addition larger numbers of CC3-positive fat cells were found (F).

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Chapter 7

Phosphorylation of the ribosomal protein S6, a marker of mTOR pathway activation, is strongly increased in hypertrophic scars and keloids

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Abstract

Background Keloids and hypertrophic scars are caused by excessive cutaneous wound healing that is characterized by myofibroblast persistence and extracellular matrix accumulation. mTOR has been shown to be activated in keloids, and dual TORC1/2 inhibitors were reported to display more potent anti-fibrotic effects in keloid organ cultures than the more selective TORC1 inhibitor rapamycin.

Objectives To analyse the *in vivo* status of TORC1 and TORC2 signalling in normal scars, hypertrophic scars and keloids. To investigate if increased mTOR signalling is specific for myofibroblast.

Methods Formaldehyde fixed and paraffin embedded biopsies were investigated by immunostaining for ribosomal protein S6 phosphorylation (marker for TORC1 signalling), AKT(S473) phosphorylation (marker for TORC2 signalling), and α-smooth muscle actin (marker for myofibroblasts).

Results None of the investigated scar and keloid biopsies revealed detectable levels of AKT(S473) phosphorylation in fibroblasts. However, all hypertrophic scars and keloids revealed strongly increased S6 phosphorylation, which was prominent both in myofibroblasts and in α-smooth muscle actin negative connective tissue cells.

Conclusions Persistent signalling via mTOR complex 1 rather than mTOR complex 2 drives extracellular matrix over-production in hypertrophic scars and keloids.

What is already known about this topic?

- mTOR is activated in keloids, which are cutaneous overgrowths that display excessive extracellular matrix deposition by myofibroblasts.
- Both mTOR complex 1 and mTOR complex 2 signalling is thought to be required to drive fibrosis in keloid organ cultures.

What does this study add?

- Biopsies from hypertrophic scars and keloids revealed strongly increased signalling of mTOR complex 1, but not of mTOR complex 2.
- Increased mTOR complex 1 signalling in excessive scars was most prominent in fibroblasts, but not restricted to myofibroblasts.

Introduction

Hypertrophic scars and keloids are fibrous overgrowths, which may develop in response to skin injury, but spontaneous forms also exist. It has been reported that tissue extracts from keloids contain elevated levels of activated mTOR (mammalian target of rapamycin) and the downstream mTOR signalling component p70KDa S6 kinase (p70S6K)[1]. mTOR is a serin/threonine kinase which is involved in the regulation of fundamental cellular processes, such as growth, proliferation, protein synthesis and autophagy, and has emerged as an important target for the treatment of certain cancers and inflammatory diseases[2]. mTOR is part of two distinct cellular signalling complexes: mTOR complex 1 (TORC1) and mTOR complex 2 (TORC2). These complexes are differentially regulated and drive diverse cellular activities[3]. Blockade of mTOR specifically impaired expression of collagen biosynthesis genes in fibroblasts from hypertrophic scars and keloids[4]. mTOR inhibitors also showed strong anti-fibrotic activity in keloid organ cultures[5], which revealed increased TORC1 and TORC2 signalling in fibroblasts[6]. Consequently, dual TORC1/2 inhibitors displayed more potent effects in keloid organ cultures than the more selective TORC1 inhibitor rapamycin[5,6]. However, organ cultures and cell cultures may differ from the *in vivo* situation in various aspects. mTOR signalling may become up-regulated in response to tissue injury during biopsy excision, because the mTOR pathway has been found to be activated in connective tissue cells of wound granulation tissue[7]**. I**n addition, the used organ and cell culture media contain high levels of amino acids and insulin, factors which are known to activate mTOR signalling[8]. Therefore, we performed immunostaining analyses on immediately fixed biopsies to compare the *in vivo* status of mTOR activity in normal scars, hypertrophic scars and keloids. To visualize TORC1 signalling we investigated S6 phosphorylation, which is an established downstream marker of TORC1 pathway activation[9] and involved in translation initiation[10]. mTOR signalling activates p70S6 kinase which phosphorylates Ser235, Ser236, Ser240 and Ser244 of S6[11]. However, S6(Ser235) and S6(Ser236) may also be phosphorylated via the RAS/ERK pathway[12]. Therefore, we used two different anti-pS6 antibodies, which detect specifically S6(Ser235/236) or S6(Ser240) phosphorylation sites, respectively. To visualize TORC2 signalling, we stained for pAKT(S473), a downstream marker of the TORC2 pathway[3]. We also performed pS6 and alpha smooth muscle actin (αSMA) immunofluorescence double staining to investigate if the mTOR pathway is activated in myofibroblasts, a specialized contractile cell type, which plays an important role in wound healing and fibrosi[13].

Material and Methods

5µm thin paraffin sections of normal scars (n=5), hypertrophic scars (n=4), and keloids (n=6) were de-paraffinized and reacted with the following antibodies: Rabbit anti-pS6Ser(235/236) (Cell Signalling #2211; 1:400); mouse anti-pS6Ser(240) (DAKO #M7300; 1:100), rabbit anti-pAKT(S473) antibody (Cell Signaling, #4006; 1:25), and mouse anti-αSMA (Novocastra, #NCL-SMA; 1:100). Slides were then staining with Alexa488 and/or Alexa594 labelled secondary antibodies (Invitrogen; 1:500) and covered with Prolong Gold/DAPI (Invitrogen) mounting medium. The study was approved by the ethical committee of the University of Basel (EK.242/13).

Results

Anti-pS6 antibodies showed strong staining in all investigated hypertrophic scars and keloids (**red in Figure 1, b1-2**). In contrast, pS6 positive cells were rare in normal scars (**red in Figure 1, a1-2**). The vast majority of pS6 positive cells demonstrated fibroblast morphology (**Figure 1, b2**). While pS6 positive myofibroblasts were barely detectable in normal scars (**Figure 1, a1-2**), a subset of pS6 positive cells in hypertrophic scars and keloids stained also positive for the myofibroblast marker αSMA (**green in Figure 1, b1-2**). Double staining with antibodies specific for S6Ser(235/236) phosphorylation (**red in Figure 1, c1 and c3**) and S6Ser(240) phosphorylation (**green in Figure 1, c2 and c3**) revealed a largely overlapping staining pattern (**Figure 1, c3**), suggesting that S6 phosphorylation is mainly driven by mTOR signalling. Surprisingly, pAKT(S473) staining was neither detectable in normal scars (**red in Figure 2, a1-2**), nor in keloid biopsies (**red in Figure 2, b1-2**). However, the used antibody showed strong reactivity with a positive control (human breast cancer xenograft) (**red in Figure 2, c1-2**).

Discussion

These results demonstrated that S6 is strongly activated in hypertrophic scars and keloids *in vivo*, and supports the findings of organ culture experiments that mTOR signalling is increased in keloids[5,6]. We found that the vast majority of keloid fibroblasts exhibited pS6 staining, but only a subset of them displayed αSMA staining. As such, increased protein synthesis in keloids and hypertrophic scars seem not to be restricted to myofibroblasts, although this cell type has been suggested to play a key role in fibrotic diseases[13].

Surprisingly, we did not detect expression of the TORC2 signalling marker pAKT(S473) in any of the analysed scar biopsies, although enhanced pAKT(S473) staining has been demonstrated in keloid organ cultures[6]. Keloid fibroblasts display enhanced proliferation in response to wounding[14]. Therefore it is tempting to speculate that increased TORC2 signalling in organ cultures is the result of insult stimuli and subsequent tissue repair processes. Matured keloid lesions may contain only a small number of proliferating fibroblasts despite sustained matrix protein over-production for a long period of time. This may be reflected by high TORC1 and low TORC2 signalling *in vivo*, because the TORC1 pathway is important for protein synthesis, whereas dual TORC1/TORC2 signalling seems to endow cells with increased proliferative and invasion potential[2]. TORC2 signalling in non-malignant cells has been suggested to require continuous stimulation by growth factors, such as insulin like growth factor[8]. Since keloid fibroblasts have been shown to over-express insulin like growth factor receptor 1[15], they may display an increased sensitive to the mitogenic activity of insulin present at high concentrations in organ and cell culture media. Consequently, keloid organ cultures have only limited value to predict clinical effects of mTOR inhibitors.

In conclusion, we could clearly demonstrate that the TORC1 pathway is strongly activated in mature keloids, and TORC1 signalling may thus contribute to persistently elevated protein synthesis and extra-cellular matrix deposition in established lesions. These findings support the idea that mTOR inhibitors might provide therapeutic benefit for the treatment of hypertrophic scars and keloids. Whether or not, and at which stage of the disease, dual TORC1/2 inhibitors may be more effective than TORC1 selective inhibitors needs to be tested in clinical studies.

Table

Table 1: Details of investigated clinical samples

Scar type	Sex	Age (years)	Localization
Excessive scar	F	29	Shoulder
Excessive scar	F	25	Earlobe
Excessive scar	F	18	Shoulder
Excessive scar	F	52	Ear
Excessive scar	M	62	Décolleté
Excessive scar	M	70	Cheek
Excessive scar	M	64	Back
Excessive scar	M	42	Back
Excessive scar	W	56	Chest
Excessive scar	M	58	Belly
Normal scar	F	32	Lower leg
Normal scar	F	83	Cheek
Normal scar	M	65	Nose
Normal scar	M	63	Trunk
Normal scar	F	69	Forehead

Figure

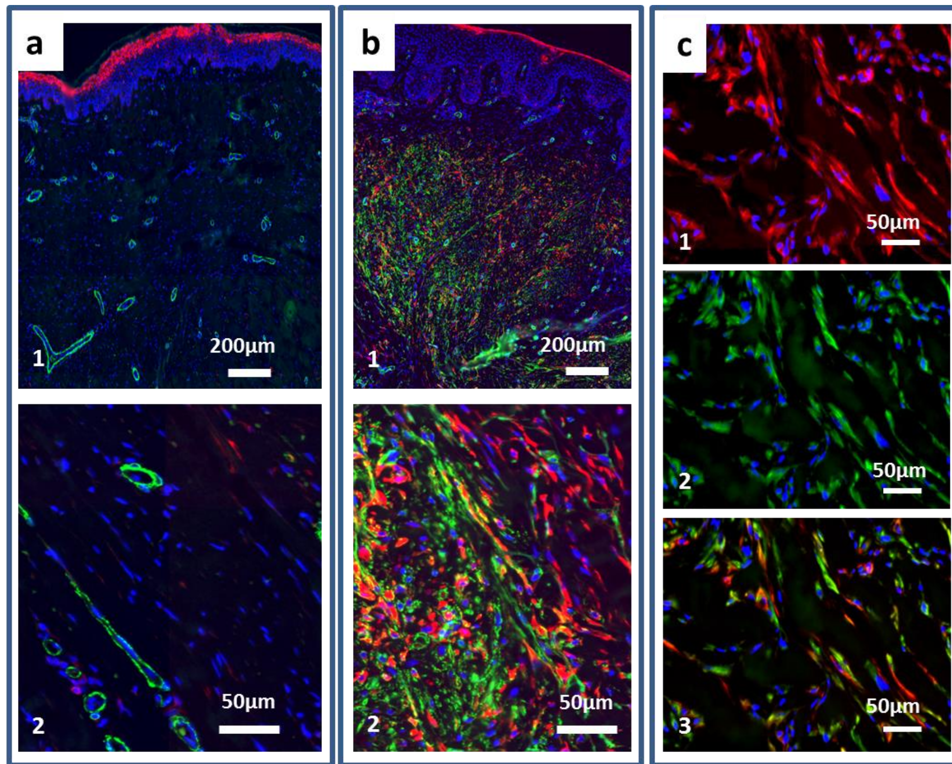

Figure 1: Immunofluorescence staining of phosphorylated S6 and alpha smooth muscle actin in normal and keloid scars.

An overview of S6(Ser235/236) (red) and αSMA (green) staining in a normal scar is shown in (**a1**), and in a keloid in (**b1**). High magnification images to visualize co-localisation of S6(Ser235/236) (red) and αSMA (green) in a normal scar are shown in (**a2**), and in a keloid in (**b2**). Overview and high magnification images in (**a**) and (**b**) are from different patients. The images in (**C**) illustrate double staining of pS6(Ser235/236) (red) and pS6(Ser240) (green) in a keloid scar – pS6(Ser235/236) staining (red) is shown in (**c1**), pS6(Ser240) (green) staining in (**c2**), and the overlay of both stainings in (**c3**).

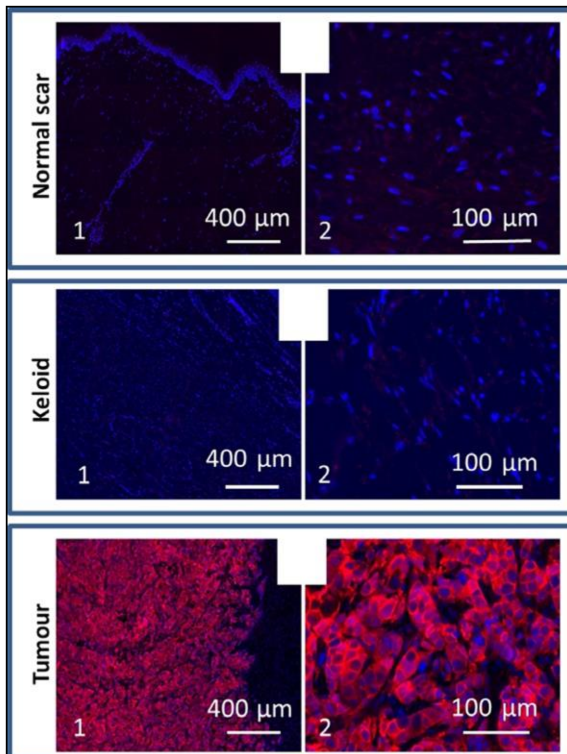

Figure 2: Immunofluorescence staining of phosphorylated AKT(S473) in a normal scar, in a keloid scar, and in a tumour xenograft.

Examples of normal scars (**a**), keloid scars (**b**), and tumour xenograft serving as positive control (**c**) are shown at low magnifications (**a1, b1, c1**), and at high magnification (**a2, b2, c2**). Overview and high magnification images in (**a**) and (**b**) are from different patients. Specific pAKT(S473) immunofluorescence signals were neither detected in normal scars (**a**), nor in keloids (**b**), whereas the anti-pAKT(S473) antibody revealed very strong immune reactivity in paraffin sections of a tumour xenograft (**c**).

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Chapter 8

Ribosomal protein S6 is hyper-activated and differentially phosphorylated in epidermal lesions of patients with psoriasis and atopic dermatitis

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Summary

Background The ribosomal protein S6 (S6) is part of the translation machinery and activated by phosphorylation via the mammalian target of rapamycin (mTOR) pathway, which is activated in psoriatic skin.

Objectives To investigate which S6 sites are phosphorylated in psoriasis and atopic dermatitis (AD), and to study whether S6 phosphorylation is associated with inflammation and/or keratinocyte hyper-proliferation.

Methods Healthy skin and skin lesions of patients with psoriasis and AD were investigated by immunostaining using antibodies which stain proliferating cells, leukocytes, and distinct phosphorylated sites of S6.

Results All psoriasis and AD lesions revealed abnormal S6 phosphorylation in the epidermis. Extent of S6 phosphorylation was diverse, generally stronger in psoriasis, and correlated in both diseases with inflammation. S6 showed differential phosphorylation in distinct epidermal layers, which was most pronounced in hyper-proliferative regions.

Conclusions Differential S6 phosphorylation may have a role in abnormal keratinocyte proliferation/differentiation.

What is already known about this topic?

- Phosphorylation of ribosomal protein S6 (S6) is important for translation initiation.
- S6 becomes phosphorylated via the mammalian target of rapamycin (mTOR) pathway, which is activated in psoriatic skin.

What does this study add?

- In psoriasis and atopic dermatitis S6 phosphorylation is associated with inflammation and epidermal hyper-proliferation.
- S6 is differentially phosphorylated in distinct layers of the diseased epidermis, which may impact keratinocyte proliferation/differentiation

Introduction

Ribosomal protein S6 (S6) is part of the 40S ribosomal subunit of the translation machinery[1]. Phosphorylation of S6 is required for translation initiation and occurs on a cluster of five serine residues at the carboxyl terminus of the protein[2,3]. The mammalian target of rapamycin (mTOR) pathway seems to be the predominant regulator of S6 activity. mTOR is a serin/threonine kinase that is part of a protein complex involved in the regulation of cell growth, proliferation, protein synthesis and autophagy, and has emerged as an important target for the treatment of certain cancers and inflammatory diseases. mTOR signalling activates S6 kinase which phosphorylates Ser235, Ser236, Ser240 and Ser244 of S6, and inhibition of mTOR causes a drastic reduction in S6 activation[4]. However, Ser235 and Ser236 can also be phosphorylated via the RAS/ERK pathway[5]. Ser247 seems to be a target of the casein kinase 1 (CK1) family of protein kinases[6].
Psoriasis and atopic dermatitis (AD) are inflammatory skin diseases with different aetiology, but often similar clinical appearance due to keratinocyte hyper-proliferation and incomplete differentiation of the epidermis. Clinical data suggest that mTOR inhibitors provide therapeutic benefit for psoriasis[7,8]. Moreover, mTOR signalling and S6Ser(235/236) phosphorylation was found to be increased in psoriatic skin.[9]
We have investigated S6 activation in depth by using antibodies, which detect specifically S6Ser(235/236), S6Ser(240/244) or S6Ser(240) phosphorylation sites. To evaluate if S6 activation is psoriasis specific or a more general characteristic of epidermal inflammation, we also analysed AD biopsies. In addition, we investigated, whether S6 phosphorylation is associated with inflammation and/or hyper-proliferation.

Material and Methods

The study was approved by the ethical committee of the University Basel (EK.242/13). All analysed biopsies were from patients that have not undergone therapy known to affect the phosphorylation status of S6.

5µm thin paraffin sections from lesional (psoriasis n=10; AD n=5) and healthy skin were de-waxed, heat pre-treated with 0.1m citrate buffer (Ph 6.0) and stained with the following antibodies.. Rabbit anti-phospho-S6Ser(235/236) (Cell Signalling #2211; 1:400); rabbit anti-phospho-S6Ser(240/244) (Cell Signalling #2215; 1:400); mouse anti-phospho-S6Ser(240) (DAKO #M7300; 1:100); rabbit anti-Ki67 (DAKO #A0067; 1:200); mouse anti-CD45 (DAKO M0701; 1:100). Immunofluorescence staining was performed by using secondary antibodies coupled to Alexa-fluor488 or Alexa-fluor594 (Invitrogen; 1:500) and mounted with Prolong® Gold/DAPI (Invitrogen).

Control immunostaining experiments with tumour xenografts treated with a selective mTOR inhibitor or placebo confirmed that the used phospho-S6 antibodies are highly specific for mTOR regulated phosphorylation sites (data not shown).

Results

While healthy human skin revealed detectable S6 activation only in the stratum granulosum (**Fig.1, a1-3**), all investigated psoriatic lesions showed abnormal phosphorylation of S6 in supra-basal and differentiating cell layers of the epidermis (**Fig.1, a4-6 and a7-9**). The staining pattern of S6 phosphorylation was diverse amongst patients with psoriasis. While some lesions revealed staining in all epidermal cell layers (**Fig.1, a4-6**), others showed staining of individual cells or cell clusters (**Fig.1, a7-9**). Psoriasis I and II illustrate extreme examples of different patients; such differences were generally less pronounced within lesions. Abnormal S6 phosphorylation was also observed in all investigated AD samples. The staining pattern showed also variation (**Fig.2, a1-3 and a4-6**), but the extent of S6 activation was generally less pronounced.

Abnormal S6 phosphorylation was associated with inflammation: double immunofluorescence staining of phosphorylated S6 and the pan-lymphocyte marker CD45, revealed that the extent of S6 phosphorylation was generally much stronger in severely inflamed areas than in those containing low numbers of inflammatory cells, both in psoriasis (**Fig.1, b1 and b2**) and in AD (**Fig.2, b1 and b2**).

Supra-basal cell layers showing abnormal S6 phosphorylation in inflamed areas contained many Ki67 positive keratinocytes (**Fig.1, c2**), whereas proliferating keratinocytes in basal layers were mostly devoid of activated S6. Phosphorylated S6 was not detected in proliferating keratinocytes of healthy human epidermis (**Fig.1, c1**).

Most notably, antibodies against different phosphorylation sites of S6 exhibited distinct staining patterns. This was most evident in strongly inflamed, highly active areas where all supra-basal epidermal layers showed S6 activation (**Fig.1, a4-6**). Such lesions revealed S6Ser(240) phosphorylation predominantly in lower epidermal layers (**Fig.1, a5 and a6**) containing many proliferating cells (**Fig.1, c2**), whereas S6Ser(235/236) phosphorylation was found to be also strong in differentiating compartments (**Fig.1, a4 and a6**). S6 phosphorylation in basal epidermal cells was rare and restricted to small areas. Weakly inflamed areas revealed supra-basal co-phosphorylation of S6Ser(240) and S6Ser(235/236) in only a subset of cells, but S6Ser(240) phosphorylation also in differentiating layers of the epidermis (**Fig.1, a7-9**).

An antibody recognizing both S6Ser(240) and S6Ser(244) phosphorylation also revealed a staining pattern which differed from that obtained with the S6Ser(240) selective antibody, and indicated stronger S6Ser(244) phosphorylation in differentiating compartments of the hyper-proliferative epidermis (**Fig.1, d1-2**).

Discussion

Our findings confirm that the mTOR pathway is activated in psoriatic skin, but do not support the conclusion that S6 is predominantly activated in upper epidermal layers[9]. Buerger *et al.* used S6Ser(235/236) phosphorylation as downstream marker for mTOR activity, but phosphorylation of this site may also be stimulated by RAS/ERK signalling[5], known to be active in psoriasis[10]. Notably, Wang et al.[11] reported increased activated ERK1 immunostaining in differentiating compartments of the psoriatic epidermis, which suggests that RAS/ERK signalling actually contributes to S6Ser(235/236) phosphorylation.

We found that S6 is differentially phosphorylated in the psoriatic epidermis. The S6Ser(240) site is peculiar with respect to its predominant phosphorylation in proliferating compartments of highly active lesions. In contrast, weakly inflamed lesions, which showed scattered S6 phosphorylation in proliferating compartments, revealed S6Ser(240) phosphorylation also in differentiating compartments. This may indicate normalization of epidermal differentiation in regressing lesions, because S6Ser(240) is phosphorylated in the stratum granulosum of healthy skin. Therefore it is tempting to speculate that activation of S6 has a role in terminal differentiation of keratinocytes.

We found increased S6 phosphorylation also in AD, suggesting that mTOR is generally activated in inflammatory skin diseases with epidermal hyper-proliferation. Inflammatory cells secrete growth factors, such as insulin like growth fator-1 (IGF-1), known to activate the mTOR pathway[12], and to inhibit keratinocyte differentiation[13]. Beside this, clinical improvement of psoriasis was found to be associated with decreased IGF-1 expression[14].

S6 activation seems to be dispensable for normal keratinocyte proliferation, because S6 phosphorylation is not evident in dividing keratinocytes of healthy skin. Thus, mTOR inhibitors may selectively target pathologically activated keratinocytes, a hypothesis supported by the observation that mTOR inhibition suppresses UVB-induced keratinocyte hyper-proliferation[15], but does not affect normal epidermal turnover.

mTOR inhibition might help to treat hyper-proliferative skin diseases. Analysis of site specific S6 phosphorylation in skin biopsies could be useful to assess pharmacodynamics of mTOR inhibitors in clinical trials. Finally, differential phosphorylation of S6 may have implications for tumour growth, which should be taken into account when analysing tumour samples.

Figure

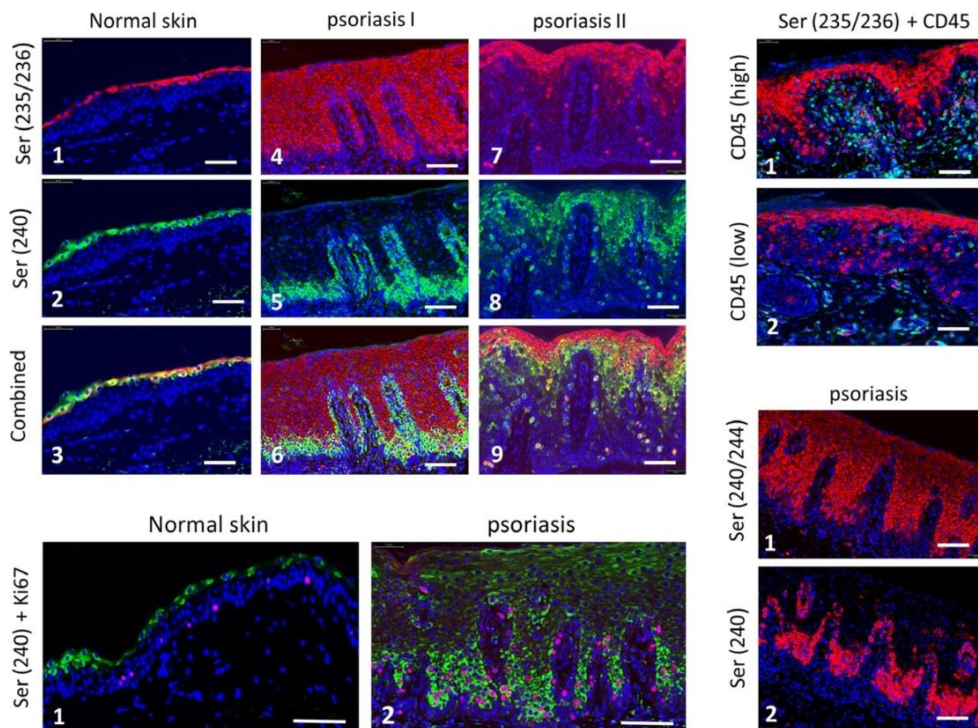

Figure 1: Immunofluorescence images showing differential S6 phosphorylation, CD45 expression and Ki67 positive cells in psoriatic lesions.

In (**a**) staining of phospho-S6Ser(235/236) is shown in red (**a1, a4 and a7**) and staining of phospho-S6Ser(240) in green (**a2, a5 and a8**); overlays of both stainings are shown in **a3, a6 and a9**. In normal skin (**a1-3**) S6 phosphorylation was restricted to the stratum granulosum. In contrast, psoriatic lesions also revealed S6 phosphorylation in suprabasal cell layers (**a4-6 and a7-9**). The staining patter was highly variable between patients as shown by the following two examples. Psoriasis lesion I (**a4-6**): staining of phospho-S6Ser(235/236) (**a4**) was visible in all supra-basal epidermal layers and more prominent in differentiating compartments, whereas staining of phospho-S6Ser(240) (**a5**) was mostly restricted to proliferating compartments. Psoriasis lesion II (**a7-9**): Ser235/236 (**a7**) and Ser240 (**a8**) were co-phosphorylated in a small subset of supra-basal cells, but showed distinct phosphorylation patterns in differentiating layers. Double staining of CD45 (**green in b1 and b2**) and phospho-S6Ser(235/236) (**red in b1 and b2**) showed S6 phosphorylation in all epidermal layers of a strongly inflamed lesion (**b1**), but restricted S6 phosphorylation in a weakly inflamed lesion (**b2**). Double staining of phospho-S6(Ser240) (**green in c2**) and Ki67 (**red in c2**) showed that S6 was activated in many proliferating keratinocytes. Most double stained cells were located in supra-basal compartments of the psoriatic epidermis (**c2**), but S6 activation was not detectable in proliferating keratinocytes of normal skin (**c1**). Ser240/244 and Ser240 were also differentially phosphorylated in a highly active psoriatic lesion, where Ser240/244 phosphorylation was strong in both supra-basal and differentiating layers (**d1**), whereas Ser240 phosphorylation was mostly restricted to proliferating epidermal layers (**d2**). Scale bars: 50µm.

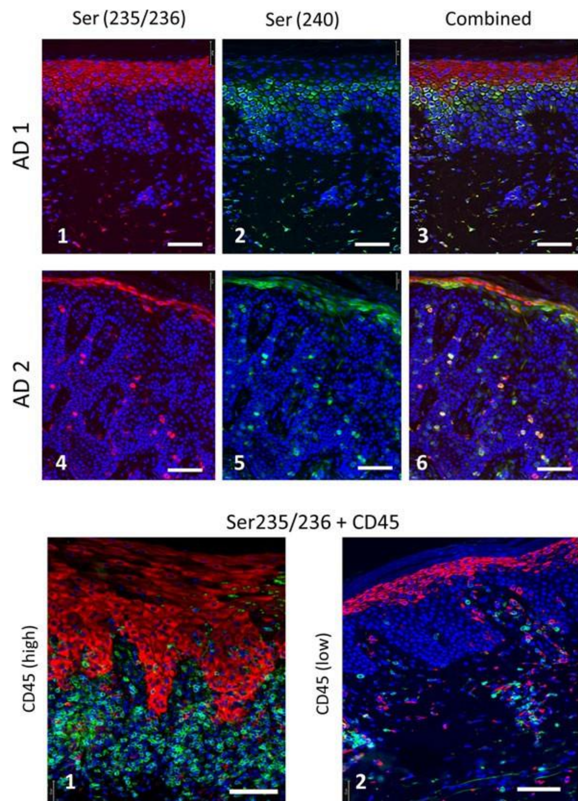

Figure 2: Immunofluorescence images showing differential S6 phosphorylation and inflammation in AD lesions.

S6 phosphorylation patterns found in AD lesions (**a1-3 and a4-6**). AD lesion I (**a1-3**): staining of phospho-S6Ser(235/236) (**red in a1**) was most prominent in differentiating compartments, whereas staining of phospho-S6Ser(240) (**green in a2**) was mostly restricted to proliferating compartments. AD lesion II (**a4-6**): staining of phospho-S6Ser(235/236) (**red in a4**) was observed in a subset of epidermal cells scattered throughout the epidermis, and in the stratum granulosum. Most phospho-S6Ser(235/236) positive cells also revealed phospho-S6Ser(240) staining (**green in a5**). Overlays are shown in **a3 and a6**. Double staining of CD45 (**green in b1 and b2**) and phospho-S6Ser(235/236) (**red in b1 and b2**) showed S6 phosphorylation in all epidermal layers of strongly inflamed AD lesions (**b1**), and restricted S6 phosphorylation in weakly inflamed lesions (**b2**). Scale bars: 50µm.

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Chapter 9

General remarks and Discussion

9.1 General remarks

Buruli ulcer (BU) is one of the 17 neglected tropical diseases (NTDs) currently listed by the WHO, which affect more than one billion people, mainly populations living in poverty [1]. All these diseases inflict a great suffering on the affected population resulting in concrete and substantial public health problems [2]. Since the Yamoussoukro conference in 1998 major research efforts and effective control interventions for BU have been undertaken in order to gain deeper insights into the disease and to prevent the population at risk from sufferings [3].

Indeed, in the recent years the number of publications and consequently the knowledge acquired on BU disease, have substantially increased. However, some aspects still remain unclear. Specifically major questions like the nature of the natural reservoir and mode of transmission are still unanswered; as well, the mechanisms leading to the different clinical forms and also the mode of action of mycolactone all remain to be clarified. Beside these still open research questions, over the years and thanks to lessons learned from the field, numerous aspects of BU care and management/control of the disease have been improved. However, even if the early case detection and an improved differential diagnosis in order to properly manage the patient and to administrate the right treatment have been implemented over the past years, they still remain to be optimized.

Within the framework of this thesis, we have contribute to strengthen the BU treatment and research site in the Bankim Health District (HD) of Cameroon [4]. A surveillance system has been implemented to promote a continuous case detection and follow up of patients to investigate the pathway of transmission (Chapter 3) and to support a comprehensive epidemiological and spatial distribution analysis of the BU evolution over time in the described region (Chapter 4). Additionally, the remarkable case series observed over the years, led to the disclosure of relevant associations between BU disease and local beliefs (Chapter 5).

Furthermore, we investigated the BU treatment outcome to gain deeper insights into wound healing (Chapter 6) and the scarring process (Chapter 7). As a comparator, we additionally examined other inflammatory and proliferative skin diseases (Chapter 8).

9.2 BU control and transmission

In 1998, the World Health Organization (WHO) established the Global Buruli Ulcer Initiative to raise awareness and to coordinate the BU control and research activities [5]. In 2014 around 2200 new cases were reported from 12 out of the 33 BU endemic countries. It has been noticed that since 2010 the number of new cases has been declining in countries actively reporting to the WHO [6]. The reasons behind this observed decline are not clear and hypotheses range from the lack of data collection and bad reporting systems, especially in remote areas, to a lack of awareness and reduced knowledge [7]. On the other hand, especially in African countries, this trend at least partially, may be also due to the establishment of effective national BU control programs which led to a reduction of untreated cases and therefore also a reduction in patients with open active lesions which could be involved in the transmission by spreading the bacteria into the environment. In our study area, the Bankim Health District (HD), we observed over 5 years of activities - from 2010 till 2014 - a progressive decrease in suspected BU cases. At the beginning (2010) of the control activities an active case-search was conducted with trained teams from the Bankim hospital and the foundation FAIRMED carrying-out screening and sensitization campaigns targeting communities suspected to be at risk of BU. At the same time training for community volunteers has been provided to increase participation and responsibilities in the villages. Volunteers acted for example as interface between the patients and the treatment centers. Progressively with the increase of the communities' awareness, the active case finding approach was converted into passive case finding, with cases coming on their own to the treatment centers. In late 2015 an active in depth survey was realized in the Bankim area, in the frame of an integrated campaign for the surveillance and control of major skin diseases including BU, yaws and leprosy. In this survey four suspected BU cases were identified and out of them one was a RT-PCR reconfirmed (personal communication J. C. Minyem and A. Um Boock). On the basis of these last observations which indicate a very low BU incidence in the area, it appears that in the Bankim HD we are experiencing a real reduction in BU cases.

The Bankim HD, in the Mapé dam region, was originally discovered as BU endemic area in 2007. In this area, the damming of the Mapé in 1989 and the resulting formation of an artificial lake were regarded as possible causes for an increase in BU incidence. In 2010, in collaboration with the FAIRMED, a new BU research site was build up in the Bankim HD. After the first survey on BU realized in the area [4], a steady flow of new cases (passive case finding) has been seen, which indicated that the BU control measures initiated were well

accepted by the local people and the personnel employed. On the basis of these good initial results, the surveillance system was strengthened and new control approaches were implemented. Beside the passive case finding, the health personnel of the Bankim hospital started to involve and train new actors such as community health workers and traditional healers in BU control activities [8]. Additionally, local populations were sensitized using health promotion videos in schools, local markets, churches and in general in places where people aggregate. The effects of these strategies needed time to be seen and despite the fact that the impact on BU control is still under evaluation, these interventions are likely to be less time and money consuming then in-depth surveys. A first evaluation showed an increased awareness and involvement of the communities at risk, as well as an increased number of cases referred to treatment centers by community health workers and traditional healers (personal communication F. Mou and A. Um Boock).

Social interventions and community participation can facilitate early BU recognition and referral of cases for early diagnosis and treatment which is fundamental to prevent the complications associated with advanced stages of the disease [6]. Patients with advanced BU are usually hospitalized for a longer period and have an increased need for extensive interventions, like surgery and skin graft [9,10]. These kind of complications can also lead to life-long sequelae and deformities which can impair the quality of life of the patients and indirectly also of their families.

The time-delay in seeking medical care for BU patients is a relevant issue for patient management but also for the health care system overall [11]. Although antimicrobial treatment and wound care are free of costs for the patients, several other expenses arise, causing delay or later potentially abandonment of treatment. A number of papers have discussed socio-cultural aspects which can affect the early case detection and the treatment seeking behaviors [12–14]. Streptomycin requires daily injections which are done at the health center every day meaning that the patients have to travel regularly or they have to stay at or close to the health center when the distances to travel are too long. In some cases the lesions are so severe that hospitalization is required. In addition, the proportion of affected children between 5 and 15 years is very high (Chapter 4) and they often require the help of a care taker especially if they have to stay at the hospital for wound care and antibiotic treatment. This implies that a member of the family has to abandon the rest of the family and their daily work. During the stay both the caretakers and the patients have to provide their own meal which adds additional costs beside the travelling efforts. A concrete solution to

minimize the stay at the health center would be a completely oral antibiotic treatment replacing streptomycin with other compounds. Currently, a valid alternative with clarithromycin - replacing streptomycin - has been identified and the results of ongoing studies appear to be promising [15]. On the other hand, proper wound care which is fundamental for a favorable wound healing progress, can be done so far only in the health center itself and a solution to this problem has to be found independently of an oral treatment. In order to solve the above indicated problems in the Bankim HD, so called “half-way houses” close to the health centers were built, for patients and relatives who live far away. Potential costs of the modern antibiotic treatment may also drive patients to consult traditional healers which are generally closer to the home village and where services can be paid for with natural goods. Traditional healing is linked to deep beliefs and remains central to the lives of many African rural communities [16]. Because of this strong cultural component people consult, as first approach [10], often traditional healers whether or not they can afford medical services. In the Bankim area traditional healers appear to play a relevant role in BU care and a system of collaboration between them and the local health system may be a good strategy [17] for a better patient management. With this collaboration, patients have the support of the traditional healers and might be less reticent to seek medical care and the health care system can benefit from receiving patients with less delay. During the past two years such a collaborative approach with traditional healers has been established in the Bankim HD, thanks to the work of anthropologists actively present in the communities and even if the benefits of this intervention are still to be formally proved, some promising results have already shown (personal communication P. Awah).

Beside the early case detection another relevant aspect associated with effective BU control is the urgent need of new diagnostic tools which are highly sensitive and specific but also easy to use in the field. Currently diagnosis in remote rural areas is mainly done on the basis of clinical signs, but because BU can be confused with many other diseases, the accuracy of clinical diagnosis may be limited [18,19]. Conditions such as tropical ulcers, venous or diabetic ulcer, yaws, tuberculosis, cutaneous leishmaniosis and also cancerous conditions, maybe miss-diagnosed as BU [20–22]. Additionally, less experienced health workers can also mistake common wounds or mycoses of the skin for BU disease. Wrongly diagnosed patients are unnecessarily treated with antibiotics and this has to be avoided in order to prevent the development of resistant mycobacteria and to avoid side effects of the medication itself. The reconfirmation of BU suspected cases clinically diagnosed, is essential

for the following reasons: to confirm that the disease is BU (1), and therefore to be able to determine the precise prevalence and incidence of BU in a given area (2), to confirm new foci (3), to appropriately manage the disease using anti-mycobacterial therapy with or without surgery (4) and to confirm the failure of treatment, relapse or reinfection after treatment (5) [19].

The current gold standard diagnostic method for BU, IS2404 qPCR, which has a very high sensitivity and specificity, requires sophisticated laboratory equipment and highly trained personal and it is not applicable in the field. Nevertheless the recent guidelines of WHO recommend that at least 70% of the suspected cases should be laboratory reconfirmed by a positive PCR result [6]. The proposed goal, even if extremely important, is also particularly challenging considering the cost of the method and the limited resources within the countries to perform such tests while assuring the necessary quality standards. Reference laboratories, which are capable of performing qPCR or other methods for the reconfirmation, such as cultivation and histopathology, are rare in BU endemic African countries and therefore samples often have to be transported to other countries for the reconfirmation, implying high cost and logistic issues. For instance in the Bankim HD, swab samples were transported to the Swiss TPH in Basel, Switzerland for laboratory reconfirmation. Over the past years during our activities, we considered the possibility to set up qPCR in Bankim, but the poor infrastructure conditions, the lack of trained personnel and also the frequent power failures hindered the sustainable establishment of this method in the area.

Additionally, building up this kind of advanced infrastructure without reinforcing the local health facilities and even more the general diagnostic laboratory platform should be avoided. Currently the only laboratory method suitable for rural health facilities and so also in Bankim, is the direct microscopy for the presence of AFBs after Ziehl-Neelsen staining of smears from ulcerative lesions or fine-needle aspirations (FNA) from nodules [23,24]. This method is easy and rapid but has a low sensitivity and a limited specificity [19]. In order to strengthen the performance and consequently the reliability of the results of the direct smear examination with Ziehl-Neelsen staining, a new microscope was installed in Bankim and training of the lab personal as well as teaching material was provided to the local laboratory technicians. A reduction of false positive results has been noticed after this intervention, indicating that indeed the training of local technician as well as adequate instrumentation are very important for the quality of BU diagnosis in the local laboratories. In 2014, FAIRMED has promoted a project to improve the health conditions of the population of the Bankim HD, by constructing a new pavilion for diagnosis and treatment of BU at the local hospital. Therein space was

dedicated for the establishment of a new laboratory. In the frame of this intervention, together with the public health system represented by the Bankim hospital, we decided to improve the laboratory infrastructure with the aim not only to strengthen research capacity in BU, but also to help clinicians and health workers in the management of BU patients and of patients suffering from other infectious and non-infectious diseases. In these premises we enabled the laboratory to perform a basic set of hematological and biochemistry tests relevant for the diagnosis and the treatment of many conditions and also to monitor the BU treatment effects. At present, the laboratory in Bankim is the only one in which basic laboratory analyses can be performed within the HD.

Despite the high number of research activities, BU is often considered a “mysterious disease” because the natural reservoir and the mode of transmission of *M. ulcerans* are still not identified. Already from studies realized during the 1970’s, the hypothesis of human-to-human spread was considered improbable so it is generally assumed that *M. ulcerans* is acquired from environmental sources [25]. BU foci in Africa and Australia are typically associated with water bodies or aquatic environment, supporting the idea that proximity and contact with flowing or stagnant water are risk factors for BU [25–27]. Other risk factors such as failure to wear protective clothes, poor wound care, and agriculture activities were identified but definitive indications were not yet obtained [28–30]. In some endemic areas it is believed that, the emergence of BU cases is related to ecological and environmental alterations which include damming of rivers or water bodies, agriculture activities, creating irrigation system and modification of water sources [26,31,32]. Such changes were suspected to be responsible for an increase in BU incidence in the Mapé dam region, where in 1989 the artificial lake was created, which led to a modification of the pre-existent environment [33] (personal communication A. Um Book). The identification of the distribution of BU cases in a given area can be very useful to elucidate the pathway of transmission and the possible origin of the infection. During the 5 years of activities carried out in the Bankim HD described here, we obtained information on the exact origin of 136 BU RT-PCR positive patients through geo-referencing of their houses. A cluster of patients was observed in the area between the Mbam river and the Mapé Dam while another cluster was located in an area upstream of the Mbam river. For these patients farms were additionally mapped in order to gather more information about people’s movements. The patients in the upstream part of the Mbam river, all except one, had their farms close to their houses and the river while for the patients living in the area between the Mapé dam and the Mbam river, the farms were far away from their

houses and again close to the Mbam river (Chapter 4). According to these findings, it is tempting to speculate that the close relation and the regular contacts between the patients and the river are associated with BU infection but so far no definite evidences were found to support this speculation.

While studies consistently associated BU with water bodies, conclusive reservoirs could not be established so far although *M. ulcerans* DNA was detected in many biological systems including insects, aquatic plants, biofilms, invertebrates, snails and soil [34–36]. Nevertheless, the presence of *M. ulcerans* DNA in the environment proofs only the presence of the mycobacteria in a given ecosystem but is not indicative of a reservoir [25]. Several studies suggest that *M. ulcerans* is unlikely to live free in the environment while it is more probable that it is associated with other protective organisms or bio-elements of water body ecosystems [37–39]. IS2404 qPCR can detect DNA in environmental samples but because there are non-human pathogenic mycobacteria that are IS2404 positive, this make the interpretation of PCR results very difficult [25,40]. The successful culture of *M. ulcerans* from an environmental sample is challenging also due to the presence of many other organisms which grow faster and so far it was achieved only once from an aquatic water strider collected in Benin [41].

In south-east Australia, in feces of possums high concentrations of *M. ulcrans* DNA were found and the possums themselves showed BU skin lesions suggesting their potential role as a reservoir [42]. In order to gain deeper insights into the transmission and the possible way of infection in the Bankim HD, we collected environmental and fecal samples from villages and farms related to BU reconfirmed patients. These samples were tested by qPCR for the presence of IS2404, IS2606 and KR. Out of the collected specimens, three village water locations and one duck fecal sample tested positive. Particularly, in one of the identified water sites we observed longitudinal persistence of *M. ulcerans* DNA in underwater detritus which may indicate the presence of a special micro-environment (Chapter 3). Hypotheses of the acquisition of the bacteria from the environment have been largely investigated and different possibilities, including skin trauma or bites from water bugs and mosquitos were suggested [43–46].

Recently, a new approach to gain deeper insights into transmission has been identified in the potential association between *M. ulcerans* and age. The results of our longitudinal observations in the Bankim HD (Chapter 4) showed that very young children below the age of 5 are underrepresented among the BU cases [4]. Data from a sero-epidemiological study, in which antibody titers against the 18kDa-small heath shock protein of *M. ulcerans* were determined in sera from Cameroonian and Ghanaian healthy people living in a BU endemic

area, suggested that children below 5 years are less exposed to *M. ulcerans* when compared to older children and adults [47]. This reduced exposure may be due to the smaller movement radius of younger children or the fact that usually babies and very young children are carried by a caregiver and therefore might be less in direct contact with the environment. Conversely, children around the age of 5 years start to be more independent as well as more involved in the family's activities or in playful activities which can be associated with displacements from home and closer contact with the environment.

Because it is likely that the increase in exposure to *M. ulcerans* is related to behavioral changes at this age, in-depth investigations of the behavior and activities of children may help to give deeper insights into *M. ulcerans* transmission. For this reason, an analysis of water contact and other environmental contact patterns of children aged between 2 and 10 years, can be a new and valid approach to capture the behavioral changes critical for the increase in exposure to *M. ulcerans.*

2.3 BU treatment and monitoring of wound healing

Until 2004, the surgical excision of *M. ulcerans* infected tissue including a healthy rim, often followed by skin grafting to facilitate the healing, was the only recommended treatment for BU [48,49]. However, these surgical interventions were particularly complicated due to the inadequate facilities in the remote rural areas and they were associated with high recurrence rates of up to 28% [49–51]. After first mouse studies showed promising treatment results by combining the two antibiotics Rifampicin and Streptomycin (RIF/STR) a pilot study on pre-ulcerative lesions realized in Ghana proved the efficacy of the combination also in human BU patients [52,53]. Based on these results, the WHO released provisional guidelines for treatment of BU by daily administration of RIF/STR for 8 weeks [49]. The routine introduction of this combination therapy in the endemic countries reduced the recurrence rates to around 2% [15,54,55]. Despite the fact that the antibiotic treatment is highly effective, it has also several limitations and complications. The daily injection of streptomycin at the health center represents one of the major burdens for patients since it requires the daily presence for 8 weeks which in turn also increases the costs and is a big obstacle for the decentralization of the care. Streptomycin is also associated with side effects including ototoxicity and nephrotoxicity especially in children [56]. Moreover, a worsening of the wound appearance is frequently observed during antibiotic treatment and even if this is not due to a treatment failure but rather a strong immune response, patients hardly accept it [57,58].

The evaluation of the efficacy of an all-oral treatment which would omit these limitations is currently ongoing and clarithromycin seems to be a good alternative, which Australia and French Guyana have already adopted as treatment for BU in combination with rifampicin [49,59]. Rifampicin is currently the most effective drug in BU treatment, but it is also one of the front line drugs used in the treatment of tuberculosis which is also highly endemic in most of the BU endemic countries. Because rifampicin is given for at least 6 months against tuberculosis, the short treatment duration for BU might foster the development of rifampicin resistance [48]. Therefore the identification of new or alternative drugs as well as alternative therapeutic approaches for BU treatment are of great relevance [60,61]. Since development of a drug is very expensive hope relies here primarily on the potential use of new tuberculosis drugs for BU.

An alternative treatment for BU is the application of heat which is based on the fact that *M. ulcerans* has a low optimal growth temperature from 28-32 °C. Based on this finding it was assumed that the application of a higher temperature onto the lesion might effectively kill the bacteria. Successful results using this rational were already reported during the seventies and later in 2009 when a thermotherapy trial was successfully performed by Junghanss et al. on six patients treated with bags filled with a phase change material (PCM) applied onto the lesion for up to 56 days [62,63]. On the basis of the promising results obtained, another bigger thermotherapy phase II trial with 53 patients was initiated in 2010 and results showed the efficacy of the local hyperthermia with a cure rate around 90 % [64]. Because the application of such PMC has only minor side effects, mainly mild blisters and skin reactions, and they were also used to treat, with positive outcome, unconfirmed skin lesion, this approach could be advantageous in rural setting where the treatment decisions are mainly taken based on clinical diagnosis only [64].

Some cases of BU that do not receive any of the established treatments discussed above, are speculated to heal spontaneously or after the application of traditional treatment [65,66]. In the course of our activities a young patient of 11 years with a laboratory reconfirmed (qPCR) BU lesion had been identified in 2011. The family refused the standard antibiotic therapy in favour of a traditional approach which consisted in the daily use of only household remedies prepared from the bark of two trees, *E. suaveolen* and *S. micranthus*. In 2013 the patient was encountered again and the ulceration at the right elbow had completely healed without disabilities. In 2014, the patient presented with a very good scar and without any

signs of relapse (Chapter 5). It is known that products derived from these trees can be used to manage wounds in general, however we report here only a single patient and more studies are needed to assess the pharmacological properties of the extracts used. On the other hand it has been shown that the application of herbal preparations or other traditional practices can cause a worsening of BU lesions [67–69], therefore to seek care at the formal health system should always be the first choice [70].

Cases of healing without standard medical treatment are likely to be rare but have been reported. In the presented case we speculate that plant derivatives can play a role in the resolution of the lesion but of course many others aspects like the immune response, the wound care quality or other aspects of care such as feeding or psychosocial support can all be important for a favourable outcome. The presented case points out two interesting aspects: the first is the importance of a good follow up and a close observation of patients enrolled in the cohort, and the second is the growing attention on alternative therapeutic approaches for the treatment of BU. Once a patient is registered at a treatment centre, it is of great importance for the public health and research purposes that he/she is carefully documented, with detailed and reliable information.
During the last few years the number of publications showing the potential therapeutic properties of derivatives and compounds originating from plants has appreciably increased and indeed many drugs currently used for the treatment of different diseases are derivatives of plants: this is for example the case for digoxin against certain heart conditions or quinine and artemisinin derivatives to cure malaria [71,72]. Additionally, in traditional medicine, plants are commonly used to manage wounds. Studies on plants from BU endemic areas in West Africa belonging to the traditional medicine repertoire are ongoing and *in vitro* experiments on their anti-inflammatory and antibacterial activities, show promising results [73–76].

Another relevant component of the clinical management of BU cases is the wound care practice. Basic principles of wound care are to maintain the wound moist and clean, to prevent the wound from additional trauma and to control infections [77]; these basic guidelines can be very helpful to reduce the time to healing and promote good treatment outcome by limiting the occurrence of sequelae [78]. Despite its fundamental role, wound care has been neglected for a long time, probably also because it substantially contributes to the BU treatment costs. Unlike the costs of drugs, it is difficult to calculate how much wound care material will be required per patient or how much time the health staff will need to assure good wound care

quality. Measures to reduce costs such as the not uncommon practice of letting the patients wash their own bandages instead of using each time a new one, are highly problematic. In order to perform wound cleaning and dressing, a clean environment and dedicated rooms are required and health staff has to be appropriately trained not only on the medical techniques, but also in cleaning and the basic principles of hygiene. In recent years, WHO underlined the importance of this wound care to obtain a better treatment outcome and standardized guidelines were developed and implemented on how BU wounds, according to size or presence of secondary infection, should be handled [78]. In this line, since the beginning of our activities at the Bankim hospital the wound care practice was improved: dedicated staff was trained by local experts and dedicated rooms for BU wound care became available.
Together with wound care, prevention of disability (POD) activities are required to prevent sequaelae, such as contractures and stiffness of joints [78]. Because BU it is often associated with deformities and disabilities which can affect the life of the patient in many aspects including restriction of social participation due to stigma, the early rehabilitation already right after the diagnosis should be an integral part of the case management [79,80]. The combined approach of antibiotic treatment, wound care and POD activities can lead to reduced stress and difficulties for the patient and can help the health system to save money and resources by having less complicated cases.

While it is often stated that BU lesions are painless, patients often complain about pain during wound care. A recent study by Alferink *et al*. investigated the pain associated with the wound care practice and other factors associated with pain in general in BU patients. Three categories of pain were identified ranging from the pain which appears right before and during the wound care which is mostly attributed to fear, and a third category of pain experienced during the removal of the bandages and during the cleaning of the wound [81]. It is likely that secondary infections which are very common in BU lesions, especially when already in advanced stages, are often responsible for the pain [82]. Several photogenic species such as *S. aureus*, *P. aeruginosa*, *S. haemolyticus* and *K. pneumonia* were isolated from BU lesions showing signs of inflammation, including pain [83,84]. While secondary infections and inflammation may contribute to delays in wound healing [85], inflammation is also physiological part of the wound healing process important to remove the affecting microorganisms [86,87].

The management of the wound is a crucial step to promote the wound healing process but different lesions can behave differently and there are wounds which heal easily, while others exhibit an impaired wound healing. Such wounds are often arrested in a state of pathological inflammation and enter a chronic state. Wounds can persist for a very long time in this stage before resolving. The wound healing process itself is very complicated and it consist of different phases which overlap each other and involve many different elements [88]. The factors affecting tissue repair are still not completely elucidated and the potential involvement of cellular and molecular mechanisms is under investigation [86]. In general, the factors leading to impaired healing can be classified into local and systemic factors; the local factors such as edema, necrosis, infection or low oxygen directly influence the wound while the systemic factors act on the general health status of the patient and can be for instance age, stress, co-morbidities or medications [86,89]. They are not mutually exclusive but in general they act simultaneously contributing to the final outcome of the wound.

Advances in cellular and molecular biology have contributed to an expanded knowledge on wound healing pathophysiology, followed by investigations whether biomarkers can be used to assess the condition of a wound and also predict its evolution. Pro-inflammatory cytokines, proteases and protease inhibitors seem to have the greatest potential as biomarkers in order to study the status of a wound [90]. Extracellular or intracellular proteins including cytokines and ECM proteins can also be used as histopathological markers to monitor the events during the healing process in tissue biopsies.

Despite the fact that also acute wounds can be very problematic, the major focus of research activities, concerning wound healing, lies on chronic non healing wounds, such as diabetic or venous ulcers which are responsible for a high morbidity [88]. BU lesions are indeed chronic wounds associated with high morbidity and in the worst cases also mortality [91]. Considering the literature available on other chronic wounds and using the histopathological approach already well-established for the diagnosis and monitoring of the treatment response in BU, we have analyzed markers of cell activation, myofibroblast formation and matrix deposition in tissue biopsies from BU lesions before, during and after treatment (Chapter 6). We were able to identify a set of markers which are appropriate to evaluate formation of granulation tissue (alpha-smooth muscle positive fibroblasts), matrix deposition (pro-collagen 1, fibronectin and tenascin C), cell activation (phosphorylated S6), hyper proliferation of the epidermis (cytokeratin 16) and apoptosis (cleaved caspase 3) during wound healing.

In BU lesions, beside the wound healing delay, scarring problems such as the formation of keloids and hypertrophic scars caused by an over production of collagen, have been reported.

In order to evaluate the suitability of our biomarkers we applied part of them to hypertrophic scars and keloid samples from non-BU samples (Chapter 7) and noticed a persistent signalling via mTOR complex 1 which induces an extracellular matrix over-production. Additionally we used our markers to evaluate the status of skin samples obtained from patients suffering of chronic inflammatory diseases such as psoriasis and atopic dermatitis to verify if our markers are broadly applicable (Chapter 8). Considering these findings the identified markers are likely to be suitable to evaluate the progression of tissue repair and also to study the functional basis of impaired wound healing conditions.

Other biomarkers currently under investigation are the matrix metalloproteinases (MMPs) which are present in the wound fluid and are considered to be good predictors for the healing process [92]. High levels of those proteins in wound fluids are characteristic for an ongoing inflammatory response which leads to a disruption of the tissue through a constant breakdown of extracellular matrix elements (ECMs) and the arrestment of a wound in a chronic state [93]. The MMP family is composed of many different metallomatrixproteinases but in regard to chronic wounds and their persistent healing problem, MMP-9 and MMP-2 are the key players [94]. Currently several techniques are available for the identification of those proteins in wound fluids but the commonly used method is a substrate zymography which identifies the presence and amount of MMPs by measuring the degradation ability of gelatin [95].

In collaboration with the University hospital of Basel a study is currently ongoing to evaluate the amount of MMP-9 in the wound fluids from venous, diabetic or decubitus ulcers. This study aims to test the (I) efficacy of the zymography technique to detect MMP-9 in the described samples, (II) stability of MMP9 in wound fluid (III) to estimate the level of MMP-9 in the different samples and to link the amount of MMP9 with the clinical outcome and (IV) to evaluate the suitability of this method in order to use it for the analysis of BU wound exudates. If MMPs are also playing a role during wound healing of BU lesions needs to be evaluated.

9.4 Conclusion

BU disease is a major public health problem in the affected communities. Despite the fact that major questions such as the natural reservoir of *M. ulcerans* and how it is transmitted are not yet fully understood, many improvements have been achieved in terms of disease control. From the lesson learned from 5 years of BU control and research activities in the Bankim HD, it is clear that it is of great importance to reduce the burden of BU on the individual patient and communities at risk by sensitization and active involvement of communities' members including the traditional healers. Efforts towards early case detection led to a decrease of severe cases and an earlier treatment in turn is associated with shorter healing times and a reduced danger to develop disabilities.
Furthermore, to strengthen the local diagnosis capabilities and to promote a good management of the patients, proper wound care practice and early rehabilitation, have been recognized to significantly improve the good outcome of the disease assuring the best quality of life possible for the patients.

In this thesis, in particular, aspects of BU clinical epidemiology and transmission were investigated:

- We identified environmental contact sites of BU patients which are potentially involved in the transmission of the disease; *M. ulcerans* DNA was found in three village water sites and at one of these, an in depth investigation revealed a persistent presence of *M. ulcerans* DNA over more than two years suggesting that this location may represent a natural reservoir of the pathogen.

- In depth analyses of the geographical distribution of households and farms were realized on PCR reconfirmed BU cases. The results of the longitudinal observations confirmed that BU is predominantly a pediatric disease in Africa and that the lesions occur mainly at the limbs without differences among sex. Furthermore, the analysis of the geographic distribution of cases in the district revealed an association with the Mbam river leading to speculations on its role in BU transmission.

- Cultural and traditional aspects are deeply rooted in the Bankim HD and they are also linked to the treatment of BU disease, as shown in the case of a BU patient that healed after receiving only household remedies.

Additionally in this thesis, histopathological analyses monitoring the healing and scarring process of BU lesions were performed. Biomarkers suitable to monitor cell activation, myofibroblast formation and matrix deposition in tissue biopsies from BU lesions before, during and after treatment were identified and used to characterize tissue regeneration, not only in BU lesions, but more broadly also in other proliferative and inflammatory skin diseases.

Overall, the results obtained from our research projects and the activities carried out by the national BU control program demonstrate that a combined and multidisciplinary approach can contribute both to a better understanding and improved control of BU disease.

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