Final Publishable Summary Report

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Coordinator:	Prof. Dr. Bernhard Fleischer
Organization:	Bernhard-Nocht-Institut fuer Tropenmedizin
Tel:	+49 40 42818 401
Fax:	+49 40 42818 400
E-mail:	Fleischer@bni-hamburg.de

Executive summary

Buruli ulcer disease (BUD), caused by *Mycobacterium ulcerans*, is a severe neglected infection occurring mostly in African rural areas affecting predominantly children. The mode of transmission is still unknown. Based on evidence for a protective immunity the general objectives of BuruliVac were to identify and characterize possible mechanisms of immunity and of pathogenesis, to develop novel vaccine candidates suitable for translation into clinical application and to obtain new preventive and therapeutic tools that will be of direct benefit for the patients. A major objective of the project was capacity building in 4 African countries. Partner institutions should be enabled to perform immunological analyses on BUD patients and to use standardized procedures for active case finding, for laboratory confirmation, treatment, and data management. An efficient dissemination and exploitation strategy was to be implemented and it was to be ensured that all procedures were carried out in accordance with international standards of the ethical conduct of research in humans as well as in experimental animal studies.

Seminal discoveries about the action of mycolactone were made and important results on the immunity against *M. ulcerans* in patients and in animal systems. We have made a big step in understanding of the pathophysiology of BUD by identifying molecular targets of the bacterial toxin. It was found that the Wiskott-Aldrich syndrome protein (WASp) and WAS-like neural WASp (N-WASp) are major targets of the bacterial mycolactone (ML) toxin. Using a combination of biochemical assays, cellular imaging and animal models, we found that ML mimics physiological signals normally delivered by Rho GTPases to deviate N-WASp-dependent actin polymerization. We showed that ML-induced activation of N-WASp in epithelial cells and the consequent dynamic rearrangements of the actin cytoskeleton dramatically impair the integrity of the epidermis, thus providing a molecular mechanism underpinning Buruli ulcer pathogenesis. We could demonstrate that the toxin is present in

tissue and blood of BUD patients enabling us to characterize its kinetics during disease and treatment.

Several live, attenuated *M. ulcerans* strains were evaluated and their residual virulence was assessed. These results provide proof of concept that mycolactone-negative strains have the potential to confer a significant degree of protection against infection with virulent *M. ulcerans* bacteria. Whole genomes of four different attenuated *M. ulcerans* strains were sequenced and one mutant was further attenuated by deleting the transcriptional regulator PhoP. A prime-boost strategy was established and showed a highly significant protective efficacy in animal models. A new model was established in guinea pigs and was shown to be a suitable correlate of the human infection. Moreover based on a multi-dimensional selection process, four subunit target antigens were selected and the production protocols for large quantities of these were established.

The project has succeeded in extensive capacity building on immunology and diagnostics in the field of Buruli ulcer in 4 African institutions. Quality System Procedures and Standard Operating Procedures for patient management, collection of diagnostic samples and laboratory confirmation are in place. At the end of the project sustainable structures have been established in the institutes of the African partners that can be used in future diagnostic and immunological research. Furthermore a web-based database was set-up which will be provided to the WHO.

The project has also substantially increased the awareness for this important but neglected disease world-wide both in the scientific community and in the population of endemic countries. The scientific results have been documented in many excellent publications, many with joint authorships among different partners of the consortium proving the extensive cooperation during the BuruliVac project.

Project context and main objectives

Context

Buruli ulcer disease (BUD) has emerged since 1980 as an important cause of human suffering. It is a poverty-related mutilating disease caused by Mycobacterium ulcerans. BUD is the third most common mycobacterial disease in humans after tuberculosis and leprosy and the most poorly understood of these three diseases. Though mortality is low, morbidity and subsequent disability are very high. The disease presents as an indolent necrotizing disease of the skin, subcutaneous tissue and bone and can afflict all age groups but children under 15 years represent the largest part of the BUD disease burden. The disease remained largely ignored by many national public health programs for decades. In 1998, the World Health Organization recognized BUD as an emerging health problem, primarily because of its frequent disabling and stigmatizing complications.

The disease is most endemic in West Africa with incidences as high as 280 / 100,000 but cases have been reported from more than 30 countries around the globe. It is endemic in rural wetlands of tropical countries of Africa, America, Asia and Australia. Cases have also been reported in non-tropical areas of Australia, Japan and China. Known incidence rates are highest in West Africa, particularly in Benin, Côte d'Ivoire and Ghana where between 1000 and 2000 cases are reported annually. In some West African countries, the number of BUD cases may even exceed those of tuberculosis and leprosy. There is evidence of enormous under-reporting of the disease. Little is known about the focal epidemiology of BUD. Incidence, prevalence, and other data are usually reported at the national or district level. These data show the importance of the disease but do not reveal the wide variations that often exist at the village level within a given district.

BUD is an infectious disease but not contagious. There is now sufficient evidence from microbiological and epidemiological data, including studies of risk factors, to consider BUD a water-related disease. However, the exact mode(s) of transmission from the environment and the ultimate natural reservoir(s) of infection remain obscure. Humans probably become infected by traumatic introduction of M. ulcerans into skin from the overlying M. ulcerans-contaminated surface. Contamination of the skin could result from direct exposure to stagnant water, aerosols arising from ponds and swamp surfaces or fomites.

In tropical rural settings where BUD is endemic and scantily dressed people play and work, avoiding contact with the M. ulcerans contaminated environment is virtually impossible. Wearing protective clothing when farming and immediate cleansing of any skin injury may reduce rates of infection, but achieving these measures is seldom feasible.

Use of protected sources of water for domestic purposes reduces exposure to M. ulcerans contaminated water and consequently may reduce prevalence rates of BUD. The problem of reducing risk factors for basic agricultural workers, fishermen and others who must put themselves at risk, remains, however, a serious concern.

M. ulcerans is genetically very close to M. marinum, an intracellular pathogen that triggers inflammatory responses and cell-mediated immunity but it is unique among pathogenic mycobacteria since it produces a family of toxic macrolides, the mycolactones, which are required for virulence. Mycolactones are secreted and diffuse into the infected tissues and surrounding areas, but the amount and precise distribution of the toxin in the lesions is not known. Mycolactones have a potent cytotoxic activity that induces apoptosis and necrosis of several cell types including adipocytes, fibroblasts and leukocytes, and participate in the tissue necrosis typical of the disease. Recently, studies using animal models have shown that mycolactone distributes beyond the sphere of its cytocidal action and gains access to the blood and lymphoid organs, where it concentrates in mononuclear cell subsets. However, the mechanisms of action in these cells and functional consequences of these interactions are still unclear. Moreover, the amount and precise distribution of the toxin in human hosts during the course of infection remains to be investigated.

Resistance to M. ulcerans has been associated with the development of Th1 type responses and as BUD disease progresses to healing, granuloma formation has been reported and the DTH burulin (which is a crude, heat-killed bacterial sonicate) skin test tends to change from negative to positive. In contrast, disseminated BUD disease and bone involvement have been reported to be associated with defects in granuloma formation.

As in tuberculosis and leprosy, the macrophage activating cytokine IFN-g seems to play a pivotal role in the control of M. ulcerans infection, and PBMC from BUD patients display a reduced capacity to produce this cytokine upon in vitro stimulation with whole M. ulcerans bacilli. An extensive real-time PCR analysis on skin biopsies of patients with early nodules and patients with late-stage ulcers showed a significantly higher expression of IL-8 and other pro-inflammatory cytokines in 32 biopsies with neutrophilia than in those from biopsies without acute inflammation. M. ulcerans infection-associated reduction of IFN-g responses is not restricted to mycobacterial antigens and resolves after surgical excision of the lesion, suggesting that bacterial factors such as mycolactone may diffuse from bacillar colonies and exert immunosuppressive effects at the systemic level. This hypothesis is supported by observations that non-cytotoxic doses of mycolactone efficiently suppress the functions of several types of mononuclear cells in vitro. At nanomolar concentrations, mycolactone inhibits the activation-induced production of IL-2 by human lymphocytes and of TNF by monocytes and macrophages. Mycolactone also blocks the capacity of dendritic cells to prime cellular responses and to produce chemotactic signals of inflammation. Lymphocytes, monocytes, DCs and macrophages compose the mononuclear cell fraction of blood and lymphoid organs. The fact that mycolactone targets mononuclear cells in mice infected with M. ulcerans thus strongly suggests that these cell subsets are immunosuppressed in infected hosts, and that mycolactone impairs the development of cellular immunity. In this model, neutralizing the immunosuppression imposed by mycolactone using inhibitors of its biosynthesis, or ablating its biological activity in vivo, would considerably enhance the efficacy of therapeutic vaccines and antibiotic treatments.

Recently treatment with antibiotics has had considerable success but it has major resource implications. It requires 8 weeks treatment with oral rifampicin and intramuscular streptomycin and the economic and humanitarian costs are high, with loss of workforce and children missing school. There is a risk of treatment failure and a potential for the development of drug resistance. Since the mode of transmission is unknown, the strategy for prevention which is most likely to be successful is development of a vaccine against M. ulcerans which would protect persons at risk in highly endemic areas. There is also the potential for using some of the novel types of vaccine proposed as therapeutic vaccines to shorten duration of treatment and to prevent or attenuate sequelae and relapses.

Concept

The BuruliVac project is based on the concept based on evidence for a protective immunity that Buruli Ulcer Disease can be prevented or positively influenced by the immune response, and that both a protective and a therapeutic vaccine will be of benefit for people at risk and

for patients in the endemic regions. This concept is supported by substantial scientific evidence.

To generate a vaccine that can be used in humans it is necessary to study the immune response to the causing agent (to define correlates of protection) and the immunopathology of the disease. As no vaccine lead candidate was available, the consortium had to identify and develop different types of new vaccine candidates and to evaluate those using bioinformatics, applied genomics and proteomics. Essential pre-clinical testing in vitro and in vivo was required and new models of protective studies had to be established and possible vaccine candidates had to be extensively characterized. To be able to evaluate vaccine candidates the consortium had to study the main pathogenicity factor, the mycolactone toxin, of the causing agent Mycobacterium ulcerans, the disease immunopathology and the specific immune response in animal model systems and in patients.

Moreover, a major objective of the project was capacity building in 4 African countries. Institutions of the African beneficiaries should be enabled to perform immunological analyses on BUD patients and to use standardized procedures for active case finding, for laboratory confirmation, treatment, and data management. An efficient dissemination and exploitation strategy should be implemented to build up awareness and to disseminate knowledge about this neglected but important infection. Finally, it should be ensured that all procedures were carried out in accordance with international standards of the ethical conduct of research in humans as well as in experimental animal studies. These objectives should be achieved by a multidisciplinary approach involving among others basic and applied research in immunology, bioinformatics, molecular genetics, tropical medicine, microbiology and clinical bacteriology.

Planned strategy for the work of the consortium

As currently no existing vaccine lead candidate is available, the consortium had to identify and develop different types of new vaccine candidates, to evaluate those using bioinformatics, applied genomics and proteomics and narrow down their number in consecutive test systems. Essential pre-clinical testing in vitro and in vivo had to select a small number of candidates that were amenable to be introduced into clinical studies. To be able to evaluate vaccine candidates that can be used in humans the consortium studied the immune response to the causing agent and the disease immunopathology to define correlates of protection.

The validity of the concept was investigated as follows: Vaccine candidates of different types were developed in three work packages and were pre-selected for their protective capacity in the mouse model. Simultaneously, studies of correlates of protection and disease immunopathology defined biomarkers that correlate with immunological resistance or susceptibility, information required to assess if a vaccine candidate will stimulate a protective response in humans. This knowledge was then applied to test pre-selected vaccine candidates for their association with biomarkers for protection in patients. Finally, the

candidates were tested in the guinea pig model for preventive and therapeutic capacity. This model has more similarities to the human infection and therefore provides a better correlate of vaccine efficacy in vivo than the mouse model. The number of vaccine candidates was thus be reduced consecutively to those suitable for clinical studies.

Since the unequivocal assignment and classification of patients to different stages and courses of BUD is essential for BuruliVac, a work package was devoted to patient management – including accrual of study participants, collection of clinical samples, laboratory confirmation, treatment, and data management – that provided a pool of samples of well characterized patients and healthy controls. Considering the extensive work with patients an entire work package was devoted to ethical issues. These studies were accompanied by extensive capacity building in the 5 African beneficiary institutions in four countries that led to establishment of immunological and microbiological expertise in these institutions.

Main S & T results/foregrounds

Summary of results and progress

We have made a big step in our understanding of the mechanisms by which ML mediates its biological effects with the identification of molecular targets of ML. We have demonstrated that ML is present in tissue specimens and blood of BU patients, and can now characterize its kinetics of variation during disease and treatment.

First monoclonal antibodies with specificity for ML have been tested for their toxin-neutralising activity.

Structure-activity relation studies with truncated synthetic MLs are now underpinning development of a strategy for the development of additional monoclonal antibodies with different fine specificities.

As a proof of principle, in experimental mouse infection models partial protection was observed with some of the candidate vaccine formulations. Based on these selected vaccine platforms, development of candidate vaccines with higher efficacy is envisioned. For live attenuated vaccines genetic modification of the vaccine strains and for subunit vaccines identification of additional target antigens and further optimization of the formulation would be the way forward.

A novel model system for vaccine testing and pathophysiological investigations of Buruli ulcer in guinea pigs was successfully established according to plan. Infection of the guinea pig was demonstrated as a useful animal model to test vaccine candidates. Using this model we could again provide proof of concept that live attenuated mycolactone-negative M. ulcerans strains are a viable option to provide protection against virulent M. ulcerans infection.

The immune response of patients with BUD was characterized. We found that BU patients displayed a broad, yet selective profile of immune suppression, marked by the down-modulation of multiple chemokines, cytokines and immunoglobulins and concurrent induction of the pro-inflammatory protein EN-RAGE. Since recent reports indicate that immune responses are metabolically regulated, we complemented this proteomic study with a metabolomic analysis of serum from patients and controls. The resulting analysis led to the finding that Mycobacterium ulcerans infection is marked by disruptions in glycolytic and oxidative metabolism. Extensive investigations on the antigen-specific response did not allow discriminating between M. ulcerans infected BUD patients and healthy non-infected patient contacts that would show immunity that would protect them from developing disease upon infection with M. ulcerans.

Capacity building in 4 African partner laboratories was successfully performed. Laboratories and facilities were prepared and are now in use. All 5 African beneficiary sites have been equipped with operating procedures and the necessary instrumentation and reagents. Extensive on-site training was performed. Quality System Procedures and Standard Operating Procedures for patient management, collection of diagnostic samples and laboratory confirmation are in place. At the end of the project sustainable structures have been established in the institutes of the African partners that can be used in future diagnostic and immunological research.

Throughout the project we ensured that all procedures of the proposed research are carried out in accordance with ethical requirements in accordance with rules and regulations in the participating countries throughout all stages of the project; and generally, that high ethics standards in all research conducted were maintained, including research in laboratory animals. We ensured that vulnerable populations were protected - with respect to the high proportion of children among BUD patients, and that specific ethical issues concerning children and their parents and / or custodians (or legal representatives) were considered in all procedures of the proposed research. We ensured that research on biological samples was carried out in accordance with local access and benefit sharing strategies and mutual benefit of all aspects of scientific medical research, including research capacity building in the BU-endemic areas in the South was secured.

Elucidating the mechanism of action of mycolactone toxin (ML)

We found that ML binds selectively and high affinity to the Wiskott-Aldrich syndrome protein (WASp), and WAS-like neural WASp (N-WASp) in vitro, to stimulate their actin-polymerizing activity. Recombinant domains of the WAS proteins and synthetic modules of ML helped us identify the interacting regions and suggest a physical mechanism for activation. We found that ML suppressed epithelial cell capacity to make adhesive contacts, with complete loss of anchorage coinciding with cell death. Further, ML-exposed cells adopted rapid and random movements, loosing capacity to heal wounds. Strikingly, both adhesion and migration defects were neutralized by N-WASp inhibition or ectopic expression of the ML binding domain. We

conclude that WAS proteins are major targets of ML and propose that BU results from the uncontrolled activation of N-WASp in infected skin.

Following up on these finding, we investigated the impact of ML-induced activation of N-WASp on the biology of epithelial cells. Using a combination of biochemical assays, cellular imaging and animal models, we found that ML mimics physiological signals normally delivered by Rho GTPases to deviate N-WASp-dependent actin polymerization. We show that ML-induced activation of N-WASp in epithelial cells and the consequent dynamic rearrangements of the actin cytoskeleton dramatically impair the integrity of the epidermis, thus providing a molecular mechanism underpinning Buruli ulcer pathogenesis.

These results are fully detailed in our latest publication (Guenin-Macé et al., J Clin Invest, 2013). In summary, we showed that ML-induced activation of N-WASp in cultivated epithelial cells resulted in defective adhesion and directional migration. Consistently in vivo, injection of ML into mouse ears altered the junctional organization and stratification of keratinocytes, leading to thinning then rupture of the epidermis. This degradation process was efficiently suppressed by co-administration of the N-WASp inhibitor wiskostatin. Our study therefore elucidated the molecular basis of ML ulcerative activity in skin tissues.

Kinetics of ML toxin vivo

In order to design immunotherapeutic vaccines based on ML neutralisation, it was important to extract and determine the concentration of mycolactone in infected human tissues and their kinetics during therapy. We have obtained ethical approval for this component of the work and mycolactone has already been extracted from infected human tissues for the first time. Recruitment of patients for this study is ongoing.

We have estimated the ML concentration in human tissues using a cytotoxicity assay in which human embryonic lung fibroblasts were exposed to acetone soluble lipids (ASL) extracted from human tissue biopsies. Synthetic ML A/B supplied by Prof Kishi was used to construct a dose response curve after 24, 48 and 72 hours incubation. ASL from normal human skin induced 20% cytotoxicity within 2 hours but mycolactone associated cytotoxicity occurred at 48-72 hours. IC-50 for the cytotoxicity assay was 4.8ng/ml of mycolactone standard. ASL were extracted from 96 skin biopsies taken from subjects with untreated Mu infection, as proven by PCR for IS2404. Cytotoxicity above 20% was detected in ASL from 91 out of 96 lesions. The median (range) concentration of mycolactone was 423.9 (113.5 – 3020 ng/ml). There was a wide range of concentration in all lesion types with a tendency to a higher concentration in 4 oedematous lesions. In 3 nodules and 3 plaques, paired biopsies were taken from the centre and the periphery of the lesion. The mean ± SEM concentration at the centre of these lesions was 397.6 ± 127ng/ml compared with 263.7 ± 49.8ng/ml at periphery ($p>0.05$). Further biopsies were taken from 23 patients 6 weeks after starting standard antibiotic therapy. In these subjects the tissue ML concentration had declined to 385.0 ng/ml (152.6 – 1160). Four weeks after the end of the 8 week course of antibiotics, 7

subjects were re-biopsied because of poor healing. The mean tissue ML concentration in these biopsies was 320.2ng/ml (168.8 – 604.1).

Design of immunotherapeutic vaccines based on ML neutralization

We have established flow cytometry assays to assess the potential ML neutralizing activity of anti-ML antibodies. We tested cell death by apoptosis and necrosis. These differ from each other in that cell membranes become leaky during necrotic cell death, while they maintain their integrity during initial stages of apoptosis. We used Annexin V as a probe to detect cells that have expressed phosphatidylserine on the cell surface, a feature found in apoptosis and other forms of cell death. In addition a dye exclusion test with propidium iodide was performed to establish whether membrane integrity has been conserved. This test combination allows discrimination between intact, apoptotic and necrotic cells. Both synthetic and extracted ML A/B was employed, yielding comparable results. In a first step a set of cell lines was assessed for sensitivity to ML and L929 fibroblasts were selected for assay development. The time course of ML action was analyzed. After 24 hours the effect was still moderate, while at 48 hours it was close to maximum. Standard assays were therefore performed with a time span for ML action of 48 hours. At a concentration of 30 ng/ml the effect was at a maximum with <10% of the cells being both propidium iodide and Annexin V negative. A monoclonal antibody raised against extracted ML coupled to reconstituted influenza virosomes as antigen delivery system was tested for toxin neutralizing activity. Up to a final concentration of 40 µg/ml no consistent toxin neutralizing activity was observed with this particular antibody, which has demonstrated binding to the core of ML in BiaCore analysis. For the development of a strategy to generate additional monoclonal antibodies with different affinity and fine specificity we have tested the biological activity of truncated synthetic derivatives of mycolactone.

Since the only ML-specific monoclonal antibody currently available has shown no toxin neutralizing activity, structure-activity relation studies with synthetic MLs have been performed to guide design of a ML-conjugate vaccine. In order to design immunotherapeutic vaccines based on ML neutralisation, it is important to extract and determine the concentration of ML in infected human tissues and their kinetics during therapy. We have obtained ethical approval for this component of the work and ML has already been extracted from infected human tissues for the first time. Patients have been recruited for this study. Systematic structure-activity relation studies have led to the design of ML-carrier conjugates that have the capacity to elicit anti-ML antibody responses.

Design of vaccines based on enzymes involved in ML biosynthesis

Plasmid vectors encoding genes involved in ML biosynthesis were constructed and were tested for immunogenicity and protective efficacy in the mouse model.

12 PKS sequences were received from Dr. Stinear in expression vectors. 3 of these genes have been cloned into a DNA vaccination vector – ACPI, KRA and KSalt. 6 of these proteins have been purified in significant quantities – ACPI, KRA, KSalt, KSc, ATacII and ER.

C57BL/6 mice were vaccinated 2x with DNA and 1x with recombinant protein with Ag85A (positive control), ACPI, KRA and KSalt. KRA and KSalt induce both IL2 and IFN-□ responses, as measured by ELISA. KSalt induced the strongest cytokine responses while KRA induced the strongest antibody responses. Little immune response was detected against ACPI. Ag85A induced both cytokine and antibody responses, as predicted.

Vaccinated mice have been challenged and protection studies are underway.

This work will be repeated using Balb/c mice, as epitope prediction software indicates that the PKS are presented better in Balb/c mice. The mice have been vaccinated with the first round of DNA.

Plasmids, containing twelve gene fragments encoding enzymatic modules of the polyketide synthase genes that are critical for ML biosynthesis were given to us by Dr. T. Stinear (University of Melbourne, Australia) The gene fragments were initially in pET-DEST42 vectors that are used for protein expression and we have cloned the fragments in V1j.ns-tPa vector for DNA vaccination and tested the vaccine potential in a DNA prime/protein boost approach.

Characterization of vaccine potential of ML deficient mutants of M. ulcerans.

Five strains of live, attenuated *M. ulcerans* on the MU 1615 background were used (provided by Dr. P. Small). The bacteria were generated by random transposon insertion, rendering the bacteria deficient in mycolactone production.

- MlsB (MU1615::*Tn84 and* MU1615::*Tn85)*
- Adh (MU1615::*Tn110)*
- FabH (MU1615::*Tn118)*
- (MU1615::*Tn120)*

These mutants were injected in BALB/c and C57BL/6 mice with 10^5 CFU of bacteria. Mutants were tested for immunogenicity, as measured by splenocyte induction of IL-2 and IFN-g in response to *M. ulcerans* antigens. Residual virulence of the mutants was assessed by monitoring vaccinated mice over the course of a year for survival and footpad swelling.

Spleen cell - IL-2 and IFN-gamma responses following stimulation with *M. ulcerans* specific antigens at month 11 post vaccination were highest in mice vaccinated with mutants 84 and 85. Significant cytokine levels were detected in response to culture filtrate, PPD and recAg85A from *M. ulcerans*. We also isolated three spontaneous mutants of *M. ulcerans* 980912 that had lost the plasmid and did not produce the mycolactone any longer. These mutants were injected in mouse footpad of B6 mice (10^5 AFB) and did not induce any

footpad swelling over a period of 100 days as compared to control WT 1615 strain for which the mice had to be sacrificed at day 63. C57BL/6 and BALB/c mice were vaccinated with the live, attenuated *M. ulcerans* mutants.10^5 CFU were injected into the left footpad of the mice. The vaccinated mice were subsequently challenged with 10^5 CFU virulent *M. ulcerans* 1615 in the right footpad and - monitored for footpad swelling as compared to infected, unvaccinated mice. According to the guidelines of the Ethical committee, mice developing footpad swelling > 4 mm were sacrificed. Mutants 84 and 85 conferred the best protection both in BALB/c and in C57BL/6 mice. These two mutants were sent to partner 15 for sequencing.

These results provide proof of concept that mycolactone-negative strains have the potential to confer protection against infection with virulent *M. ulcerans*.

Identification of potential subunit proteins target antigens

Available genomic information (public *M. ulcerans and M. marinum* genome databases, unpublished comparative genomic hybridisation and genome sequencing data and data from ongoing genome sequencing projects), proteomic data (proteome databases and data from ongoing analyses) and information on properties of orthologues (if existing) in *M. tuberculosis* were used to select a panel of potential target antigens. Presence and diversity of the corresponding genes in the genomes of *M. ulcerans* isolates belonging to different geographical lineages was assessed. Potential target antigens were expressed as his-tagged proteins in *E. coli*. Vaccine formulations of the target proteins were used to immunize mice to assess i. immunogenicity of these formulations and ii. protective efficacy after footpad challenge with *M. ulcerans.*

By combining available information with newly generated experimental evidence and *in silico* analyses, four target antigens were selected: MUL1970, MUL3720, MUL2232, MUL4987.

Selection criteria included:

- high expression level in *in vitro* cultivated *M. ulcerans*
- detection of antigens in human Buruli ulcer lesions by Western blotting
- published evidence for vaccine potential
- predicted functional relevance of target protein
- accessibility of target antigen on the surface of bacterial micro-colonies to antibodies
- genetic conservation of the protein
- ease of recombinant expression

All four selected candidate antigens could be produced in *E. coli* and the possibility of a large scale purification and production was shown.

For the formulation of the subunit vaccines two major strategies are pursued in the first phase:

- formulation of recombinant proteins with an adjuvant
- Vesicular Stomatitis Virus (VSV)-based virus replicon particles as delivery system

Virus replicon particles (VRPs) derived from VSV are virus particles in which one gene encoding an essential structural protein in the negative strand RNA genome has been depleted. Propagated on a transgenic helper cell line providing the lacking protein for particle assembly, these particles are infecting cells once, leading to amplification of the genomic information encoded on the genome of the virus but unable to produce infectious progeny. Therefore high levels of intracellular antigen expression can be reached in the infected cell - respectively the immunized animal - and such RNA replicons have the potential to efficiently stimulate both, humoral as well as cellular immune responses. Cellular responses are thought to be induced as antigens expressed by the infected cells can be partially degraded and if this is taking place in antigen presenting cells (APCs) presented to the immune system via MHC class-I molecules. At the same time apoptosis is relatively quickly induced in replicon infected cells and apoptotic vesicles are taken up by APCs that degrade antigens contained in the apoptotic vesicles and present it via MHC class-II molecules or via the so called cross-presentation via MHC class-I molecules as well. It is not necessary to administer VRPs together with adjuvants, as the particles provide for example RNA recognized by pattern recognition receptors of the immune system that replaces the costimulatory function of adjuvants.

The bases for generation of VRPs encoding the *M. ulcerans* target antigens is pVSV*ΔG, a plasmid encoding the RNA genome of VSV in anti-genomic (positive) orientation. All genes on this plasmid are under the control of a T7 promoter, required restriction sites are abundant and the envelope glycoprotein coding gene (G) is replaced by a gene fore green fluorescent protein (GFP). Synthetic genes encoding the target antigens and optimized for human cell expression were directly ligated into pVSV*ΔG at the fourth position between the matrix protein (M) coding region and the gene for GFP. For production of virus particles from these plasmids transgenic BHK21 cells providing the G protein *in trans* were infected with a modified vaccinia virus expressing the T7 phage RNA polymerase. In a second step the infected cells were transfected with pVSV*ΔG and three helper plasmids encoding for viral proteins under the control of a T7 promoter. These viral proteins were required to be encoded on separate plasmids only for virus particle generation from genomic information alone but were not needed further on in the experiments. As all components needed for virus assembly were then present in the infected cells the negative strand RNA was autonomously packed into VRPs that could be harvested in the cell culture supernatant and further propagated on the transgenic BHK21 cell line. Virus titres were determined on a non transgenic cell line by counting infected GFP expressing cells after serial dilution infection. In this way four different VRP types were produced, each one encoding for one of the selected target antigens beside GFP and the four remaining viral genes in the genome

VSV*ΔG(MUL2232)

VSV*ΔG(MUL3720)

VSV*ΔG(MUL1970)

VSV*ΔG(tPA-MUL4987)

For all different VRPs *M. ulcerans* protein expression levels, localization in the infected cells and size confirmation of the antigen was assessed *in vitro*. BHK21 or MARC-145 cells were infected with VRPs fixed for immunofluorescence and confocal microscopy or harvested and lysed for Western Blotting. Concept validation was obtained from first immunization experiments in BALB/c mice, which demonstrated that immunization with the replicons can induce immune responses against the *M. ulcerans* target antigens. Further experiments are now planned to optimize VRP delivery with respect to the dose of VRPs, number of immunizations and route of administration. Both adjuvanted formulations as well as vesicular stomatits virus replicon particle-based formulations showed good immunogenicity.

Adjuvanted recombinant protein formulations

The recombinant proteins we have expressed in E. coli were formulated with different adjuvants with the rationale to elicit differentially polarized immune responses. Together with adjuvant 20ug of each recombinant protein was administered sub-cutaneously (s.c.) to groups of BALB/c mice. Immunizations were done three times in three week intervals and after every immunization blood was taken to assess antibody responses in serum. Sigma Adjuvant served as positive control but is only possible to use in mice. All protein – adjuvant formulations tested were immunogenic and led to antibody responses in sera. Compared to Sigma Adjuvant the IDRI adjuvant leads to similar antibody titers already after second immunization (Fig. 1) that do not change much after third immunization. In contrast immunizations with Telormedix adjuvant need three injections to lead to similar antibody levels as Sigma Adjuvant. To investigate whether polarization of the antibody response upon immunization with different adjuvants was actually different we performed ELISA experiments on recombinant protein to determine Ig subclasses of the generated antibodies in the immunized animals. We observed the generation of a broad spectrum of antibody subclasses in sera after third immunization but no marked differences between mice that had received the same protein but formulated with different adjuvants. To estimate how long the increased levels of M. ulcerans specific antibodies would persist in sera of immunized mice we compared serum antibody levels 14 days and 7 month after third immunization. We found a drop of serum antibodies against the previously administered proteins in all groups but antibodies were still detectable in all mice.

Vesicular stomatits virus replicon particles

We have evaluated the number of VRPs that have to be administered per immunization as well as route and number of immunizations needed and we found two suitable immunization schedules for the replicons we chose as second delivery system for the candidate vaccine antigens. Current protocols for active immune protection compare administration of two times 10^7 VRPs i.m. and once 10^7 VRPs i.m. followed by a boost with non-adjuvanted recombinant protein. Typically we do not see antibody responses for the regimen with replicons only but we have good indication that a primarily cellular immune response is triggered by this way of immunization. In contrast a prime-boost regimen leads to the production of antibodies in immunized animals.

Immune protection experiments

Macroscopic appearance as well as bacterial load measured by RT-PCR indicates that a strong humoral response we have seen upon immunization does not beneficially influence the course of infection. These results confirm what we have seen already in passive protection experiments, namely that humoral immune responses against the selected vaccine candidate antigens seem to play no major role in immune protection against *M. ulcerans* disease. In contrast cellular immune responses against MUL3720 could be protection-relevant as seen in animals immunized with VSV replicons that are designed to mainly elicit such responses.

Establishment of a novel animal model

We have established the standard course of *M. ulcerans* infection in the guinea pig model. Cutaneous lesions were monitored daily and at specific time points lesions were photographed and excised for microbiological and histological studies. The obtained results underline the similarities between human BU disease and the course of infection in the guinea pig, supporting the suitability of this animal model for the validation of vaccines designed to prevent the human disease. In that sense, we performed experiments to optimize the immunization protocol in the guinea pig model. Once the protocol was optimized, we carried out a comparative analysis of the protection conferred by vaccination with two live attenuated vaccine candidates: spontaneous mycolactone negative mutant strains 5114 and 98-912. In this established guinea pig model vaccine-candidates from WP2 could be tested. Vaccination with two live attenuated vaccine candidates (spontaneous mycolactone negative mutant strains 5114 and 98-912) conferred a significant degree of protection.

Collectively, these results provide proof of concept that live attenuated mycolactone negative *M. ulcerans* strains are a viable option to provide protection against virulent *M. ulcerans* infection.

Determination of *M ulcerans* specific antibodies and immune cell markers

Serological analysis was performed for novel immune biomarkers as well *M ulcerans* specific antibodies such as the 18kD heat shock protein. In addition an analysis of the T cell repertoire will be carried out for possible markers of immune evasion as shown in severe childhood tuberculosis. In preliminary work we have shown that chemokines such as IL-8, MCP-1, MIP-1β, MIPα, eotaxin, fractalkine and T cell cytokines (IL-4, GM-CSF) were augmented 10 months after completion of antibiotics suggesting a protective role. Despite promising expectations the analysis of 18 kDa shsp-specific IgG in the current investigated cohorts was not meaningful enough to identify putative immune contacts of BUD patients.

To further identify novel biological correlates of BUD and prognostic markers for a good clinical response, we performed a multi-analyte of serum proteins in patients at various stages of treatment and healing. Our results validate the potential to use previously-identified immunological mediators as correlates of infection status. Twenty (20) patients with confirmed Buruli ulcer of less than 10 cm in maximum diameter provided 2 mls of serum at the time of BU diagnosis (Week 0) and 4 weeks after completion of antibiotic therapy (e.g. Week 12) were compared to those of age and sex matched healthy controls (n=15) from the same endemic zone.

Multi-analyte profiling: Serum samples were sent to Rules-Based Medicine Inc. (Austin, USA) for quantitative measurement of 88 proteins (Human Multi-Analyte Profile (MAP) v1.6). Only proteins with 80% or more values above the least detectable dose (LDD, determined as mean + 3 SD of 20 blank readings) for at least one of the control or patient groups were considered.

BUD patients display a broad but selective anti-inflammatory profile

BUD patients displayed significantly lower circulating levels of Macrophage Inflammatory Protein (MIP)-1β and Monocyte Chemotactic Protein (MCP)-1. Although statistical significance was not reached in this study for Interleukin (IL)-8, a marked decrease in the serum concentrations of other inflammatory chemokines such as the Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES), Macrophage-Derived *Chemokine* (*MDC)* and Epithelial Neutrophil-Activating Protein 78 (*ENA-78*) were observed. The circulating levels of many cytokines were comparably suppressed: IL-1a, IL-3, IL-5, IL-7, IL-10, IL-$12_{p40/p70}$, IL-13, IL-15, IL-18, Tumor necrosis factor (TNF)-α, IL-10 and Stem Cell Factor (SCF). We also observed a significant decrease in the concentrations of soluble CD40, CD40 Ligand (CD40L) and beta-2-microglobulin (ß2m), suggesting systemic defects in T cell activation in BU patients. Immune modulation also affected the B cell compartment, as shown by the reduced levels of IgA and IgM. As previously reported for MIP-1b and MCP-1, down-modulation of the aforementioned molecules persisted after completion of antibiotic treatment. Intriguingly, BU-associated immune modulation was sustained but selective, as inflammatory mediators such as IgE, IL-16 or Myeloperoxidase (MPO) were unaffected by

the disease, and the pro-inflammatory EN-RAGE (also known as *S100A12* and Calgranulin C) was up-regulated.

Expanding on the scope of previous findings, we found that BU patients displayed a broad, yet selective profile of immune suppression, marked by the down-modulation of multiple chemokines, cytokines and immunoglobulins and concurrent induction of the pro-inflammatory protein EN-RAGE.

Metabolomic profiling: Since recent reports indicate that immune responses are metabolically regulated, we complemented this proteomic study with a metabolomic analysis of serum from patients and controls. Samples were sent to Metabolon (Durham, USA), for the semi-quantitative determination of >400 biochemical parameters. The resulting analysis led to the finding that *Mycobacterium ulcerans* infection is marked by disruptions in glycolytic and oxidative metabolism.

While heterogeneity was observed within the patient populations, principal component analysis revealed separate clustering between control and infected individuals. Notably, our study suggested that *Mycobacterium ulcerans* infection is marked by disruptions in glycolytic and oxidative metabolism that may be induced by the generation of steroid mediators. Furthermore, alterations in glutathione metabolism, antioxidant mobilization, and purine catabolism may reflect a change in redox homeostasis, while reduced heme catabolism may increase the supply of this metabolite for mycobacterium growth. Finally, differences in bile acid levels may also reflect minor differences in the micro flora of these individuals.

Identification of novel biomarkers of protection by gene expression profiling

Recent studies identified miRNA (miR)-29 as a central non-redundant suppressor of IFNγ and increased miR-29 expression promoting susceptibility against mycobacterial infections. We determined the expression profile of immune-related miRNAs in PBMCs from Buruli ulcer patients at baseline and on therapy. Initial experiments showed no distinct pattern in expression of miR-21, miR-26a, miR29a, miR-21, miR142-3 and miR155 when samples obtained at baseline were compared with those obtained at week 6 and week 12 (4 weeks post treatment). Subsequent analysis of PBMCs of additional Buruli ulcer patients showed a slight but not significant increase in the expression levels of miR-21, miR-26a, miR29a, miR-21, miR142-3 and miR155 compared to the housekeeping gene at week 6 of treatment which slightly decreased at week 12 i.e. four weeks after treatment. These results suggest an effect of therapy on the expression of different miRNAs in BUD patients. Obtained results are promising but for statistical power larger number of patents has to be investigated.

Immune response to M. ulcerans antigens in patients, healthy contacts and controls

In vitro analyses of healthy non-infected contact persons were performed to find non-diseased individuals with specifically high reactivity to specific antigens of *M. ulcerans*. The assays worked very well and high reactivity could be demonstrated. However, it was not

possible to identify unequivocally such putatively immune persons. Thus the evaluation of a correlation of reactivity to vaccine candidates and protection was not yet possible.

Both T cell (IFN-γ) and humoral (IgG and IgM) responses to subunit proteins of *M. ulcerans* were tested in patients and controls. MUL4987 and MUL3720 and MUL4987- or MUL3720-specific IgM and IgG titers did not allow discriminating between *M. ulcerans* infected and non-infected BUD patient contacts. However, to evaluate biomarkers of protection and for the selection of promising vaccine candidates the identification of healthy infected individuals (contacts of BUD) is essential. It can be expected that the definition of biomarkers for protection and the selection of vaccine candidates will be successful, once a clear definition of patient cohorts is possible.

We conclude that the subunit protein vaccine candidates MUL3720 and MUL4978 are well immunogenic. Differential T-cell responses against these antigens in BUD patients and healthy contacts were not detected. Under therapy T-cell immunity against vaccine candidates is increased when compared to baseline. This argues for T-cell suppression in BUD patients before onset of therapy.

The polyketide syntheses enzymatic domains of *M. ulcerans* could not discriminate between patients and endemic controls at baseline. Analyses of antibodies against different mycobacterial antigens (e.g. MUL3720 and MUL4978) and description of immunological reactivity of live attenuated vaccines likewise did not discriminate. Both T cell responses (IFN-γ) to MUL4987 and MUL3720 and MUL4987- or MUL3720-specific IgM and IgG titers did not allow to discriminate between *M. ulcerans* infected and non-infected BUD patient contacts. But to evaluate biomarkers of protection and for the selection of promising vaccine candidates the identification of healthy infected individuals (contacts of BUD) is essential. It can be expected that the definition of biomarkers for protection and the selection of vaccine candidates will be successful, once a clear definition of patient cohorts is possible.

Production procedures for the three promising vaccine candidates for Buruli ulcer MUL2232, MU3720, and MUL4987 were established and several mg of highly pure proteins were delivered to STPHI for initial experiments. All process and purification data were stored. Using the standard optimized conditions we are now in an excellent position to produce of 3 subunit vaccines large quantities for vaccine development.

Determination of M ulcerans-specific T cell cytokine expression pattern

This work involves determination of the evolution of T cell cytokine response of BUD patients' lymphocytes after restimulation with M. ulcerans antigens such as M ulcerans sonicate and Ag85 which will be compared with those of *M. tuberculosis* (CFP-10, ESAT-6, PPD) and *M. avium*. Preliminary work done on the evolution of the IFN-gamma and Interleukin 10 responses in BUD subjects in an ex vivo whole blood assay showed an increase in IFN-gamma response after 4 weeks of antibiotics and a further increase after 8 weeks of antibiotics. Responses were greater for larger ulcers compared with smaller ulcers. This

information is useful suggesting that IFN-gamma production is a marker of protection but this was further tested in a larger BUD patient group.

There were 102 with confirmed Buruli ulcer of which there were 28 (27.5%) nodules, 18 (17.6%) plaques, 8 (7.8%) oedema and 48 (47.1%) ulcers. 44 (43.1%) were category 1, 26(25.5%) were category II and 32 (31.3%) were category III. 51 age-matched healthy individuals from the same districts were recruited to serve as controls. ELISA's have been carried out on these.

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

We showed that under successful antibiotic treatment a significant increased TH1 response to mycobacterial antigens could be observed in BUD patients. Analysis of IFN-gamma expression of BUD patients whole blood prior to treatment, under treatment (6 and 12 weeks after treatment onset) against MUL3720 and MUL4978 revealed a significant increase (p=0,03; p=0,005) after six weeks of therapy. At week 12 similar levels were detected as compared to week 6. The same was found after 5 days stimulation of PBMC with MU lysate when patients were on treatment: significantly higher IFN-gamma secretion was produced at 12 weeks compared to baseline to MU lysate1 (p=0.005). Similarly after stimulation of whole blood with 5ug/ml of mycobacterial antigens for 18 hours significantly higher IFN-gamma was produced after 6 weeks for MU lysates and also at 12 weeks.

Immune response of patients with Buruli ulcer of different categories, ulcerative and non-ulcerative forms

When PBMC were stimulated for 5 days there was no difference in IFN-gamma response across the different categories. However in the whole blood assay there was statistically significant increase in IFN-gamma (p=0.01) response in the category II lesions compared with category I or II lesions. In addition patients with ulcerated forms of disease produced significantly higher IFN-gamma responses to Mu lysate1 compared to non-ulcerated forms. (p=0.008) in the 5 day stimulation and not in the short term assay. Ulcerative form of Buruli ulcers made significantly higher IFN-gamma compared to non-ulcerative forms of disease.

In conclusion, we have described the IFN-gamma response of patients with Buruli ulcer and age and sex matched controls living in the endemic area with the 5 day longer term PBMC stimulation and also with the short term 18 hour whole blood stimulation. Patients produced statistically significant increase in IFN-gamma to *M ulcerans* antigens - MU lysate and Ag85Aulz and not to the M tuberculosis antigen Ag85A tub suggesting some specificity for the infecting agent.

The different categories of Buruli ulcer disease could not be discriminated with their IFN-gamma response in the 5 day PBMC stimulation but category II lesions appeared to respond with higher IFN-gamma levels in the short term whole blood assay but the numbers were

small. Ulcerated forms of the disease responded with significantly higher IFN-gamma levels compared with non-ulcerated forms after PBMC stimulation.

Capacity building in BUD case finding, diagnostics and research

Before the work on vaccine candidates could start we had performed extensive preparations. Most importantly, capacity building was performed in the laboratories of the African beneficiaries. Materials had been purchased and shipped to the African beneficiary institutes and SOPs for collection, transport and storage of clinical samples had been prepared. Intensive training sessions in laboratory immunology followed in all 5 African beneficiary laboratories involving all African beneficiaries.

Throughout the project regular training activities for different levels of health staff were conducted in all four African countries. All patient-related and laboratory processes followed standard operating procedures. Standardization of data management was achieved by project-specific study forms and the project-specific web-based data base. Case finding, laboratory confirmation and clinical management of BUD patients according to BuruliVac standards are continued beyond the end of the project. On-site evaluation of all procedures (followed by re-training if required) has been conducted in all four countries by LMU and ITM. A National Reference Laboratory for BUD was implemented at INH, Togo. And laboratories in Togo, Benin, and DRC were equipped with real time PCR. EQA for diagnostic laboratory procedures and data management was conducted throughout the project, EQA results indicate that laboratory analyses are delivered at high standards. Local GCP training workshops have been conducted in Benin and Ghana.

At the end of the project sustainable structures have been established for case finding, diagnostic verification as well as for future vaccine trials through training in epidemiological field research and the performance of clinical trials and for immunological investigations in BUD research.

Ethical issues

An entire work package dedicated to Ethics was implemented to ensure that all procedures of the research in the BuruliVac Consortium are carried out in accordance with international standards of the ethical conduct of research in humans as well as in experimental animal studies. The BuruliVac consortium members have decided to devote an entire work package to address ethical issues. The challenge for the consortium was to conduct high-tech immunological research on the one hand and on the other hand to address the needs of poor-resource countries to improve health among afflicted populations, and build research capacity in the endemic regions with limited research infrastructure and resources. In this WP, we address ethical issues beyond the present standards, and touch upon emerging aspects of ethical implications of research in poor-resource settings, as is typically the case for Buruli ulcer-endemic areas. Just one example illustrating the aspect of Ethics concerns is

the fact that in the various studies among humans in the Buruli ulcer-endemic regions, most of participants appear unable to write: most give informed consent by thumb-print.

In the beginning ethics clearances were checked of all human and animal studies and it was ensured that requirements are met in accordance with rules and regulations in the participating countries.

Moreover, we have continued to sensitize all partners to protect vulnerable populations - with respect to the high proportion of children among BUD patients, to ensure that specific ethical issues concerning children and their parents and / or custodians (or legal representatives) are considered in all procedures of the current as well as future research. Apart from these basic ethical requirements, and following internationally accepted novel standards for medical research in less affluent countries, this WP8 has urged all partners from affluent countries to ensure that research on biological samples is carried out in accordance with local access and benefit sharing strategies and to enhance and secure mutual benefit of all aspects of scientific medical research, including research capacity building in the BU-endemic areas in the South. We encouraged research teams to involve researchers in the South, to offer positions for participation in research, training in research, and authorship positions reflecting the mutuality of this consortium. A Good Clinical Practice course was designed and held in Allada, Benin in June, 2011, for all workers involved in the studies by the consortium involving human subjects. A second similar but now improved course was prepared. This second course held in Kumasi, June 19-20. It was attended by over 45 people from Ghana and Benin at the occasion of the launch of a new WHO drug trial for Buruli ulcer, with WP8 in the lead as the principal investigator. A large proportion of time was dedicated to designing the study is such way as to meet beyond state-of-the-art Ethics standards; discussions with the ethics Committee, WHO, Geneva, and with the Ethics Committee, Ghana Health Research Unit Accra, as well as the Ethics Committee of Benin in Cotonou were intense but fruitful. Several different versions were exchanged, and now, all three committees have approved the protocol plus consent/assent forms.

Finally, we have started exploring a novel study beyond state-of-the-art in Ethics research. We study aspects of Ethics approval by participants in poor-resource settings in the context of medical research in Buruli ulcer; we have designed a questionnaire exploring to what extent participants really understand the nature of what they have consented to, in a formal study protocol to be conducted in Ghana and Benin.

In summary, by devotion of a whole work package to ethical issues it was ensured that all procedures of the research were carried out in compliance with ethical requirements and regulations in the participating countries and that the research was conducted in accordance with local access and benefit sharing strategies.

Protection of vulnerable populations

Much time and effort was dedicated to designing the protocol of a large, WHO-initiated drug study. We made an effort to meet beyond state-of-the-art Ethics standards; discussions with the ethics Committee, WHO, Geneva, and with the Ethics Committee, Ghana Health Research Unit Accra, as well as the Ethics Committee of Benin in Cotonou were intense but fruitful. Several different versions were exchanged, and finally, all three committees approved the protocol plus consent/assent forms. A large proportion of time and effort was dedicated to discuss the concept of assent by minors, who by definition should be seen as vulnerable groups. Discussions with various experts as well as with stakeholders of the national Programs in Ghana and Benin, Ethics experts in the various different Ethics Committees but also endemic community representatives were held.

Informed consent of children beyond 10 years of age, and consent of parents or custodians – or legal representatives were covered separately. Rights of women with participation in Ethics Committees were monitored, evaluated and – if needed – improved. Genuine interest for the young children (age 5-10; who do not give informed consent themselves) to benefit from research aiming at improving their health were balanced against potential discomfort. Blood draws were limited in sample size (≤10 ml), and punch biopsies for research purposes will not be collected. Those below age 5 will only participate in observational, not in experimental studies; and only with their parents' / custodians' consent.

In 2012, September, a kick-off meeting was held in Kumasi, Ghana, with over 50 people in attendance from Benin and Ghana. GCP Certificate training was held, somewhat similar but also different from the training one year earlier in Allada, Benin. The training was once more adapted to help start the drug trial. Several international and national WHO officials were in attendance.

In all, over 60 people passed the GCP test and received the GCP Certificate. The training was combined with practical issues e.g., Prevention of Disabilities training with physiotherapists; discussing and hand-on training of dressing changes using a new protocol, developed in close collaboration with Wound Care experts from the WHO and MAP international; discussions about Counselling & Testing for HIV and pregnancy testing, especially among young female participants. Again, ethical and cultural discussions were held.

In February, 2013, a field trip was organized to monitor the trials at the five different trial sites in Ghana and Benin. Several different official visits to regional WHO offices, key stakeholders from the national BU programs, and hospital officials were combined with visits to BU clinics, trial sites, and satellite sites including dressing stations and health centers.

For the drug trial, field trips for monitoring and troubleshooting were conducted in the fall of 2012. Ethics and GCP training greatly advanced the work, and currently near 100 participants have been enrolled in that study.

Both studies have been registered with Clinicaltrials.gov: the surgical trial under number NCT01432925, and the drug trial under number NCT01659437; this registration is part of GCP standards.

Potential impact, main dissemination activities and exploitation of results

Impact on understanding BUD pathogenesis

We have made major advances in understanding the pathogenesis of BUD by elucidating the molecular action of ML toxin. This will undoubtedly help to develop new therapeutic strategies. Moreover, the immunosuppressive properties of the ML toxin may be of interest for the therapy of other diseases.

We have also made large steps in understanding the immune response to various antigens of *M. ulcerans* that also will have an impact on management of the disease. Important is also the establishment of a novel animal model system that is more closely related to the human disease as models used so far and will help to better characterize the pathologic features if the infection.

Progress towards a vaccine against BUD

We have obtained proof of principle in several experimental systems that a significant protection by vaccination is possible in BUD. We have developed several vaccine candidates and are in the capacity to evaluate lead vaccine candidates that can in the future progress to clinical studies.

Capacity building

At the end of the project sustainable structures have been established for case finding, diagnostic verification as well as for future vaccine trials through training in epidemiological field research and the performance of clinical trials and for immunological investigations in Buruli ulcer research.

Capacity building in 4 African partner laboratories was successfully performed. Laboratories and facilities were prepared and are now in use. All 5 African beneficiary sites have been equipped with operating procedures and the necessary instrumentation and reagents. Extensive on-site training was performed. Quality System Procedures and Standard Operating Procedures for patient management, collection of diagnostic samples and laboratory confirmation are in place.

Dissemination

The scientific results of the work of the consortium have been documented in many excellent publications, many with joint authorships among different partners of the consortium proving the extensive cooperation during the BuruliVac project. A large number of publication is still to be published. Major scientific results have been presented at different international conferences and workshops, in particular at the international meeting on Buruli ulcer that is organised by WHO as the most important platform for BUD research and management. BuruliVac was widely disseminated to both the scientific community and the general public. It has substantially increased the awareness for the Buruli ulcer disease world wide in the scientific community and in the population of endemic countries. The BuruliVac project leaves a modern and efficient databank and a precious collections of strains and patient cells for further scientific studies.

List of beneficiaries

Beneficiary Number	Beneficiary name	Beneficiary short name	Contact persons	Country
P1 (coo)	BERNHARD-NOCHT-INSTITUT FUER TROPENMEDIZIN	BNITM	Bernhard Fleischer	Germany
P2	LUDWIG-MAXIMILIANS-UNIVERSITAET MUENCHEN	LMU	Gisela Bretzel	Germany
P3	PRINS LEOPOLD INSTITUUT VOOR TROPISCHE GENEESKUNDE	ITM	Bouke de Jong	Belgium
P4	SCHWEIZERISCHES TROPENINSTITUT	STI	Gerd Pluschke	Switzerland
P5	ST GEORGE'S HOSPITAL MEDICAL SCHOOL	SGUL	Mark Wansbrough Jones	United Kingdom
P6	ACADEMISCH ZIEKENHUIS GRONINGEN	UMCG	Tjip S van der Werf	Netherlands
P7	INSTITUT PASTEUR	IPP	Caroline Demangel	France
P8	UNIVERSIDADE DO MINHO	UMINHO	Jorge Pedrosa	Portugal
P9	DEUTSCHE LEPRA- UND TUBERKULOSEHILFE e.V.	DAHWT	Adolf Diefenhardt	Germany
P10	KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY	KNUST	Ohene Adjei Richard Phillips	Ghana
P11	MINISTERE DE LA SANTE	PNLLUB	Ghislain Sopoh	Benin
P12	CENTRE NATIONAL HOSPITALIER DE PNEUMO-PHTISIOLOGIE	LRM	Dissou Affolabi	Benin
P13	INSTITUT MEDICAL EVANGELIQUE DE KIMPESE	IME	Delphin Mavinga Phanzu	DR Congo
P14	INSTITUT SCIENTIFIQUE DE SANTE PUBLIQUE	WIV-ISP	Kris Huygen	Belgium
P15	LIONEX GMBH	LIONEX	Mahavir Singh	Germany
P16	EUROPEAN RESEARCH AND PROJECT OFFICE GMBH	EUR	Vera Schneider Julia Buech	Germany