Immunoparasitology of Neglected Helminth Infections

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
Patrick Voßberg

Tübingen
2019

Gedruckt mit der Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen

Tag der mündlichen Qualifikation:	18.07.2019
Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	Prof. Dr. Peter Soboslay
2. Berichterstatter:	Prof. Dr. Hans-Georg Rammensee

Table of Contents

Table of Contents

Zusammenfassung

Infektionen durch Helminthen werden durch eine Vielzahl an Wurmarten ausgelöst und zeigen im Verlauf der Erkrankung verschiedenartigste Symptome, die bis zum Tod führen können. Eine große Mehrzahl der Helmintheninfektionen zählen zu den vernachlässigten Tropenkrankheiten, da sie mit Armut assoziiert sind. Trotz der lebensbeeinflussenden Folgen einer Wurm-erkrankung finden Sie jedoch nur wenig Beachtung und nur sehr wenig Forschungsgelder werden in Projekte, die diese Infektionen betreffen investiert.

Anhand parasitologischer, serologischer, ophthalmologischer und entomologischer Untersuchungen konnte nachgewiesen werden, dass eine Übertragung von *Onchocerca volvulus* in Nord- und Zentraltogo noch immer vorkommt. Sowohl Kinder, wie auch Erwachsene wiesen Antikörper gegen *O. volvulus* Antigene auf. Auch Augenpathologien, die auf eine aktive Infektion mit *O. volvulus* hindeuten wurden gefunden. Die Prävalenz in den Untersuchungsgebieten lag unter 5%, jedoch wurden in einigen Dörfern auch Prävalenzen zwischen 5% und 10% gefunden. Durch starke saisonale Migration in und aus den endemischen Gebieten, insbesondere auch in und aus Nachbarländern, werden viele Personen während den Massenbehandlungen nicht angetroffen und stellen ein mögliches Reservoir für den Parasiten dar. Um die Onchozerkose auszurotten reichen die Standardmaßnahmen der Massenbehandlung mit Ivermectin nicht aus. Insbesondere sollte auf die saisonale länderübergreifende Migration der Bevölkerung eingegangen und zukünftige Ivermectin-behandlungen mit benachbarten nationalen Onchozerkose-Kontrollprogrammen zeitlich abgestimmt werden.

Durch die Massenbehandlung mit Ivermectin ist die Mikrofilarienanzahl unter der Haut drastisch gesunken und der „Gold-Standard“ nicht mehr empfindlich genug, um eine Infektion mit *O. volvulus* zuverlässig nachzuweisen. Zum ersten Mal wurden durch eine Immunpräzipitation *O. volvulus*-spezifische Peptide von Antigen-präsentierenden Zellen in Onchozerkomen isoliert und anschließend mittels Massenspektrometrie identifiziert. Die gefundenen Peptide entstammten dem immuno-dominanten Ov33 Protein, P-Glykoprotein, Onchocystatin und *Wolbachia*, dem Endosymbionten von *O. volvulus*. Mithilfe einer Epitopen-Vorhersage und der Synthese von überlappenden Peptiden des immundominanten Antigens Ov33 wurden weitere *O. volvulus*-spezifische Peptide identifiziert. In Tests wurden die Peptide mit einer hohen Empfindlichkeit von Onchozerkose-patienten erkannt. Mittels ROC Analyse wurden 4

Peptide identifiziert, die eine Sensitivität von 100% und eine Spezifität von über 80% zeigten. Eine kombinierte Anwendung dieser Peptide könnte die Sensitivität und Spezifität von serologischen Tests steigern.

Eine weitere vernachlässigte Erkrankung stellt die Alveoläre Echinokokkose dar. Unbehandelt kann diese Wurmerkrankung zum Tod führen. Benzimidazole, die einzigen therapeutischen Medikamente, die zurzeit erhältlich sind haben nur eine parasitostatische Wirkung auf den Parasiten. Manche Patienten zeigen sich jedoch schwerwiegende Nebenwirkungen auf diese Medikamente. Bei der Analyse von *Echinococcus multilocularis* Metacestoden cDNA auf humane Microarrays zeigte sich eine starke Expression von Genen, die mit Krebs assoziiert sind. Auf dieser Basis wurden zytostatische Krebsmedikamente ausgewählt und sowohl *in vitro* als auch *in vivo* getestet. Das Parasitenwachstum wurde durch Docetaxel gehemmt, während Paclitaxel und Navelbine das Wachstum nur für bis zu 3 Monate verlangsamte. Vorinostat und Doxorubicin hatten keine nachweisbare Wirkung. Eine Anwendung dieser Medikamente in weiteren vorklinischen Studien könnte dazu beitragen neue Behandlungsmethoden für eine Infektion mit *Echinococcus multilocularis* zu entwickeln.

Summary

Helminth infections are triggered by a variety of worm species and show a variety of symptoms which can lead to death. A large majority of helminth infections are considered as neglected tropical diseases which are associated with poverty receive little attention and very little research funding.

Based on parasitological, serological, ophthalmological and entomological examinations it could be proven that transmission of *Onchocerca volvulus* in north and central Togo is still ongoing. Both, children and adults had antibodies against *O. volvulus* antigens. Eye pathologies suggesting an active infection with *O. volvulus* were also been found. The allover prevalence in the study areas was below 5%, but prevalences between 5% and 10% were found in some villages. Strong seasonal migration in and out of the endemic areas, especially into neighboring countries has left many people unaffected during mass treatment and may present a possible reservoir for the parasite. To reach elimination of onchocerciasis, it is necessary to include further activities beyond the treatment with ivermectin. Particular attention should be paid to the seasonal transnational migration of the population and to timely coordination of future ivermectin treatments with neighboring national onchocerciasis control programs.

Mass treatment with ivermectin has drastically reduced the number of microfilariae in the skin and the "gold standard" is no longer sensitive enough to reliably detect infections with *O. volvulus*. For the first time, immunoprecipitation was used to isolate *O. volvulus*-specific peptides from antigen-presenting cells in onchocercomata tissues and then identify them by mass spectrometry. Peptides found were derived from the immunodominant Ov33 protein, P-glycoprotein, onchocystatin and *Wolbachia*, the endosymbiont of *O. volvulus*. Using epitope prediction and synthesis of overlapping Ov33 peptides, additional *O. volvulus*-specific peptides were identified. In IgG-specific ELISAs, peptides were recognized with a high sensitivity by onchocerciasis patients. Using a ROC analysis, 4 peptides were identified which showed a sensitivity of 100% and a specificity of over 80%. The combined use of those peptides could increase the sensitivity and specificity of serological tests.

Another neglected disease is the alveolar echinococcosis. If left untreated, this worm can lead to death. Benzimidazoles, the only therapeutic drugs currently available, have only parasitostatic effects on the parasite. However, some patients show serious side effects on these medications. Analysis of *Echinococcus multilocularis* metacestoden

cDNA on human microarrays showed strong expression of genes associated with cancer. Based on these findings, cytostatic FDA-approved anti-cancer drugs have been selected and tested both in vitro and in vivo. Parasite growth was inhibited by docetaxel, whereas paclitaxel and navelbine only slowed growth for up to 3 months. Vorinostat and doxorubicin had no detectable effects. The application of these drugs in other preclinical studies could help to develop new treatments for *Echinococcus multilocularis* infection.

Published Manuscripts

This thesis is based on the following publications and manuscripts:

Komlan K, Vossberg PS, Gantin RG, Solim T, Korbmacher F, Banla M, Padjoudoum K, Karabou P, Köhler C, Soboslay PT. *Onchocerca volvulus* infection and serological prevalence, ocular onchocerciasis and parasite transmission in northern and central Togo after decades of *Simulium damnosum s.l.* vector control and mass drug administration of ivermectin. PLoS Negl Trop Dis. 2018 Mar 1;12(3):e0006312.

Contribution of PS Voßberg to this publication: Sample collection, performance of PCR, data analysis and manuscript drafting.

Voßberg PS, Stevanović S, Hotz C, Gantin RG, Komlan K, Soboslay PT, Köhler C. Identification of *Onchocerca volvulus*-specific peptide antigens for serodiagnosis of onchocerciasis. Submitted to Parasitology Research Jan. 2019.

Contribution of PS Voßberg to this manuscript: Sample collection, performance of experiments, data analysis and data interpretation, manuscript drafting and revision.

Wangala B, Gantin R, Voßberg P, Vovor A, Poutouli W, Komlan K, Banla M, Köhler C, Soboslay PT. Inflammatory and regulatory CCL and CXCL chemokine and cytokine cellular responses in patients with patent *Mansonella perstans* filariasis.

Contribution of PS Voßberg to this publication: Isolation of parasite antigens, determination of endotoxin in antigens, cell culture and quantification of cytokines and chemokines.

Huang X, Wiehr S, Wild AM, Voßberg P, Hoffmann W, Grüner B, Köhler C, Soboslay PT. The effects of taxanes, vorinostat and doxorubicin on growth and proliferation of *Echinococcus multilocularis* metacestodes assessed with magnetic resonance imaging and simultaneous positron emission tomography. Oncotarget. 2018 Jan 10;9(10):9073-9087.

Contribution of PS Voßberg to this publication: Parasite cell cultures, parasite drug exposure experiments and manuscript drafting.

Introduction

Approximately more than 2.4 billion people worldwide are infected with helminths (Hotez et al. 2007). Especially people living in poverty with miserable hygiene standards and close contact to infectious vectors are in risk of getting infected. Helminth infections are poverty-promoting and can cause disability, undernutrition, disfigurement, cognitive impairments as well as high economic loss for the affected countries (Hotez et al. 2009). Parasitic worms can be found worldwide but especially in rural or developing countries in tropical or sub-tropical regions in Africa, Asia and the Americas (Hunter 2014). Many of these helminth infections are grouped as neglected tropical diseases (NTDs). This diverse group comprises parasitic worms, protozoa, bacteria and ectoparasites that are strongly associated with poverty and receive only small research funding (Mitra and Mawson 2017). The Global Burden of Disease study of 2017 attributed 17.3 million disability-adjusted life years (DALYs) to NTDs with a mortality of 152 000 deaths per year (Molyneux, Savioli, and Engels 2017; DALYs and Collaborators 2018). Over the last years, the interest in NTDs increased and the World Health Organization (WHO) has developed defined plans for the elimination of these diseases in Africa and Asia (World Health Organization 2013, 2011).

Onchocerciasis

Onchocerciasis is one of the major NTDs prioritized by the World Health Organization (World Health Organization 2012). 187 million people are estimated to live in onchocerciasis-endemic regions and an estimated 37 million are infected (World Health Organization 2016b). More than 99% of those people live in 31 sub-Saharan African countries, representing about 120 million endangered and 21 million infected people (Coffeng et al. 2013; World Health Organization 2015; Disease, Injury, and Prevalence 2018). Onchocerciasis is caused by the filarial nematode *Onchocerca volvulus* and is transmitted by black flies of the *Simulium damnosum* complex. Once infected, the L3 larvae migrate through the subcutaneous tissue and mature into adult worms. Female worms are encapsuled into nodules (onchocercomata), which are created by the immune system. Adult males however, can migrate through these nodules and mate with the bigger female worms. Eggs mature internally into microfilaria and are released from the female's body. Pathological symptoms are caused by migrating microfilaria, especially upon their death. The most common symptom of onchocerciasis is onchodermatitis. It is characterized by pigment disorders

of the skin and pruritic rash (Murdoch 2018). Long-term infections can cause diseases of the eyes. Microfilaria can migrate the cornea, anterior eye chamber, retina and even the optic nerve causing inflammations which ultimately lead to blindness. Until now, there is no treatment against the adult worms. Microfilaria, however, can be killed by Ivermectin. For almost three decades, onchocerciasis interventions with vector control and mass drug administration of ivermectin were ongoing by the Onchocerciasis Control Programme (OCP) and the African Programme for Onchocerciasis Control (APOC) (World Health Organization 2016a). In vast parts of the initial OCP area, *O. volvulus* infection prevalence and microfilaria load in the skin have greatly been reduced (Boatin 2008; Seketeli et al. 2002).

Mansonelliasis

Onchocerciasis patients are often coinfected with *Mansonella perstans* (Simonsen, Onapa, and Asio 2011). The well-adapted filarial nematode is endemic in 33 African countries, northern South America and the Caribbean and an estimated 114 million people are infected, giving an estimated prevalence of about 20 percent (Simonsen, Onapa, and Asio 2011). Humans are infected through the bite of infective midges of the genus *Culicoides* where L3 larvae actively penetrate the host skin. The further lifecycle is still unknown, but adult worms were found in the several body cavities like the pericardium, the mesentery and connective tissues (Baird et al. 1987). Female worms are viviparous and produce unsheathed microfilariae which migrates into bloodstream. Diagnosis of *M. perstans* infections is usually done by detection and identification of these microfilaria in blood samples (Ta-Tang et al. 2018). Infections with *M. perstans* are, in contrast to other human filarial infections, not associated with specific clinical pathologies. However, some clinical reports on eosinophilia, subcutaneous swellings, aches, skin rashes and infections of the pericardial and pleural cavities have been linked to *M. perstans* infections (Baird et al. 1987), but symptoms may also be caused by coinfections with other helminths (Ta-Tang et al. 2018). The asymptomatic course of *M. perstans* infections could be attributed to chronic infections with recurring new infections which modulates the immune system. Due to the lack of specific symptoms and the high prevalence of coinfections with other filariae, it is difficult to determine the disability of *M. perstans* infections among endemic populations. In addition, effective treatment of *M. perstans* infection is lacking. Convential antifilarial drugs including ivermectin, praziquantel, albendazole and diethylcarbamatine (DEC) are ineffective (Bregani et al. 2006; Bregani, Tantardini, and

Rovellini 2007). After the identification of *Wolbachia* endosymbionts in *M. perstans*, several trials assessing the effectiveness of doxycycline showed dramatic reduction of *M. perstans* microfilaria (Coulibaly et al. 2009). The unknown life cycle paired with the absence of specific clinical symptoms and effective druf therapy results in a neglection of this parasite infection.

Echinococcosis

The human echinococcosis is a parasitic disease caused by tapeworms of the genus *Echinococcus*. The two most important zoonotic species are *E. granulosus* and *E. multilocularis*. The cystic echinococcosis (CE), caused by *E. granulosus*, occurs globally except in the Antarctica, while alveolar echinococcosis (AE), caused by *E. multilocularis* occurs predominantly on the northern hemisphere (Western China, North America and Central and Eastern Europe) (Romig, Dinkel, and Mackenstedt 2006; Thompson 2017). Worldwide, 666 434 DALYs are lost each year due to AE (Torgerson et al. 2010). In Germany a prevalence of 0.64/100 000 persons was calculated, but the public health authorities underestimate the number of cases by the factor 3-5 (Schmidberger et al. 2018; Jorgensen et al. 2008). Notably, in southern Germany, the calculated prevalence was much higher than in northern Germany (Baden-Württemberg: 2.18/100 000 inhabitants, Bavaria: 1.48/100 000 inhabitants) (Schmidberger et al. 2018).

Adult worms of *E. multilocularis* live in the intestine of canids like red foxes (*Vulpes vulpes*) which act as definitive hosts and release eggs within their feces. In parts of southern Germany, 75% of the red foxes are infected with *E. multilocularis* (Romig et al. 1999). Small rodents, such as mice, serve as intermediate hosts and are infected by fecal-oral uptake (Eckert and Deplazes 2004). Humans are accidental intermediate hosts and the main risk factors for infection is contact with foxes and dogs. Especially hunters, foresters and agricultural workers are particularly exposed (Eckert and Deplazes 2004; Kern et al. 2004; Kreidl et al. 1998; Piarroux et al. 2013). The parasite invades predominantly the liver but can also infiltrate adjacent tissues and organs. The metacestode, the larval stage of the *E. multilocularis*, show a tumor-like pattern and through budding of metacestode tissue, surrounding tissues are progressively invaded (Kern et al. 2003; Kern et al. 2017). In most cases infections with *E. multilocularis* are inconspicuous over years or even decades until first clinical symptoms occur (Ammann and Eckert 1996). Often, infections are found by chance at regular examinations. Therapeutic treatment of echinococcosis consists of radical surgery in early stages of

the infection followed by antihelminthic chemotherapy with parasitostatic benzimidazoles (Brunetti et al. 2010). Until the 1970s untreated echinococcosis had a mortilliy rate of 100% and death occurred within 15 years (Ammann and Eckert 1996). Diagnosis of echinococcosis is based on clinical findings: By means of PCR polymerase chain reaction (PCR), parasitic DNA can be detected in blood and tissue samples, while imaging techniques like Ultrasonic and Positron emission tomography - computer tomography (PET-CT) can detect in vivo the growing of cysts (Brunetti et al. 2010). Besides the initial development of benzimidazoles, the pharmaceutical industry is not interested in the support of new treatment options for echinococcosis. Thus, echinococcosis can be considered a neglected disease. Since 2010 the WHO listed CE as a Tropical Neglected Disease and AE as Neglected Zoonotic Disease, a subgroup of the NTDs (World Health Organization 2010).

Immune system

Understanding the human immune system and immunological responses to parasites and their impact on the hosts immune system is the first step in developing new diagnostic tools and vaccines.

The human immune system consists of three different mechanisms: mechanical and chemical barriers, the initiate and the adaptive immune system. The initiate immune system is a relatively old evolutionary defense strategy and includes myeloid cells like macrophages, dendritic cells (DC), natural killer cells (NK), mast cells as well as proteins of the complement system and plasma proteins. Pathogens are recognized by pathogen recognition receptors (PRR) such as toll-like receptors or Nod receptors, which are located on macrophages, neutrophil granulocytes and DCs. These receptors recognize pathogen- associated molecular patterns (PAMPs) like mannose-rich oligosaccharides, lipopolysaccharides (LPS) and other peptides as well as amino acids. Once recognized, pathogens are either attacked by cytotoxic contents of granulomas or phagocyted. Components of phagocyted pathogens are then processed and presented as peptide-antigens on Major Histocompatibility Complex (MHC) Class 2 molecules on the surface of antigen-presenting cells like native dendritic cells, eosinophil granulocytes or macrophages. These now activated cells migrate into lymph nodes and activate the adaptive immune system.

Human Leukocyte Antigen

In humans, the MHC complex is encoded by the human leukocyte antigen gene complex (HLA), located on chromosome 6 (Trowsdale 1988). HLA genes can be

divided in two groups: HLA-A, HLA-B and HLA-C correspond to MHC class I molecules and present intracellular antigens. HLA-DP, -DM, -DO, -DQ and -DR corresponding to MHC class II molecules and present extracellular antigens (Choo 2007). The encoding HLA genes are highly polymorph but within populations, frequent HLA haplotypes can be identified. Several studies indicated an influence of HLA alleles in immune responses and protective immunity in helminth and other infectious diseases (Meyer et al. 1994; Blackwell, Jamieson, and Burgner 2009).

Adaptive Immune system

Since most parasitic worms cannot be killed by their hosts initiate immune system, the adaptive immune system is induced to eliminate them. The adaptive immune system is extremely specific and consists of T- and B-lymphocytes. All T-cells originate from hematopoietic stem cells in the bone marrow and most of them develop in the thymus. T-cells can be distinguished by their Cluster of Differentiation (CD). During T-cell development, $CD4^-CD8^-$ double negative cells can differentiate into $CD4^+CD8^+$ double positive cells and finally into $CD8^+CD4^-$ and $CD8^-CD4^+$ single positive cells (D'Acquisto and Crompton 2011). $CD8^+$ cytotoxic cells recognize pathogen-specific peptide-antigens presented on MHC class I molecules on the surface of infected cells and either perforate them or induce apoptosis. Native $CD4^+$ T helper (Th) cells recognize receptor-specific peptide-antigens and differentiate into different T helper cell subclasses. These subsets can be defined by their cytokine and chemokine secretion, expression of surface receptors and different CDs and initiate different immune responses.

Th1 responses are cell-mediated. Th1 cells activate macrophages and cytotoxic cell which destroy cells infected with intracellular pathogens through phagocytosis and upregulated production of reactive oxygen radicals and nitric oxides (Bogdan, Rollinghoff, and Diefenbach 2000). Pathogens too big for phagocytosis (e.g. helminths) can also be damaged by reactive oxygens and nitric oxides (James and Glaven 1989; Malkin et al. 1987; Moncada, Palmer, and Higgs 1991). Th1 cells can further interact with native B cells and stimulate the production of IgG1 and IgG3, which act mainly against bacterial pathogens by opsonization for enhanced uptake by phagocytes (Vidarsson, Dekkers, and Rispens 2014). Humoral responses are mediated by Th2 cells and mainly react to extracellular pathogens like helminths (Harris and Gause 2011). Th2 cells activate eosinophil granulocytes as well as B-cells and are characterized by an enhanced production of IgE antibodies. Regulatory T-cells (Treg)

are responsible for the suppression of the adaptive immune response (Corthay 2009). They prevent autoimmunity and down-regulate the immune system after an infection. Interleucin-17 producing T helper cells (Th17) are pro-inflammatory and play an important role in host defense against infections (Ouyang, Kolls, and Zheng 2008). They activate local epithelial and stromal calls to recruit neutrophils and macrophages to infected tissues (Tesmer et al. 2008). A new subset of T helper cells are Interleucin-9 producing cells (Th9). Although many functional and regulatory roles of this subset of T helper cells are currently not understood, it was shown that Th9 cells are involved in allergic inflammation, tumor and parasitosis (Anuradha et al. 2013; Jia and Wu 2014).

In contrast to T-lymphocytes, B-cells mature in the bone marrow and circulate in blood and lymphatic organs. B-cell receptors (BCR) on their surface can bind a high variety of chemical structures and initiate an antibody response. B-cells can be activated either T-cell-dependent or -independent. Independent B-cell activation occurs mainly by contact with repeating polysaccharides from the surface of bacteria and results in differentiation to short-living plasmablasts which produce mainly IgM (Mond et al. 1995). Most antigens activate B-cells with the T-cell-dependent activation. Once an antigen binds to the BCR, it is taken up into the B-cell, being processed and presented on the surface of MHC class II molecules (Parker 1993). T helper cells can recognize these antigens via T-cell receptor (TCR) on their surface and lead to proliferation of B-cells into short-living plasmablasts, long-living plasma cells and memory B-cells.

Immunoglobin G

B-cells produce large amounts of antigen-specific immuno-globulins, which can be found membrane bound as B-cell receptors or secreted as antibodies. Immunoglobins are heterodimeric, Y-shaped proteins and are composed of two heavy and two light chains which are connected by a disulfide bridge (Schroeder and Cavacini 2010). They can be divided into the antibody-binding fragment (Fab) and the fragment crystallizable region (Fc) which interacts with the complement system and Fc receptors. Based on their heavy chains, human antibodies can be distinguished into IgG, IgM, IgA, IgD, IgE and several subclasses (Schroeder and Cavacini 2010). IgG is the predominant isotype found in the human body and can be distinguished in IgG1, IgG2, IgG3 and IgG4, in order of decreasing abundance in blood of normal, healthy individuals (Vidarsson, Dekkers, and Rispens 2014). Despite more than 90% of the amino acid sequences are identical in all 4 subclasses, each subclass differ in half-life, antigen

binding and immune responses (Vidarsson, Dekkers, and Rispens 2014). The binding of antibodies to a pathogen has a large spectrum of effects. Opsonization by antibodies facilitates the recognition of pathogens by neutrophils and macrophages and leads to phagocytosis. The neutralization of pathogens by binding on their surface suppresses their function and agglutination activates the complement system. Large parasites like helminths are too big to be destroyed by phagocytosis. The antibody-dependent cell cytotoxicity (ADCC) is an important mechanism against them. It mediates effector cells, such as NK cells, eosinophils and macrophages, to actively lyses targeted cells by secreting perforins and granzymes or activation of apoptosis. IgG1 encompasses more than 60% of all IgG subclasses (Vidarsson, Dekkers, and Rispens 2014). It is predominantly responsible for immune responses against soluble proteins and membrane antigens. It can bind to immunoglobulin-Fc receptor I (FcγR), FcγRII and FcγRIII on monocytes and to FcγRII and FcγRIII on neutrophils or activate the complement via binding to the C1 complex (Bruhns et al. 2009; Redpath et al. 1998). The immune response to bacterial polysaccharides and carbohydrates is almost completely restricted to antibodies of the IgG2 subclass (Smith, Bain, and Schiffman 1990). IgG2 binds only to FcγRII on monocytes (Bruhns et al. 2009). IgG3 is predominantly a proinflammatory antibody and mainly responsible for immune responses against protein or polypeptide antigens by activating the complement (Vidarsson, Dekkers, and Rispens 2014). Together with IgG1, these subclasses are also mainly responsible for viral infections (Cavacini et al. 2003). IgG4 antibodies are associated with repeated or long-term exposure to antigens, allergies and filarial infections (McSorley and Maizels 2012; Aalberse et al. 2009). IgG4 binds to FcγRI and FcγRII on monocytes and FcγRI on neutrophils (Bruhns et al. 2009). The antibody subclass switch to IgG4 is linked to a downregulation of immune responses or tolerance reduction and high titers of IgG4 can be associated with an asymptomatic infection (Kurniawan et al. 1993).

Cytokines and Chemokines

Cytokines are small (10-25 kDa) molecules which are secreted by a large variety of cells, including macrophages, dentritic cells (DC), T- and B-lymphocytes, endothelial cells and stromal cells. These proteins are responsible for cell communication and involved in cell proliferation (Legler and Thelen 2016). They can also activate immune cells, regulate hematopoiesis, promote inflammation, but also suppress immune responses by binding to specific receptors on cell surfaces. Cytokines can be classified

into different groups, according to their function and effects. Interleukins (IL) are immunomodulatory proteins, that modulate growth, differentiation and activation of cells (Brocker et al. 2010). Chemokines are a group of small (8-11 kDa) chemotactic cytokines and are classified into CC, CXC, XC, or CX3C chemokines (Zlotnik and Yoshie 2000). They promote migration of distant leukocytes toward sites of inflammation (Nedoszytko et al. 2014). Cytokines of the Tumor necrosis factor (TNF) family are associated with physiological and pathological effects like tumor cell necrosis and apoptosis (Chu 2013). Interferons (IFN) activate NK cells and macrophages and play an important role in the initiate immune response to virus infections (Belardelli 1995).

Aim of study

After more than two decades of mass treatment with ivermectin, prevalence of *Onchocherca volvulus* infections declined largely, and elimination appears achievable in certain regions. In northern and central Togo, the present epidemiological situation remains unknown. The WHO recommends that mass drug administration with ivermectin can be stopped once the interruption of *O. volvulus* has been demonstrated. The aim of this study was to evaluate the current epidemiological situation of onchocerciasis prevalence in sentinel villages in northern and central Togo and determine if transmission has been interrupted and mass drug administration with ivermectin can be stopped.

With decades of mass treatment of the population with ivermectin, the microfilaria count in the skin of onchocerciasis patients has dropped rapidly. As a result, the current "gold standard" is no longer sensitive enough to detect infection without any doubt. The aim of this study was to identify *O. volvulus*-specific peptides with different methods which can be used as highly sensitive antigens in a serological assay to clearly detect an infection with *O. volvulus*.

Infections with *Mansonella perstans* occur in large parts of sub-Saharan Africa. The lifecycle is still unknown and there is no therapeuthic drug available. However, only few studies had focused this neglected disease. The aim of this study was to evaluate the influence of *M. perstans* to the host’s immune response.

Alveolar echinococcosis is rarely diagnosed, and patients can be asymptomatic over years. At the moment, no therapeutic treatment is available and patients with alveolar echinococcosis must undergo surgery followed by chemotherapy with parasitostatic benzimidazoles which can cause severe side effects. The aim of this pre-clinical study was to assess the effects of FDA-approved cancer drugs on *E. multilocularis* metacestodes in vitro and in vivo.

Results and Discussion

O. volvulus prevalence in north and central Togo

Due to the mass drug administration with Ivermectin and the vector control with insecticides for almost three decades, the microfilaria (Mf) load decreased greatly. The WHO Guidelines recommend the stop of mass drug administration and shifting to post-treatment surveillance when the interruption of parasite transmission is demonstrated (World Health Organization 2016a). Guatemala and Mexico, as well as some regions of Mali and Senegal already achieved the elimination of *O. volvulus* (Boatin 2008; Diawara et al. 2009; Kazura 2015; Rodriguez-Perez et al. 2015; Traore et al. 2012; World Health Organization 2016c; Evans, Unnasch, and Richards 2015). In western Uganda, several areas reached the WHO criteria for elimination of *O. volvulus* and in most regions, transmission of *O. volvulus* was interrupted (Katabarwa et al. 2018). In northern Venezuela, the transmission of onchocerciasis was interrupted in 2012 and further studies showed that the elimination phase was reached (World Health Organization 2017; Convit et al. 2013).

Together with the National Onchocerciasis Control Programme (NOCP), we examined endemic populations from 11 sentinel villages which are located within 1 km of distance to the rivers Ôti, Kara or Mô during their annual survey. Skin biopsies were collected from the left and right iliac crest and incubated with physiological saline solution (Fig. 1).

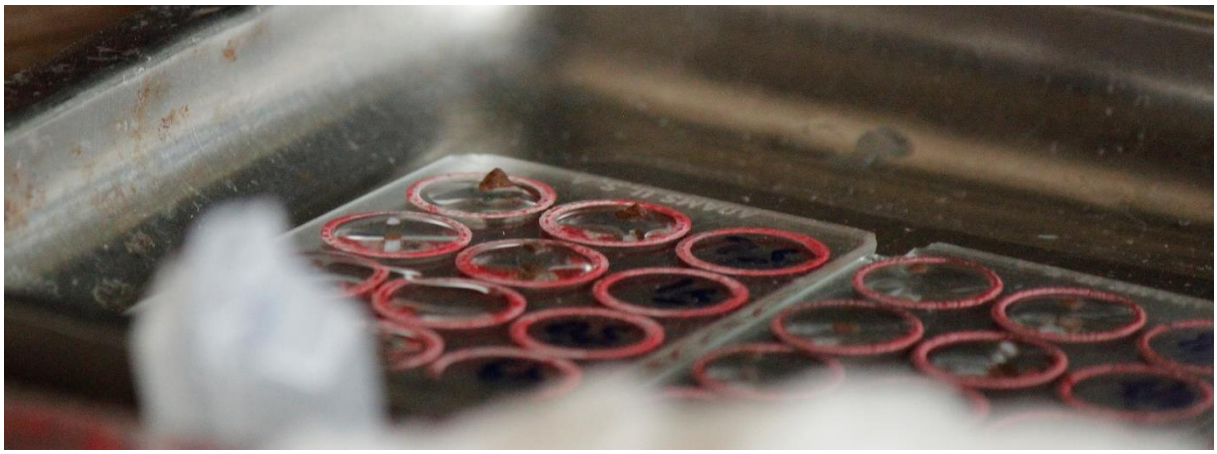


Figure 1: Skin snip from the left hip. Using a sterile 2-mm Holth corneo-scleral punch biopsy tool, skin snips were collected from the left and right iliac crest and incubated with physiological saline solution for 30 minutes.

After 30 minutes of incubation, skin snips were analyzed under a microscope for emerging microfilaria (Fig. 2).

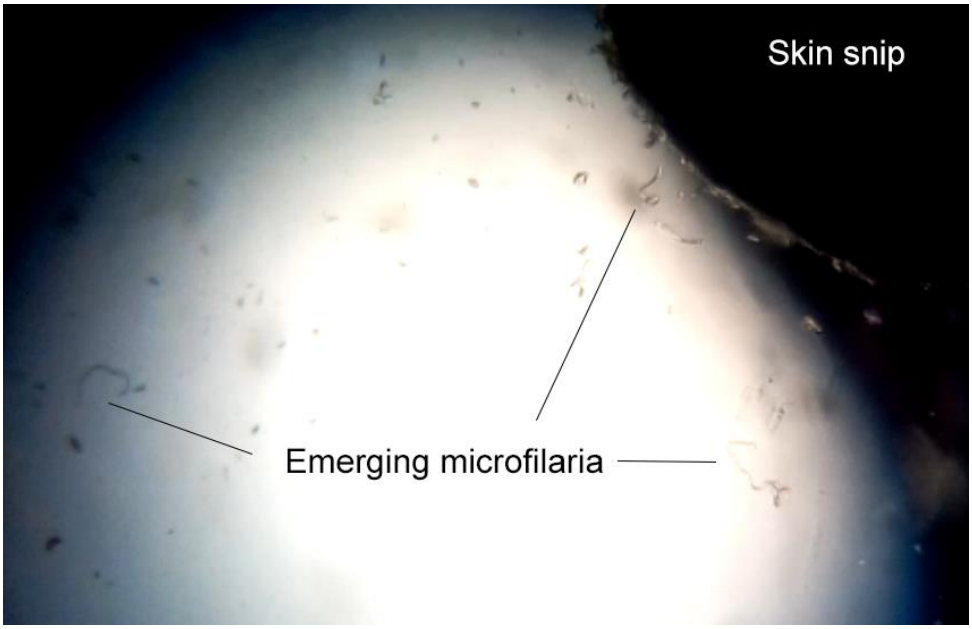


Figure 2: Skin snip with emerging microfilaria under the microscope (10-fold magnification). Living *O. volvulus* microfilaria (220-280µm) emerge from the skin snip (2mm) into saline solution.

Our results show that the overall prevalence of *O. volvulus* infection in the surveyed populations in north and central Togo decreased below 5%. In several villages however, a microfilaria positivity between 5-10% can be observed, indicating that the parasite interruption was not achieved yet.

In addition to the skin biopsy surveys, blood samples were collected from fingertip pricks with a sterile lancet on protein saver cards (Fig. 3). Dried blood samples from the protein saver cards were stamped out and eluted in the laboratory for the application in an ELISA-based serological evaluation with an *O. volvulus* adult worm antigen extract (OvAg) and the Ov16 antigen. The observed all-ages sensitivity was 89%, respectively 71%. These results support previous findings that the Ov16 IgG4 ELISA would miss many Mf-positive cases and underestimate the actual *O. volvulus* infection prevalence (Golden et al. 2016). To detect ongoing *O. volvulus* transmission, the WHO recommends the Ov16-based ELISA for testing children under 10 years (World Health Organization 2016a). Not only the sensitivity of the Ov16-based test poses a problem but also the fact that the people with the highest exposure are field workers on the river sides, often women above the primary school age (Golden et al. 2016). Due to human migration in and out of the river basins, many people did not receive ivermectin treatment and may limit the treatment coverage. Especially men

between 15 and 40 years were absent during the examination and treatment and may represent a parasite reservoir. The absence was often explained by travelling and temporary work away from the villages, often across the borders of Ghana and Benin. These persons should be selectively treated to improve the therapeutic treatment coverage with ivermectin.

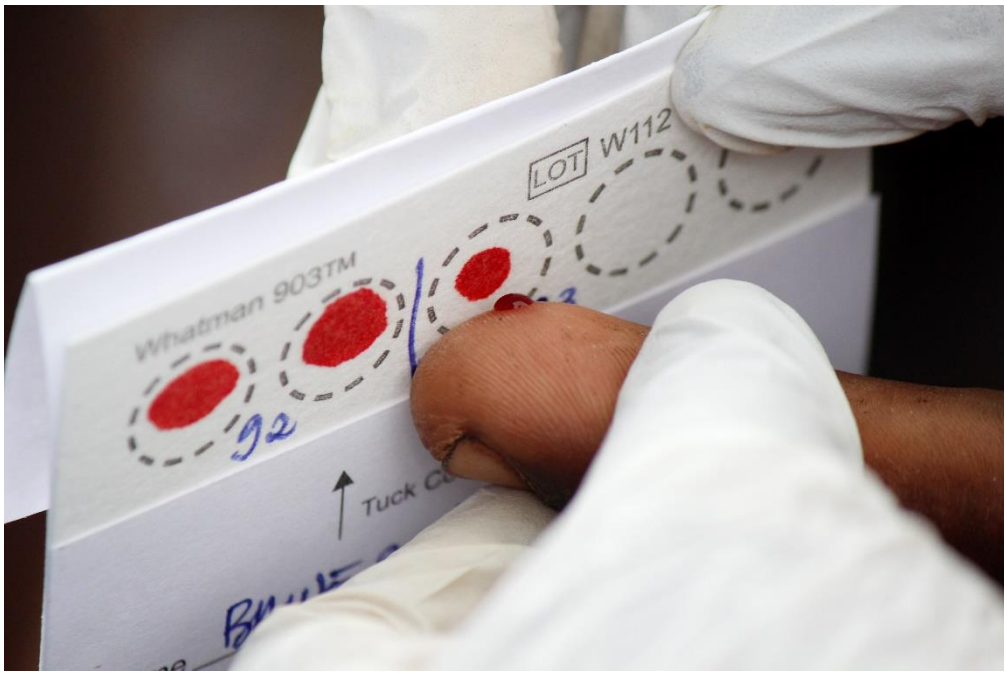


Figure 3: Blood sample from fingertip prick. Fingertips were disinfected and pricked with a sterile lancet. Blood samples were collected on protein saver cards for later analysis.

The ophthalmological assessments were performed using slit lamps and ophthalmoscopes. We identified ocular pathologies caused by active *O. volvulus* infections, indicating that parasite transmission is still ongoing (Fig. 4). In the river basin of Ôti, fewer cases of ocular pathologies were observed than in the river basins of Mô and Kéran, which could indicate a regression of ochocerciasis-induced ocular pathologies. The Ôti river basin was part of the initial Onchocerciasis Control Programme (OCP), where vector control and ivermectin mass drug administration (MDA) has been applied for almost three decades (Boatin 2008). Annual treatment with ivermectin may prevent the emerge of ocular pathology, but the present ophthalmological surveys indicate, that an interruption of *O. volvulus* transmission has not yet been achieved and MDA should not be stopped.

Figure 4: Examination of the posterior eye segment using an ophthalmoscope. The 10-year-old boy had an active *O. volvulus* infection that was confirmed by skin snip.

Transmission of *O. volvulus* has never been interrupted completely in Benin and north and central Togo. The river basins of Mô, Kéran and Ôti were Special Intervention Zones (SIZ) with intensified ivermectin distribution and vector control (Yameogo 2008). Nevertheless, a high vector density was documented in 2015 and 2016 with an annual biting rate (ABR) of 15519 bites/person/year of *Simulium damnosum s.l.* The observed biting rate was similar to that observed before launching the OCP and may favor ongoing parasite transmission (Onchocerciasis Control Programme in West Africa 2002). The Ov150 rtPCR on black flies support the ongoing transmission of *O. volvulus*. The persistent *O. volvulus* transmission can be attributed to the geographical conditions, which allow cross-border vector migration, especially during the raining seasons (Baker et al. 1990; Garms 1982). Thus, further efforts in transmission control of *O. volvulus* should be expand into cross-border collaborations and ivermectin treatment should be coordinated and applied during the same time to enhance the coverage of treatment in the community.

Identification of *O. volvulus-specific* peptides for serodiagnosis

The detection of patent *O. volvulus* infection has become increasingly challenging. Due to mass drug administration with ivermectin for more than two decades, the load of microfilaria in the skin is highly reduced and the “gold standard” for detection of active *O. volvulus* infections in skin biopsies has become more and more unsatisfied. The WHO recommends a serological test based on the Ov16 antigen, but this test shows low sensitivities (Golden et al. 2016). Diagnostic tests based on peptide antigens are already applied for various infectious diseases with good performance (Morre et al. 2002; Yoshida et al. 1992). Peptides are cheap and easy to produce and can be modified to increase the specificity by eliminating nonspecific regions or their chemical structure (Alcaro et al. 2003; Gauna et al. 2015; Gomara and Haro 2007; Noya et al. 2003; Wu et al. 1999).

In this study, several *O. volvulus*-specific peptide antigens were identified and applied for serodiagnosis of patent *O. volvulus* infection. For the first time, peptide antigens from antigen-presenting cells of onchocercomata tissues (Fig. 5+6) were isolated and identified, using immune-precipitation, HPLC and tandem mass spectrometry.

Figure 5: Onchocercomata. Active *O. volvulus* infection found on the upper leg of a woman in central Togo.

Figure 6: Isolated onchocercomata tissue from onchocerciasis patients (deep frozen).

Six *O. volvulus*-specific peptides, originating from the immunodominant Ov33 protein, P-Glycoprotein, oncho-cystatin and the *Wolbachia* endosymbiont of *O. volvulus* were identified. All antigens are well known and are already applied as vaccine candidates or recombinant antigens in immune diagnosis (Ardelli, Guerriero, and Prichard 2005; Bouchery et al. 2013; Cho-Ngwa, Liu, and Lustigman 2010; Lucius et al. 1992; Lustigman et al. 2018; Xu et al. 1998). The peptides were mainly recognized by IgG4 with high sensitivity and could thus be suitable for diagnostic application to detect a patent infection with *O. volvulus*. In contrast to previous works (Lagatie, Van Dorst, and Stuyver 2017), only moderate IgG1 and IgG3 isotype responses were observed. This switch in isotype recognition may be due to the fact that these peptides are eluted from MHC molecules of antigen-presenting cells in onchocercomata tissue. The continuous antigen presentation in the presence of active immune modulating molecules may favor the recognition of linear epitopes by IgG4 (Collins and Jackson 2013; Kurniawan et al. 1993; Maizels and Yazdanbakhsh 2003; van Riet, Hartgers, and Yazdanbakhsh 2007). Additionally, IgG4 is considered as a “non-inflammatory” immunoglobin isotype, interacting poorly with FcγRII, FcγRIII and complement and is associated with chronic persistence of helminth parasites (Davies et al. 2014).

The HLA phenotype distribution in the endemic study population showed DRB1*13, DRB1*11, DRB1*08, DRB1*03, DRB1*15 and DRB1*07 as the most frequent HLA-DRB1 alleles. Previous studies confirmed these findings (Goeury et al. 2018; Meyer et al. 1994). Based on these HLA phenotypes, the computer-based SYFPEITHI algorithm has predicted five peptide ligands from the Ov33 antigen. Four of the identified

peptides are located in the middle of the Ov33 protein. The Ov33-specific peptides eluted from the MHC molecules however were located at the N-terminal of the protein, suggesting that the N-terminal parts of the protein are more likely to be presented on MHC molecules than part of the middle of the protein. In contrast to the peptides isolated from MHC molecules which were mainly recognized by IgG4, these peptides showed immune responses mainly driven by IgG3 with sensitivities between 80% and 97.5%. Previous studies suggest that IgG3 may bind to Ov33 and activate the complement cascade but arrests before the terminal complexes are formed (Garred, Michaelsen, and Aase 1989; Meri et al. 2002).

The SYFPEITHI algorithm was used to predict peptides from the *Wolbachia* Surface Protein (WSP). *Wolbachia* are gram-negative endosymbiotic bacteria that can be found in most filarial nematodes, including *Brugia spp.*, *W. bancrofti* and *O. volvulus* (Taylor, Bandi, and Hoerauf 2005; Kozek and Marroquin 1977). Four peptides were identified by SYFPEITHI, located at the N-terminus and the middle of the protein, suggesting the location of relevant parts for immune recognition in those regions. The immune response was mainly driven by IgG3 with moderate sensitivity above 70%. In previous studies with *B. malayi* and *W. bancrofti* patients, WSP was mainly recognized by IgG1, IgG4 and total IgG (Punkosdy, Addiss, and Lammie 2003; Shiny et al. 2009). Short linear peptides we used in our studies are, in contrast to the full length recombinant WSP, not glycosylated or processed, which could result in a shift in antibody isotype recognition (Lagatie, Van Dorst, and Stuyver 2017; Lagatie et al. 2018). Targeting the improvement of peptide antigen recognition, the complete Ov33 antigen was analyzed in 24 overlapping peptides, each 20 amino acid in length, spanning the whole protein. The serological responses to the peptides were mainly mediated by IgG3. 11 peptides were 100% sensitive for accurate detection of patent *O. volvulus* infection.

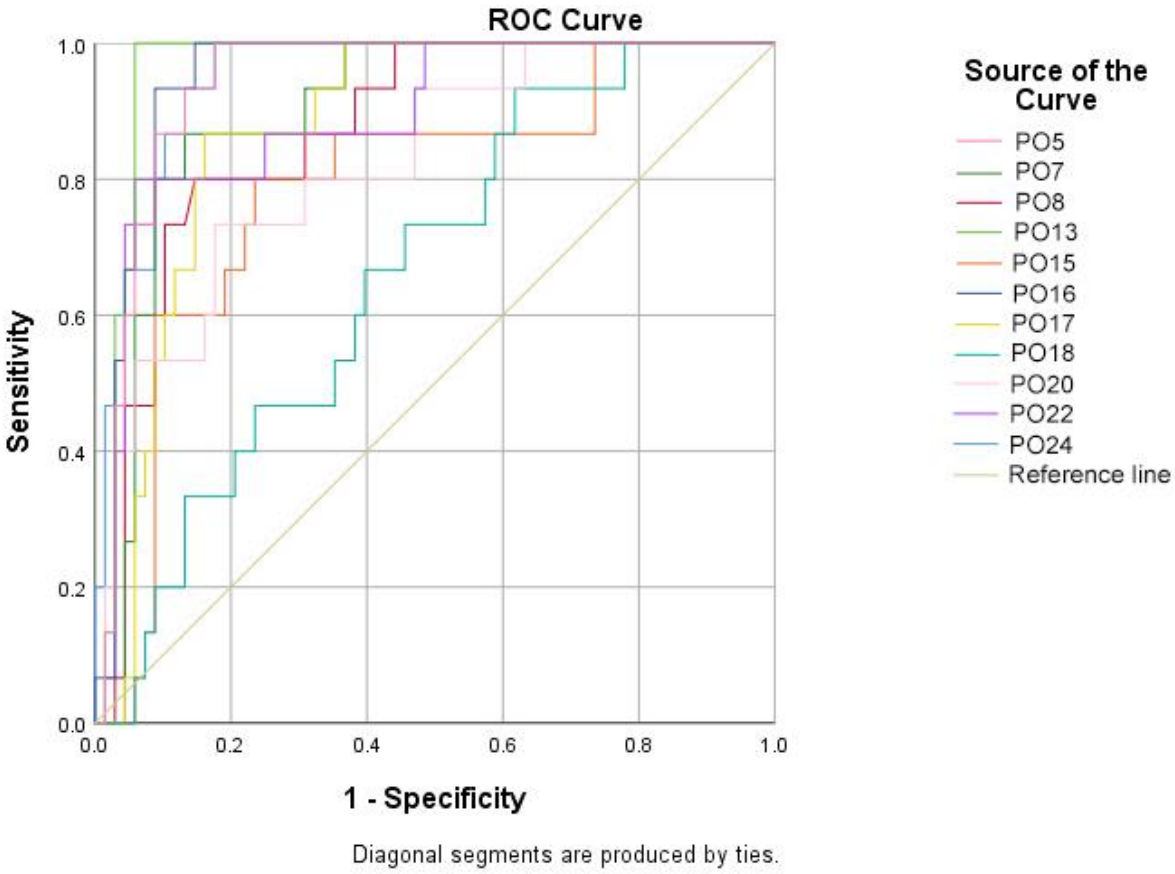


Figure 7: ROC Analysis of 11 peptides. X-axis: 1-Specificity (0.0 = 100% Specificity), Y-axis: Sensitivity (1.0=100%).

To evaluate the diagnostic performance of the peptides and cross-reactivity with other helminth infections, these 11 peptides with 100% sensitivity were applied in an IgG3 ELISA. Sera from *O. volvulus* Mf-negative participants with intestinal, intravascular helminth or intestinal protozoan monoinfection were used as controls and dry blood samples from Mf-positive onchocerciasis patients who received ivermectin treatment over several years. The Receiver Operation Curve (ROC) analysis identified four peptides with a 100% sensitivity and a specificity above 80% (Fig.7). Especially one peptide performs very good with a sensitivity of 100%, a specificity of 94% and a cutoff of 0.6.

In conclusion, the combined application of those peptides but also in combination with other peptides and proteins current tested for serodiagnosis, may enhance the sensitivity and specificity for accurate detection of patent *O. volvulus* infection.

Cytokine and chemokine responses in patients with patent *Mansonella perstans* filariasis

Although mansonelliasis is very common in sub-Saharan Africa and an estimated 20% of the population is infected, only little is known about this tropical disease (Simonsen, Onapa, and Asio 2011). The diagnosis of mansonelliasis is usually done by detection and identification of microfilaria in the blood (Fig. 8). Mansonelliasis is often co-endemic with onchocerciasis and lymphatic filariasis. Due to MDA with ivermectin, large populations become permanently negative for *O. volvulus* microfilaria (Schulz-Key et al. 1993; Korbmacher et al. 2018; Kyelem et al. 2005). Ivermectin has only small effects on microfilaria from *M. perstans* and thus, chronic infections with *M. perstans* will persist (Wanji et al. 2016). The application of ivermectin influences the host's immune system, but it is still unknown to which extent an infection with *M. perstans* and repeated ivermectin treatment may modulate the immune response (Soboslay et al. 1992; Mai et al. 2007; Lechner et al. 2012; Arndts et al. 2014; Arndts et al. 2015; Wammes et al. 2016).

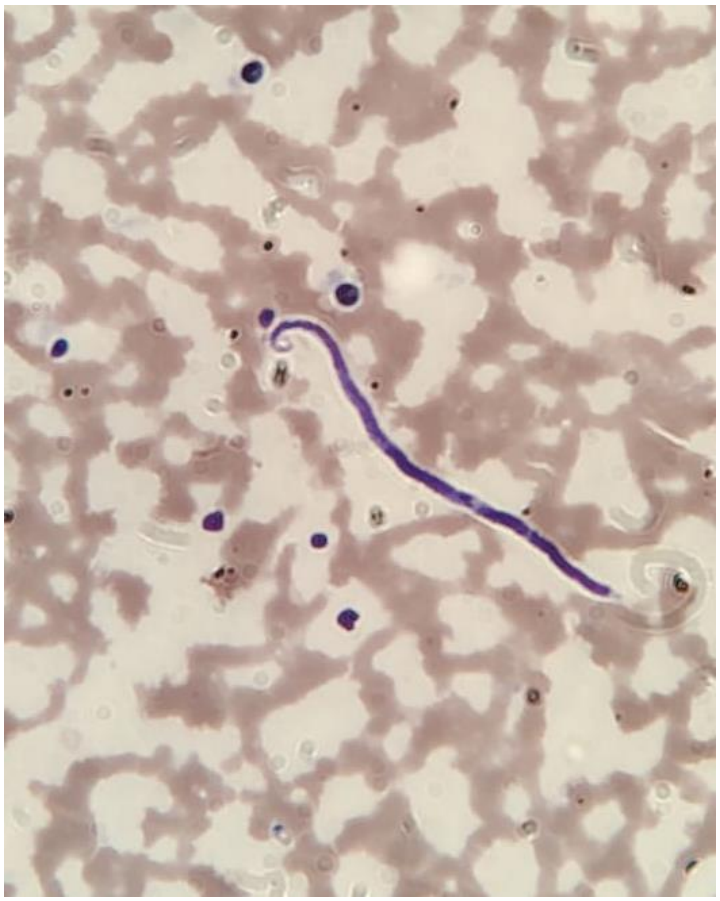


Figure 8: *Mansonella perstans* microfilaria (length ~200µm) in Giemsa-stained blood smear. 40-fold magnification.

In this study, we monitored the *in vitro* cellular responsiveness of mononuclear peripheral blood cells from mansonelliasis patients to different antigen simulations.
Spontaneous and antigen-inducible cellular production levels of several Th1 and Th2 proinflammatory and regulatory cytokines and chemokines could be observed. The levels of Th1-associated chemokine CCL13, the Th2-associated chemokines CCL22, CCL24 and CXCL8 as well as the levels of the regulatory-associated cytokine IL-27 were significantly enhanced in mansonelliasis patients in contrast to controls, while a depressed production of CXCL9 could be observed in mansonelliasis patients. This mixed immune response may indicate that there is no serious immune-mediated pathology, representing a balance between a tolerable parasite load and immune adaption which limits the parasite numbers. Microfilaria of *M. perstans* may activate neutrophil and eosinophil granulocyte-mediated mechanisms, but in parallel, *M. perstans* will dampen die inflammation by stimulating regulatory cytokines. Similar mechanisms were already found in mansonelliasis patients from Cameroon, W. bancrofti patients and hookworm-coinfected mansonelliasis patients (Ritter et al. 2018; Metenou et al. 2009; Metenou et al. 2011; Dolo et al. 2012).
High levels of the eosinophil- and neutrophil-activating chemokines CCL24 and CXCL8 may enhance the killing of microfilaria. Previous studies showed correlations between killing of microfilaria from *O. volvulus* and the activation and chemotaxis of neutrophils and macrophages by CXCL8 (Greene, Taylor, and Aikawa 1981; Brattig, Buttner, and Hoerauf 2001). Despite high levels of CCL24 and CXCL8 were observed, blood circulating *M. perstans* microfilaria were not eliminated by granulocytes.
The role of ivermectin in immune modulation of mansonelliasis patients is also not yet clear. The repeated treatment with ivermectin could facilitate or impede coinfections.
Previous studies showed a temporarily increase in CCL17 and CCL22 levels after ivermectin treatment and after reduction of *O. volvulus* microfilaria load, both cytokine levels dropped significantly (Fendt et al. 2005).This indicates that the elimination of microfilaria could be supported by the Th2-type chemokines CCL17 and CCL22. In the present study, neither mansonelliasis patients nor controls show enhanced levels of those chemokines after stimulation with helminth antigens. Only the stimulation with phytohemagglutinin (PHA), Purified protein derivative (PPD) and Streptolysin O (SLO) lead to an increased production of CCL17 and CCL22 in mansonelliasis patients, indicating, that the Th2-type cell recruitment and migration of lymphocytes is intact (Kunkel and Butcher 2002).

The Th1-type chemokine CCL13 can be found in many chronic inflammatory diseases. CCL13 can bind to several chemokine receptors and enhance the cytokine production which lead to activation of further effector cells (Mendez-Enriquez and Garcia-Zepeda 2013). This cascade could facilitate the elimination of M. perstans. In our study, a significant enhanced cellular release of CCL13 was observed after stimulation with mitogen, bacteria- and helminth antigen in mansonelliasis patients in contrast to controls.

CXCL9 and CXCL10 are also Th1-related chemokines. Both chemokines were mainly activated through IFN-γ and contribute to tissue damage by recruiting T cells, eosinophils, monocytes and NK cells to inflammation sites (Charo and Ransohoff 2006; Gotsch et al. 2007). The production of CXCL9 can be induced by stimulation with helminth extracts, protozoan and bacterial antigens and was always higher in mansonelliasis patients, indicating, that helminths can also activate Th1 chemokines.

Regulatory T cells (Treg) can suppress adaptive immune responses and are able to steer monocyte differentiation toward alternatively activated macrophages (AAM) which possess potent inhibitory activity against T cells (Tiemessen et al. 2007; Huber et al. 2010). Chronic filarial infections are associated with increased levels of regulatory cytokines produced by Treg and the presence of AAM, accompanied with increased expression of the genes encoding resistin, mannose receptor C type 1 (MRC1), macrophage galactose type C lectin (MGL), and CCL18 (Babu, Kumaraswami, and Nutman 2009). In our study, the production of CCL18 did not differ between mansonelliasis patients and controls, suggesting a equilibrated cellular production of CCL18.

IL-27 is a regulatory cytokine with diverse influences on immune responses through promoting IL-10, as well as through antagonizing Th17 responses (Yoshida and Hunter 2015). In parasitic diseases, IL-10 plays an important role in limiting infection induced inflammation (Redpath, Fonseca, and Perona-Wright 2014). Previous studies demonstrated a correlation between the number of parasites in children and the level of IL-27 (Hegewald et al. 2015). In our study, the spontaneous production of IL-27 was higher in mansonelliasis patients than in controls, but IL-27 could only be induced in controls. It is still unanswered if the high levels of CCL24 and IL-27 have an impact on the control, facilitation or prevention of patent M. perstans infection or the pathogenesis.

The non-polarized cytokine and chemokine response we observed may facilitate the persistence of *M. perstans* and is responsible for the asymptomatic infection. This immune adaption, however, could facilitate the susceptibility with other protozoan and metazoan parasites.

Cancer drugs and their ability to suppress growth and proliferation of *Echinococcus multilocularis* metacestodes

The treatment of echinococcosis is based on surgery and long-term chemotherapy with benzimidazoles (BMZ). However, benzimidazoles are parasitostatic and not parasitocidal and undetected residual metacestodes may restart growth after the chemotherapy is stopped. In addition, some patients experience severe side effects like hepatoxicity and are left without an alternative treatment (Budke et al. 2017).

Previous studies observed antigenic similarities between *E. granulosus* and tumors, suggesting anti-cancer drug could be used as treatment of echinococcosis (Alvarez Errico et al. 2001; van Knapen 1980) but this was never done with *E. multilocularis*.

In this pre-clinical study, we identify human cancer related genes in *E. multilocularis* metacestodes and applied cytostatic drugs, presently used in cancer therapy for their capacity to inhibit *E. multilocularis* metacestode growth and proliferation.

Hybridization of *E. multilocularis* cDNA to human microarrays revealed a strong expression of human cancer-related genes of the RAS oncogene family (RAB2), the folate receptor (FOLR1), the eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), tubulins (TUBA1A, TUBA1C, TUBB3), aquaporin, calreticulin and synuclein alpha, suggesting similarities between *E. multilocularis* metacestode proliferation and cancer progression. Several FDA-approved cancer drugs were selected due to their ability to disrupt cell division and DNA replication.

E. multilocularis metacestodes were exposed in vitro to docetaxel, paclitaxel, navelbine, SAHA (vorinostat) and doxorubicin and then implanted into *Meriones unguiculatus* (gerbils). The efficacy of drugs is usually determined by weighing the parasite tissue taken from *E. multilocularis* infected rats, mice or gerbils. The precise determination of the parasite weight is very difficult due to the danger of cyst rupture, followed by a release of vesicle fluid, as well as connective hosts tissue encapsulating the parasite tissue (Gorgas et al. 2017; Hemphill et al. 2014), thus these weight measurements may be not exact. Non-invasive imaging techniques like magnet resonance tomography (MRI) and ultrasound allow the assessment of parasite growth

and tissue volumes (Hubner et al. 2010; Gorgas et al. 2017). PET tracers, notably [^{18}F]FDG PET, can be used to monitor the metabolic activity of the parasite and may help to differentiate between active and inactive lesions in AE patients (Caoduro et al. 2013; Rolle et al. 2015; Yibulayin et al. 2018).

The taxanes docetaxel and paclitaxel are important drugs for the treatment of various cancers and approved by the FDA (Crown and O'Leary 2000). Both drugs stabilize the polymerization of microtubules and interfere with microtubule disassembly during mitosis. Cell cycle arrests in G_2/M phases and induces apoptosis (Montero et al. 2005; Rao et al. 1994; Zhang et al. 2014). The cytotoxicity of docetaxel is much higher than the one of paclitaxel against several tumor cell lines (Riccardi et al. 1995; Crown 2001). There are only few studies using taxanes for echinococcosis treatment. Hübner et al. demonstrated the in vitro inhibition of survival of larval cells, protoscoleces and metacestodes of *E. granulosus* when treated with paclitaxel (Pensel et al. 2014). Many problems still exist with taxanes including severe adverse events and development of resistance (Goldstein 1996; Sibaud et al. 2016; Bumbaca and Li 2018; Breen et al. 2008). Navelbine (vinorelbine) is a chemotherapy drug especially for treatment of breast cancer and non-small cell lung cancer (Degardin et al. 1994; Bertsch and Donaldson 1995; Gralla et al. 1999). As a derivate of vinca alkaloid, it inhibits microtubule polymerization (Jordan and Wilson 2004). In previous studies, navelbine showed no parasitocidal but parasitostatic effects against *E. multilocularis* (Hubner et al. 2010; Stadelmann et al. 2014). Vorinostat (SAHA) is an FDA-approved drug for the treatment of advanced cutaneous T cell lymphoma. In clinical trials SAHA was effective against hematologic malignancies like leukemia and lymphomas (Bubna 2015; Crump et al. 2008; Garcia-Manero et al. 2008; Watanabe et al. 2010). As a histone deacetylase inhibitor, SAHA has a broad spectrum of epigenetic activities; it induces cell cycle arrest by suppressing the expression and function of cell cycle-associated proteins and inhibits angionesis (Sato 2012). Until now, SAHA was not yet tested on *E. multilocularis* metacestodes. Doxorubicin is used as treatment for a wide variety of malignomes, including both, hematological and solid tumors like lymphoma, leukemia, breast cancer and bladder cancer (Tacar, Sriamornsak, and Dass 2013; Friesen et al. 2013). In cancer cells, it interacts with the DNA by intercalation and disrupts the topoisomerase-II-mediated DNA repair, resulting in apoptosis of cancer cells and inhibits DNA synthesis (Tacar, Sriamornsak, and Dass 2013). Doxorubicin also increases the production of quinone type free radicals, which damage cellular

membranes, DNA and proteins (Thorn et al. 2011). Multidirectional cytotoxic side effects like cardiotoxicity, the depression of the immune system, drug resistance and low bio-availability are big disadvantages of Doxorubicin (Tacar, Sriamornsak, and Dass 2013; Cappetta et al. 2018). In murines, infected with *E. multilocularis*, nanoparticle-bound doxorubicin inhibit hepatic larval growth and reduce viability of the parasite (Liance et al. 1993).

In our study, the in vivo growth and proliferation of *E. multilocularis* was inhibited by docetaxel. In vivo volumetric MRI measurements showed low or no parasite growth. Also no uptake of [^{18}F]FDG could be measured in Meriones, infected with docetaxel-exposed metacestodes of *E. multilocularis*. Paclitaxel and navelbine suppressed the in vivo growth only until 3 month post infection and similar to previous findings, doxorubicin and SAHA had no effect on *E. multilocularis* metacestode growth and proliferation (Liance et al. 1993; Stadelmann et al. 2014). The successful inhibition and suppression of *E. multilocularis* metacestodes growth and proliferation by docetaxel, paclitaxel and navelbine nominate them for further pre-clinical studies on *E. multilocularis* focusing on the drug metabolics and may result in new treatments of alveolar echinococcosis.

References

Aalberse, R. C., S. O. Stapel, J. Schuurman, and T. Rispens. 2009. 'Immunoglobulin G4: an odd antibody', *Clin Exp Allergy*, 39: 469-77.

Alcaro, M. C., E. Peroni, P. Rovero, and A. M. Papini. 2003. 'Synthetic peptides in the diagnosis of HIV infection', *Curr Protein Pept Sci*, 4: 285-90.

Alvarez Errico, D., A. Medeiros, M. Miguez, C. Casaravilla, R. Malgor, C. Carmona, A. Nieto, and E. Osinaga. 2001. 'O-glycosylation in Echinococcus granulosus: identification and characterization of the carcinoma-associated Tn antigen', *Exp Parasitol*, 98: 100-9.

Ammann, R. W., and J. Eckert. 1996. 'Cestodes. Echinococcus', *Gastroenterol Clin North Am*, 25: 655-89.

Anuradha, R., P. J. George, L. E. Hanna, V. Chandrasekaran, P. Kumaran, T. B. Nutman, and S. Babu. 2013. 'IL-4-, TGF-beta-, and IL-1-dependent expansion of parasite antigen-specific Th9 cells is associated with clinical pathology in human lymphatic filariasis', *J Immunol*, 191: 2466-73.

Ardelli, B. F., S. B. Guerriero, and R. K. Prichard. 2005. 'Genomic organization and effects of ivermectin selection on Onchocerca volvulus P-glycoprotein', *Molecular and Biochemical Parasitology*, 143: 58-66.

Arndts, K., U. Klarmann-Schulz, L. Batsa, A. Y. Debrah, C. Epp, R. Fimmers, S. Specht, L. E. Layland, and A. Hoerauf. 2015. 'Reductions in microfilaridermia by repeated ivermectin treatment are associated with lower Plasmodium-specific Th17 immune responses in Onchocerca volvulus-infected individuals', *Parasit Vectors*, 8: 184.

Arndts, K., S. Specht, A. Y. Debrah, F. Tamarozzi, U. Klarmann Schulz, S. Mand, L. Batsa, A. Kwarteng, M. Taylor, O. Adjei, C. Martin, L. E. Layland, and A. Hoerauf. 2014. 'Immunoepidemiological profiling of onchocerciasis patients reveals associations with microfilaria loads and ivermectin intake on both individual and community levels', *PLoS Negl Trop Dis*, 8: e2679.

Babu, S., V. Kumaraswami, and T. B. Nutman. 2009. 'Alternatively activated and immunoregulatory monocytes in human filarial infections', *J Infect Dis*, 199: 1827-37.

Baird, J. K., R. C. Neafie, L. Lanoie, and D. H. Connor. 1987. 'Adult Mansonella perstans in the abdominal cavity in nine Africans', *Am J Trop Med Hyg*, 37: 578-84.

Baker, R. H., P. Guillet, A. Seketeli, P. Poudiougo, D. Boakye, M. D. Wilson, and Y. Bissan. 1990. 'Progress in controlling the reinvasion of windborne vectors into the western area of the Onchocerciasis Control Programme in West Africa', *Philos Trans R Soc Lond B Biol Sci*, 328: 731-47, discussion 47-50.

Belardelli, F. 1995. 'Role of interferons and other cytokines in the regulation of the immune response', *APMIS*, 103: 161-79.

Bertsch, L. A., and G. Donaldson. 1995. 'Quality of life analyses from vinorelbine (Navelbine) clinical trials of women with metastatic breast cancer', *Semin Oncol*, 22: 45-53; discussion 53-4.

Blackwell, J. M., S. E. Jamieson, and D. Burgner. 2009. 'HLA and infectious diseases', *Clin Microbiol Rev*, 22: 370-85, Table of Contents.

Boatin, B. 2008. 'The Onchocerciasis Control Programme in West Africa (OCP)', *Ann Trop Med Parasitol*, 102 Suppl 1: 13-7.

Bogdan, C., M. Rollinghoff, and A. Diefenbach. 2000. 'Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity', *Curr Opin Immunol*, 12: 64-76.

Bouchery, T., E. Lefoulon, G. Karadjian, A. Nieguitsila, and C. Martin. 2013. 'The symbiotic role of Wolbachia in Onchocercidae and its impact on filariasis', *Clinical Microbiology and Infection*, 19: 131-40.

Brattig, N. W., D. W. Buttner, and A. Hoerauf. 2001. 'Neutrophil accumulation around Onchocerca worms and chemotaxis of neutrophils are dependent on Wolbachia endobacteria', *Microbes Infect*, 3: 439-46.

Breen, L., L. Murphy, J. Keenan, and M. Clynes. 2008. 'Development of taxane resistance in a panel of human lung cancer cell lines', *Toxicol In Vitro*, 22: 1234-41.

Bregani, E. R., A. Rovellini, N. Mbaidoum, and M. G. Magnini. 2006. 'Comparison of different anthelminthic drug regimens against Mansonella perstans filariasis', *Trans R Soc Trop Med Hyg*, 100: 458-63.

Bregani, E. R., F. Tantardini, and A. Rovellini. 2007. '[Mansonella perstans filariasis]', *Parassitologia*, 49: 23-6.

Brocker, C., D. Thompson, A. Matsumoto, D. W. Nebert, and V. Vasiliou. 2010. 'Evolutionary divergence and functions of the human interleukin (IL) gene family', *Hum Genomics*, 5: 30-55.

Bruhns, P., B. Iannascoli, P. England, D. A. Mancardi, N. Fernandez, S. Jorieux, and M. Daeron. 2009. 'Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses', *Blood*, 113: 3716-25.

Brunetti, E., P. Kern, D. A. Vuitton, and Who-Iwge Writing Panel for the. 2010. 'Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans', *Acta Trop*, 114: 1-16.

Bubna, A. K. 2015. 'Vorinostat-An Overview', *Indian J Dermatol*, 60: 419.

Budke, C. M., A. Casulli, P. Kern, and D. A. Vuitton. 2017. 'Cystic and alveolar echinococcosis: Successes and continuing challenges', *PLoS Negl Trop Dis*, 11: e0005477.

Bumbaca, B., and W. Li. 2018. 'Taxane resistance in castration-resistant prostate cancer: mechanisms and therapeutic strategies', *Acta Pharm Sin B*, 8: 518-29.

Caoduro, C., C. Porot, D. A. Vuitton, S. Bresson-Hadni, F. Grenouillet, C. Richou, H. Boulahdour, and O. Blagosklonov. 2013. 'The role of delayed 18F-FDG PET imaging in the follow-up of patients with alveolar echinococcosis', *J Nucl Med*, 54: 358-63.

Cappetta, D., F. Rossi, E. Piegari, F. Quaini, L. Berrino, K. Urbanek, and A. De Angelis. 2018. 'Doxorubicin targets multiple players: A new view of an old problem', *Pharmacol Res*, 127: 4-14.

Cavacini, L. A., D. Kuhrt, M. Duval, K. Mayer, and M. R. Posner. 2003. 'Binding and neutralization activity of human IgG1 and IgG3 from serum of HIV-infected individuals', *AIDS Res Hum Retroviruses*, 19: 785-92.

Charo, I. F., and R. M. Ransohoff. 2006. 'The many roles of chemokines and chemokine receptors in inflammation', *N Engl J Med*, 354: 610-21.

Cho-Ngwa, F., J. Liu, and S. Lustigman. 2010. 'The Onchocerca volvulus Cysteine Proteinase Inhibitor, Ov-CPI-2, Is a Target of Protective Antibody Response That Increases with Age', *Plos Neglected Tropical Diseases*, 4.

Choo, S. Y. 2007. 'The HLA system: genetics, immunology, clinical testing, and clinical implications', *Yonsei Med J*, 48: 11-23.

Chu, W. M. 2013. 'Tumor necrosis factor', *Cancer Lett*, 328: 222-5.

Coffeng, L. E., W. A. Stolk, H. G. Zoure, J. L. Veerman, K. B. Agblewonu, M. E. Murdoch, M. Noma, G. Fobi, J. H. Richardus, D. A. Bundy, D. Habbema, S. J. de Vlas, and U. V. Amazigo. 2013. 'African Programme For Onchocerciasis

Control 1995-2015: model-estimated health impact and cost', *PLoS Negl Trop Dis*, 7: e2032.

Collins, A. M., and K. J. Jackson. 2013. 'A Temporal Model of Human IgE and IgG Antibody Function', *Front Immunol*, 4: 235.

Convit, J., H. Schuler, R. Borges, V. Olivero, A. Dominguez-Vazquez, H. Frontado, and M. E. Grillet. 2013. 'Interruption of Onchocerca volvulus transmission in Northern Venezuela', *Parasit Vectors*, 6: 289.

Corthay, A. 2009. 'How do regulatory T cells work?', *Scand J Immunol*, 70: 326-36.

Coulibaly, Y. I., B. Dembele, A. A. Diallo, E. M. Lipner, S. S. Doumbia, S. Y. Coulibaly, S. Konate, D. A. Diallo, D. Yalcouye, J. Kubofcik, O. K. Doumbo, A. K. Traore, A. D. Keita, M. P. Fay, S. F. Traore, T. B. Nutman, and A. D. Klion. 2009. 'A randomized trial of doxycycline for Mansonella perstans infection', *N Engl J Med*, 361: 1448-58.

Crown, J. 2001. 'Docetaxel: overview of an active drug for breast cancer', *Oncologist*, 6 Suppl 3: 1-4.

Crown, J., and M. O'Leary. 2000. 'The taxanes: an update', *Lancet*, 355: 1176-8.

Crump, M., B. Coiffier, E. D. Jacobsen, L. Sun, J. L. Ricker, H. Xie, S. R. Frankel, S. S. Randolph, and B. D. Cheson. 2008. 'Phase II trial of oral vorinostat (suberoylanilide hydroxamic acid) in relapsed diffuse large-B-cell lymphoma', *Ann Oncol*, 19: 964-9.

D'Acquisto, F., and T. Crompton. 2011. 'CD3+CD4-CD8- (double negative) T cells: saviours or villains of the immune response?', *Biochem Pharmacol*, 82: 333-40.

DALYs, G. B. D., and Hale Collaborators. 2018. 'Global, regional, and national disability-adjusted life-years (DALYs) for 359 diseases and injuries and healthy life expectancy (HALE) for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017', *Lancet*, 392: 1859-922.

Davies, A. M., T. Rispens, P. Ooijevaar-de Heer, H. J. Gould, R. Jefferis, R. C. Aalberse, and B. J. Sutton. 2014. 'Structural determinants of unique properties of human IgG4-Fc', *J Mol Biol*, 426: 630-44.

Degardin, M., J. Bonneterre, B. Hecquet, J. M. Pion, A. Adenis, D. Horner, and A. Demaille. 1994. 'Vinorelbine (navelbine) as a salvage treatment for advanced breast cancer', *Ann Oncol*, 5: 423-6.

Diawara, L., M. O. Traore, A. Badji, Y. Bissan, K. Doumbia, S. F. Goita, L. Konate, K. Mounkoro, M. D. Sarr, A. F. Seck, L. Toe, S. Touree, and J. H. Remme. 2009. 'Feasibility of onchocerciasis elimination with ivermectin treatment in endemic foci in Africa: first evidence from studies in Mali and Senegal', *PLoS Negl Trop Dis*, 3: e497.

Disease, G. B. D., Incidence Injury, and Collaborators Prevalence. 2018. 'Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017', *Lancet*, 392: 1789-858.

Dolo, H., Y. I. Coulibaly, B. Dembele, S. Konate, S. Y. Coulibaly, S. S. Doumbia, A. A. Diallo, L. Soumaoro, M. E. Coulibaly, S. A. Diakite, A. Guindo, M. P. Fay, S. Metenou, T. B. Nutman, and A. D. Klion. 2012. 'Filariasis attenuates anemia and proinflammatory responses associated with clinical malaria: a matched prospective study in children and young adults', *PLoS Negl Trop Dis*, 6: e1890.

Eckert, J., and P. Deplazes. 2004. 'Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern', *Clin Microbiol Rev*, 17: 107-35.

Evans, D. S., T. R. Unnasch, and F. O. Richards. 2015. 'Onchocerciasis and lymphatic filariasis elimination in Africa: it's about time', *Lancet*, 385: 2151-2.

Fendt, J., D. M. Hamm, M. Banla, H. Schulz-Key, H. Wolf, G. Helling-Giese, C. Heuschkel, and P. T. Soboslay. 2005. 'Chemokines in onchocerciasis patients after a single dose of ivermectin', *Clin Exp Immunol*, 142: 318-26.

Friesen, C., M. Roscher, I. Hormann, I. Fichtner, A. Alt, R. A. Hilger, K. M. Debatin, and E. Miltner. 2013. 'Cell death sensitization of leukemia cells by opioid receptor activation', *Oncotarget*, 4: 677-90.

Garcia-Manero, G., H. Yang, C. Bueso-Ramos, A. Ferrajoli, J. Cortes, W. G. Wierda, S. Faderl, C. Koller, G. Morris, G. Rosner, A. Loboda, V. R. Fantin, S. S. Randolph, J. S. Hardwick, J. F. Reilly, C. Chen, J. L. Ricker, J. P. Secrist, V. M. Richon, S. R. Frankel, and H. M. Kantarjian. 2008. 'Phase 1 study of the histone deacetylase inhibitor vorinostat (suberoylanilide hydroxamic acid [SAHA]) in patients with advanced leukemias and myelodysplastic syndromes', *Blood*, 111: 1060-6.

Garms, R., Cheke, R. A., Vajime, C., Sowah, S.A. 1982. 'The occurrence and movements of different members of the Simulium damnosum complex in Togo and Benin', *Zeit Ange Zool*, 69: 219-36.

Garred, P., T. E. Michaelsen, and A. Aase. 1989. 'The IgG subclass pattern of complement activation depends on epitope density and antibody and complement concentration', *Scand J Immunol*, 30: 379-82.

Gauna, A., S. Losada, M. Lorenzo, H. Bermudez, M. Toledo, H. Perez, E. Chacon, and O. Noya. 2015. 'Synthetic peptides for the immunodiagnosis of hepatitis A virus infection', *J Immunol Methods*, 427: 1-5.

Goeury, T., L. E. Creary, L. Brunet, M. Galan, M. Pasquier, B. Kervaire, A. Langaney, J. M. Tiercy, M. A. Fernandez-Vina, J. M. Nunes, and A. Sanchez-Mazas. 2018. 'Deciphering the fine nucleotide diversity of full HLA class I and class II genes in a well-documented population from sub-Saharan Africa', *HLA*, 91: 36-51.

Golden, A., D. Faulx, M. Kalnoky, E. Stevens, L. Yokobe, R. Peck, P. Karabou, M. Banla, R. Rao, K. Adade, R. G. Gantin, K. Komlan, P. T. Soboslay, T. de Los Santos, and G. J. Domingo. 2016. 'Analysis of age-dependent trends in Ov16 IgG4 seroprevalence to onchocerciasis', *Parasit Vectors*, 9: 338.

Goldstein, L. J. 1996. 'MDR1 gene expression in solid tumours', *Eur J Cancer*, 32A: 1039-50.

Gomara, M. J., and I. Haro. 2007. 'Synthetic peptides for the immunodiagnosis of human diseases', *Curr Med Chem*, 14: 531-46.

Gorgas, D., N. Marreros, R. Rufener, A. Hemphill, and B. Lundstrom-Stadelmann. 2017. 'To see or not to see: non-invasive imaging for improved readout of drug treatment trials in the murine model of secondary alveolar echinococcosis', *Parasitology*, 144: 937-44.

Gotsch, F., R. Romero, L. Friel, J. P. Kusanovic, J. Espinoza, O. Erez, N. G. Than, P. Mittal, S. Edwin, B. H. Yoon, C. J. Kim, S. Mazaki-Tovi, T. Chaiworapongsa, and S. S. Hassan. 2007. 'CXCL10/IP-10: a missing link between inflammation and anti-angiogenesis in preeclampsia?', *J Matern Fetal Neonatal Med*, 20: 777-92.

Gralla, R., P. Harper, S. Johnson, and F. M. Delgado. 1999. 'Vinorelbine (Navelbine) in the treatment of non-small-cell lung cancer: studies with single-agent therapy and in combination with cisplatin', *Ann Oncol*, 10 Suppl 5: S41-5.

Greene, B. M., H. R. Taylor, and M. Aikawa. 1981. 'Cellular killing of microfilariae of Onchocerca volvulus: eosinophil and neutrophil-mediated immune serum-dependent destruction', *J Immunol*, 127: 1611-8.

Harris, N., and W. C. Gause. 2011. 'To B or not to B: B cells and the Th2-type immune response to helminths', *Trends Immunol*, 32: 80-8.

Hegewald, J., R. G. Gantin, C. J. Lechner, X. Huang, A. Agosssou, Y. F. Agbeko, P. T. Soboslay, and C. Kohler. 2015. 'Cellular cytokine and chemokine responses to parasite antigens and fungus and mite allergens in children co-infected with helminthes and protozoa parasites', *J Inflamm (Lond)*, 12: 5.

Hemphill, A., B. Stadelmann, R. Rufener, M. Spiliotis, G. Boubaker, J. Muller, N. Muller, D. Gorgas, and B. Gottstein. 2014. 'Treatment of echinococcosis: albendazole and mebendazole--what else?', *Parasite*, 21: 70.

Hotez, P. J., A. Fenwick, L. Savioli, and D. H. Molyneux. 2009. 'Rescuing the bottom billion through control of neglected tropical diseases', *Lancet*, 373: 1570-5.

Hotez, P. J., D. H. Molyneux, A. Fenwick, J. Kumaresan, S. E. Sachs, J. D. Sachs, and L. Savioli. 2007. 'Control of neglected tropical diseases', *N Engl J Med*, 357: 1018-27.

Huber, S., R. Hoffmann, F. Muskens, and D. Voehringer. 2010. 'Alternatively activated macrophages inhibit T-cell proliferation by Stat6-dependent expression of PD-L2', *Blood*, 116: 3311-20.

Hubner, C., S. Wiehr, L. Kocherscheidt, H. Wehrl, B. J. Pichler, A. Schmid, P. Kern, and P. T. Soboslay. 2010. 'Effects of in vitro exposure of Echinococcus multilocularis metacestodes to cytostatic drugs on in vivo growth and proliferation of the parasite', *Parasitol Res*, 107: 459-63.

Hunter, P. 2014. 'Tropical diseases and the poor: Neglected tropical diseases are a public health problem for developing and developed countries alike', *EMBO Rep*, 15: 347-50.

James, S. L., and J. Glaven. 1989. 'Macrophage cytotoxicity against schistosomula of Schistosoma mansoni involves arginine-dependent production of reactive nitrogen intermediates', *J Immunol*, 143: 4208-12.

Jia, L., and C. Wu. 2014. 'Differentiation, regulation and function of Th9 cells', *Adv Exp Med Biol*, 841: 181-207.

Jordan, M. A., and L. Wilson. 2004. 'Microtubules as a target for anticancer drugs', *Nat Rev Cancer*, 4: 253-65.

Jorgensen, P., M. an der Heiden, P. Kern, I. Schoneberg, G. Krause, and K. Alpers. 2008. 'Underreporting of human alveolar echinococcosis, Germany', *Emerg Infect Dis*, 14: 935-7.

Katabarwa, M. N., T. Lakwo, P. Habomugisha, T. R. Unnasch, R. Garms, L. Hudson-Davis, E. Byamukama, A. Khainza, J. Ngorok, E. Tukahebwa, and F. O. Richards. 2018. 'After 70 years of fighting an age-old scourge, onchocerciasis in Uganda, the end is in sight', *Int Health*, 10: i79-i88.

Kazura, J. W. 2015. 'More Progress in Eliminating Transmission of Onchocerca volvulus and Wuchereria bancrofti in the Americas: A Portent of Global Eradication', *Am J Trop Med Hyg*, 93: 1128-9.

Kern, P., A. Ammon, M. Kron, G. Sinn, S. Sander, L. R. Petersen, W. Gaus, and P. Kern. 2004. 'Risk factors for alveolar echinococcosis in humans', *Emerg Infect Dis*, 10: 2088-93.

Kern, P., K. Bardonnet, E. Renner, H. Auer, Z. Pawlowski, R. W. Ammann, D. A. Vuitton, P. Kern, and Registry European Echinococcosis. 2003. 'European echinococcosis registry: human alveolar echinococcosis, Europe, 1982-2000', *Emerg Infect Dis*, 9: 343-9.

Kern, P., A. Menezes da Silva, O. Akhan, B. Mullhaupt, K. A. Vizcaychipi, C. Budke, and D. A. Vuitton. 2017. 'The Echinococcoses: Diagnosis, Clinical Management and Burden of Disease', *Adv Parasitol*, 96: 259-369.

Korbmacher, F., K. Komlan, R. G. Gantin, W. P. Poutouli, K. Padjoudoum, P. Karabou, P. T. Soboslay, and C. Kohler. 2018. 'Mansonella perstans, Onchocerca volvulus and Strongyloides stercoralis infections in rural populations in central and southern Togo', *Parasite Epidemiol Control*, 3: 77-87.

Kozek, W. J., and H. F. Marroquin. 1977. 'Intracytoplasmic bacteria in Onchocerca volvulus', *Am J Trop Med Hyg*, 26: 663-78.

Kreidl, P., F. Allerberger, G. Judmaier, H. Auer, H. Aspock, and A. J. Hall. 1998. 'Domestic pets as risk factors for alveolar hydatid disease in Austria', *Am J Epidemiol*, 147: 978-81.

Kunkel, E. J., and E. C. Butcher. 2002. 'Chemokines and the tissue-specific migration of lymphocytes', *Immunity*, 16: 1-4.

Kurniawan, A., M. Yazdanbakhsh, R. van Ree, R. Aalberse, M. E. Selkirk, F. Partono, and R. M. Maizels. 1993. 'Differential expression of IgE and IgG4 specific antibody responses in asymptomatic and chronic human filariasis', *J Immunol*, 150: 3941-50.

Kyelem, D., J. Medlock, S. Sanou, M. Bonkoungou, B. Boatin, and D. H. Molyneux. 2005. 'Short communication: impact of long-term (14 years) bi-annual ivermectin treatment on Wuchereria bancrofti microfilaraemia', *Trop Med Int Health*, 10: 1002-4.

Lagatie, O., B. Van Dorst, and L. J. Stuyver. 2017. 'Identification of three immunodominant motifs with atypical isotype profile scattered over the Onchocerca volvulus proteome', *PLoS Negl Trop Dis*, 11: e0005330.

Lagatie, O., A. Verheyen, E. Nijs, B. Van Dorst, L. Batsa Debrah, A. Debrah, T. Supali, E. Sartono, and L. J. Stuyver. 2018. 'Evaluation of the Diagnostic Performance of Onchocerca volvulus Linear Epitopes in a Peptide Enzyme-Linked Immunosorbent Assay', *Am J Trop Med Hyg*.

Lechner, C. J., R. G. Gantin, T. Seeger, A. Sarnecka, J. Portillo, H. Schulz-Key, P. K. Karabou, G. Helling-Giese, C. Heuschkel, M. Banla, and P. T. Soboslay. 2012. 'Chemokines and cytokines in patients with an occult Onchocerca volvulus infection', *Microbes Infect*, 14: 438-46.

Legler, D. F., and M. Thelen. 2016. 'Chemokines: Chemistry, Biochemistry and Biological Function', *Chimia (Aarau)*, 70: 856-59.

Liance, M., F. Nemati, C. Bories, and P. Couvreur. 1993. 'Experience with doxorubicin-bound polyisohexylcyanoacrylate nanoparticles on murine alveolar echinococcosis of the liver', *Int J Parasitol*, 23: 427-9.

Lucius, R., A. Kern, F. Seeber, T. Pogonka, J. Willenbucher, H. R. Taylor, M. Pinder, H. W. Ghalib, H. Schulzkey, and P. Soboslay. 1992. 'Specific and Sensitive Igg4 Immunodiagnosis of Onchocerciasis with a Recombinant 33kd Onchocerca-Volvulus Protein (Ov33)', *Tropical Medicine and Parasitology*, 43: 139-45.

Lustigman, S., B. L. Makepeace, T. R. Klei, S. A. Babayan, P. Hotez, D. Abraham, and M. E. Bottazzi. 2018. 'Onchocerca volvulus: The Road from Basic Biology to a Vaccine', *Trends in Parasitology*, 34: 64-79.

Mai, C. S., D. M. Hamm, M. Banla, A. Agossou, H. Schulz-Key, C. Heuschkel, and P. T. Soboslay. 2007. 'Onchocerca volvulus-specific antibody and cytokine responses in onchocerciasis patients after 16 years of repeated ivermectin therapy', *Clin Exp Immunol*, 147: 504-12.

Maizels, R. M., and M. Yazdanbakhsh. 2003. 'Immune regulation by helminth parasites: cellular and molecular mechanisms', *Nat Rev Immunol*, 3: 733-44.

Malkin, R., E. Flescher, J. Lengy, and Y. Keisari. 1987. 'On the interactions between macrophages and developmental stages of Schistosoma mansoni: the cytotoxic

mechanisms involved in macrophage-mediated killing of schistosomula in vitro', *Immunobiology*, 176: 63-72.

McSorley, H. J., and R. M. Maizels. 2012. 'Helminth infections and host immune regulation', *Clin Microbiol Rev*, 25: 585-608.

Mendez-Enriquez, E., and E. A. Garcia-Zepeda. 2013. 'The multiple faces of CCL13 in immunity and inflammation', *Inflammopharmacology*, 21: 397-406.

Meri, T., T. S. Jokiranta, J. Hellwage, A. Bialonski, P. F. Zipfel, and S. Meri. 2002. 'Onchocerca volvulus microfilariae avoid complement attack by direct binding of factor H', *J Infect Dis*, 185: 1786-93.

Metenou, S., B. Dembele, S. Konate, H. Dolo, S. Y. Coulibaly, Y. I. Coulibaly, A. A. Diallo, L. Soumaoro, M. E. Coulibaly, D. Sanogo, S. S. Doumbia, M. Wagner, S. F. Traore, A. Klion, S. Mahanty, and T. B. Nutman. 2009. 'Patent filarial infection modulates malaria-specific type 1 cytokine responses in an IL-10-dependent manner in a filaria/malaria-coinfected population', *J Immunol*, 183: 916-24.

Metenou, S., B. Dembele, S. Konate, H. Dolo, Y. I. Coulibaly, A. A. Diallo, L. Soumaoro, M. E. Coulibaly, S. Y. Coulibaly, D. Sanogo, S. S. Doumbia, S. F. Traore, S. Mahanty, A. Klion, and T. B. Nutman. 2011. 'Filarial infection suppresses malaria-specific multifunctional Th1 and Th17 responses in malaria and filarial coinfections', *J Immunol*, 186: 4725-33.

Meyer, C. G., M. Gallin, K. D. Erttmann, N. Brattig, L. Schnittger, A. Gelhaus, E. Tannich, A. B. Begovich, H. A. Erlich, and R. D. Horstmann. 1994. 'HLA-D alleles associated with generalized disease, localized disease, and putative immunity in Onchocerca volvulus infection', *Proc Natl Acad Sci U S A*, 91: 7515-9.

Mitra, A. K., and A. R. Mawson. 2017. 'Neglected Tropical Diseases: Epidemiology and Global Burden', *Trop Med Infect Dis*, 2.

Molyneux, D. H., L. Savioli, and D. Engels. 2017. 'Neglected tropical diseases: progress towards addressing the chronic pandemic', *Lancet*, 389: 312-25.

Moncada, S., R. M. Palmer, and E. A. Higgs. 1991. 'Nitric oxide: physiology, pathophysiology, and pharmacology', *Pharmacol Rev*, 43: 109-42.

Mond, J. J., Q. Vos, A. Lees, and C. M. Snapper. 1995. 'T cell independent antigens', *Curr Opin Immunol*, 7: 349-54.

Montero, A., F. Fossella, G. Hortobagyi, and V. Valero. 2005. 'Docetaxel for treatment of solid tumours: a systematic review of clinical data', *Lancet Oncol*, 6: 229-39.

Morre, S. A., C. Munk, K. Persson, S. Kruger-Kjaer, R. van Dijk, C. J. Meijer, and A. J. van Den Brule. 2002. 'Comparison of three commercially available peptide-based immunoglobulin G (IgG) and IgA assays to microimmunofluorescence assay for detection of Chlamydia trachomatis antibodies', *J Clin Microbiol*, 40: 584-7.

Murdoch, M. E. 2018. 'Onchodermatitis: Where Are We Now?', *Trop Med Infect Dis*, 3.

Nedoszytko, B., M. Sokolowska-Wojdylo, K. Ruckemann-Dziurdzinska, J. Roszkiewicz, and R. J. Nowicki. 2014. 'Chemokines and cytokines network in the pathogenesis of the inflammatory skin diseases: atopic dermatitis, psoriasis and skin mastocytosis', *Postepy Dermatol Alergol*, 31: 84-91.

Noya, O., M. E. Patarroyo, F. Guzman, and B. Alarcon de Noya. 2003. 'Immunodiagnosis of parasitic diseases with synthetic peptides', *Curr Protein Pept Sci*, 4: 299-308.

Onchocerciasis Control Programme in West Africa. 2002. "Joint Program Committee. Country-specific Onchocerciasis Control Issues." In *JPC23/INF/DOC.1*. Ouagadougou.

Ouyang, W., J. K. Kolls, and Y. Zheng. 2008. 'The biological functions of T helper 17 cell effector cytokines in inflammation', *Immunity*, 28: 454-67.

Parker, D. C. 1993. 'T cell-dependent B cell activation', *Annu Rev Immunol*, 11: 331-60.

Pensel, P. E., C. Albani, G. U. Gamboa, J. P. Benoit, and M. C. Elissondo. 2014. 'In vitro effect of 5-fluorouracil and paclitaxel on Echinococcus granulosus larvae and cells', *Acta Trop*, 140: 1-9.

Piarroux, M., R. Piarroux, J. Knapp, K. Bardonnet, J. Dumortier, J. Watelet, A. Gerard, J. Beytout, A. Abergel, S. Bresson-Hadni, J. Gaudart, and Network FrancEchino Surveillance. 2013. 'Populations at risk for alveolar echinococcosis, France', *Emerg Infect Dis*, 19: 721-8.

Punkosdy, G. A., D. G. Addiss, and P. J. Lammie. 2003. 'Characterization of antibody responses to Wolbachia surface protein in humans with lymphatic filariasis', *Infect Immun*, 71: 5104-14.

Rao, S., N. E. Krauss, J. M. Heerding, C. S. Swindell, I. Ringel, G. A. Orr, and S. B. Horwitz. 1994. '3'-(p-azidobenzamido)taxol photolabels the N-terminal 31 amino acids of beta-tubulin', *J Biol Chem*, 269: 3132-4.

Redpath, S. A., N. M. Fonseca, and G. Perona-Wright. 2014. 'Protection and pathology during parasite infection: IL-10 strikes the balance', *Parasite Immunol*, 36: 233-52.

Redpath, S., T. Michaelsen, I. Sandlie, and M. R. Clark. 1998. 'Activation of complement by human IgG1 and human IgG3 antibodies against the human leucocyte antigen CD52', *Immunology*, 93: 595-600.

Riccardi, A., T. Servidei, A. Tornesello, P. Puggioni, S. Mastrangelo, C. Rumi, and R. Riccardi. 1995. 'Cytotoxicity of paclitaxel and docetaxel in human neuroblastoma cell lines', *Eur J Cancer*, 31A: 494-9.

Ritter, M., W. P. C. Ndongmo, A. J. Njouendou, N. N. Nghochuzie, L. C. Nchang, D. B. Tayong, K. Arndts, N. Nausch, M. Jacobsen, S. Wanji, L. E. Layland, and A. Hoerauf. 2018. 'Mansonella perstans microfilaremic individuals are characterized by enhanced type 2 helper T and regulatory T and B cell subsets and dampened systemic innate and adaptive immune responses', *PLoS Negl Trop Dis*, 12: e0006184.

Rodriguez-Perez, M. A., N. A. Fernandez-Santos, M. E. Orozco-Algarra, J. A. Rodriguez-Atanacio, A. Dominguez-Vazquez, K. B. Rodriguez-Morales, O. Real-Najarro, F. G. Prado-Velasco, E. W. Cupp, F. O. Richards, Jr., H. K. Hassan, J. F. Gonzalez-Roldan, P. A. Kuri-Morales, and T. R. Unnasch. 2015. 'Elimination of Onchocerciasis from Mexico', *PLoS Negl Trop Dis*, 9: e0003922.

Rolle, A. M., P. T. Soboslay, G. Reischl, W. H. Hoffmann, B. J. Pichler, and S. Wiehr. 2015. 'Evaluation of the Metabolic Activity of Echinococcus multilocularis in Rodents Using Positron Emission Tomography Tracers', *Mol Imaging Biol*, 17: 512-20.

Romig, T., A. Dinkel, and U. Mackenstedt. 2006. 'The present situation of echinococcosis in Europe', *Parasitol Int*, 55 Suppl: S187-91.

Romig, T., W. Kratzer, P. Kimmig, M. Frosch, W. Gaus, W. A. Flegel, B. Gottstein, R. Lucius, K. Beckh, and P. Kern. 1999. 'An epidemiologic survey of human alveolar echinococcosis in southwestern Germany. Romerstein Study Group', *Am J Trop Med Hyg*, 61: 566-73.

Sato, A. 2012. 'Vorinostat approved in Japan for treatment of cutaneous T-cell lymphomas: status and prospects', *Onco Targets Ther*, 5: 67-76.

Schmidberger, J., W. Kratzer, K. Stark, B. Gruner, and Group Echinococcosis Working. 2018. 'Alveolar echinococcosis in Germany, 1992-2016. An update based on the newly established national AE database', *Infection*, 46: 197-206.

Schroeder, H. W., Jr., and L. Cavacini. 2010. 'Structure and function of immunoglobulins', *J Allergy Clin Immunol*, 125: S41-52.

Schulz-Key, H., W. Albrecht, C. Heuschkel, P. T. Soboslay, M. Banla, and H. Gorgen. 1993. 'Efficacy of ivermectin in the treatment of concomitant Mansonella perstans infections in onchocerciasis patients', *Trans R Soc Trop Med Hyg*, 87: 227-9.

Seketeli, A., G. Adeoye, A. Eyamba, E. Nnoruka, P. Drameh, U. V. Amazigo, M. Noma, F. Agboton, Y. Aholou, O. O. Kale, and K. Y. Dadzie. 2002. 'The achievements and challenges of the African Programme for Onchocerciasis Control (APOC)', *Ann Trop Med Parasitol*, 96 Suppl 1: S15-28.

Shiny, C., N. S. Krushna, B. Archana, B. Farzana, and R. B. Narayanan. 2009. 'Serum antibody responses to Wolbachia surface protein in patients with human lymphatic filariasis', *Microbiol Immunol*, 53: 685-93.

Sibaud, V., N. R. Leboeuf, H. Roche, V. R. Belum, L. Gladieff, M. Deslandres, M. Montastruc, A. Eche, E. Vigarios, F. Dalenc, and M. E. Lacouture. 2016. 'Dermatological adverse events with taxane chemotherapy', *Eur J Dermatol*, 26: 427-43.

Simonsen, P. E., A. W. Onapa, and S. M. Asio. 2011. 'Mansonella perstans filariasis in Africa', *Acta Trop*, 120 Suppl 1: S109-20.

Smith, T. F., R. P. Bain, and G. Schiffman. 1990. 'Relationship between serum IgG2 concentrations and antibody responses to pneumococcal polysaccharides in children with chronic chest symptoms', *Clin Exp Immunol*, 80: 339-43.

Soboslay, P. T., C. M. Dreweck, W. H. Hoffmann, C. G. Luder, C. Heuschkel, H. Gorgen, M. Banla, and H. Schulz-Key. 1992. 'Ivermectin-facilitated immunity in onchocerciasis. Reversal of lymphocytopenia, cellular anergy and deficient cytokine production after single treatment', *Clin Exp Immunol*, 89: 407-13.

Stadelmann, B., D. Aeschbacher, C. Huber, M. Spiliotis, J. Muller, and A. Hemphill. 2014. 'Profound activity of the anti-cancer drug bortezomib against Echinococcus multilocularis metacestodes identifies the proteasome as a novel drug target for cestodes', *PLoS Negl Trop Dis*, 8: e3352.

Ta-Tang, T. H., J. L. Crainey, R. J. Post, S. L. Luz, and J. M. Rubio. 2018. 'Mansonellosis: current perspectives', *Res Rep Trop Med*, 9: 9-24.

Tacar, O., P. Sriamornsak, and C. R. Dass. 2013. 'Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems', *J Pharm Pharmacol*, 65: 157-70.

Taylor, M. J., C. Bandi, and A. Hoerauf. 2005. 'Wolbachia bacterial endosymbionts of filarial nematodes', *Adv Parasitol*, 60: 245-84.

Tesmer, L. A., S. K. Lundy, S. Sarkar, and D. A. Fox. 2008. 'Th17 cells in human disease', *Immunol Rev*, 223: 87-113.

Thompson, R. C. 2017. 'Biology and Systematics of Echinococcus', *Adv Parasitol*, 95: 65-109.

Thorn, C. F., C. Oshiro, S. Marsh, T. Hernandez-Boussard, H. McLeod, T. E. Klein, and R. B. Altman. 2011. 'Doxorubicin pathways: pharmacodynamics and adverse effects', *Pharmacogenet Genomics*, 21: 440-6.

Tiemessen, M. M., A. L. Jagger, H. G. Evans, M. J. van Herwijnen, S. John, and L. S. Taams. 2007. 'CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages', *Proc Natl Acad Sci U S A*, 104: 19446-51.

Torgerson, P. R., K. Keller, M. Magnotta, and N. Ragland. 2010. 'The global burden of alveolar echinococcosis', *PLoS Negl Trop Dis*, 4: e722.

Traore, M. O., M. D. Sarr, A. Badji, Y. Bissan, L. Diawara, K. Doumbia, S. F. Goita, L. Konate, K. Mounkoro, A. F. Seck, L. Toe, S. Toure, and J. H. Remme. 2012. 'Proof-of-principle of onchocerciasis elimination with ivermectin treatment in endemic foci in Africa: final results of a study in Mali and Senegal', *PLoS Negl Trop Dis*, 6: e1825.

Trowsdale, J. 1988. 'Molecular genetics of the MHC', *Immunol Suppl*, 1: 21-3.

van Knapen, F. 1980. 'Echinococcus granulosus infection and malignancy', *Br Med J*, 281: 195-6.

van Riet, E., F. C. Hartgers, and M. Yazdanbakhsh. 2007. 'Chronic helminth infections induce immunomodulation: consequences and mechanisms', *Immunobiology*, 212: 475-90.

Vidarsson, G., G. Dekkers, and T. Rispens. 2014. 'IgG subclasses and allotypes: from structure to effector functions', *Front Immunol*, 5: 520.

Wammes, L. J., F. Hamid, A. E. Wiria, L. May, M. M. Kaisar, M. A. Prasetyani-Gieseler, Y. Djuardi, H. Wibowo, Y. C. Kruize, J. J. Verweij, S. E. de Jong, R. Tsonaka, J. J. Houwing-Duistermaat, E. Sartono, A. J. Luty, T. Supali, and M. Yazdanbakhsh. 2016. 'Community deworming alleviates geohelminth-induced immune hyporesponsiveness', *Proc Natl Acad Sci U S A*, 113: 12526-31.

Wanji, S., D. B. Tayong, L. E. Layland, F. R. Datchoua Poutcheu, W. P. Ndongmo, J. A. Kengne-Ouafo, M. Ritter, N. Amvongo-Adjia, F. F. Fombad, C. N. Njeshi, A. S. Nkwescheu, P. A. Enyong, and A. Hoerauf. 2016. 'Update on the distribution of Mansonella perstans in the southern part of Cameroon: influence of ecological factors and mass drug administration with ivermectin', *Parasit Vectors*, 9: 311.

Watanabe, T., H. Kato, Y. Kobayashi, S. Yamasaki, Y. Morita-Hoshi, H. Yokoyama, Y. Morishima, J. L. Ricker, T. Otsuki, A. Miyagi-Maesima, Y. Matsuno, and K. Tobinai. 2010. 'Potential efficacy of the oral histone deacetylase inhibitor vorinostat in a phase I trial in follicular and mantle cell lymphoma', *Cancer Sci*, 101: 196-200.

World Health Organization. 2010. *Working to overcome the global impact of neglected tropical diseases* (Geneva: World Health Organization).

World Health Organization. 2011. "Regional strategic plan for integrated neglected tropical diseases control in South-East Asia region." In.: Geneva: World Health Organization.

World Health Organization. 2012. "Accelerating Work to Overcome the Global Impact of Neglected Tropical Diseases: A Roadmap for Implementation." In.: Geneva: World Health Organization.

World Health Organization. 2013. "Regional strategic plan for neglected tropical diseases in the African Region 2014–2020." In.: Geneva: World Health Organization.

World Health Organization. 2015. *Investing to overcome the global impact of neglected tropical diseases : Third WHO report on neglected tropical diseases* (World Health Organization: Geneva).

World Health Organization. 2016a. 'Guidelines for Stopping Mass Drug Administration and Verifying Elimination of Human Onchocerciasis: Criteria and Procedures.' in (Geneva).

World Health Organization. 2016b. 'Progress report on the elimination of human onchocerciasis, 2015-2016', *Wkly Epidemiol Rec*, 91: 505-14.

World Health Organization. 2016c. 'Progress towards eliminating onchocerciasis in the WHO Region of the Americas: verification of elimination of transmission in Guatemala', *Wkly Epidemiol Rec*, 91: 501-5.

World Health Organization. 2017. 'Progress towards eliminating onchocerciasis in the WHO Region of the Americas: elimination of transmission in the north-east focus of the Bolivarian Republic of Venezuela', *Wkly Epidemiol Rec*, 92: 617-23.

Wu, C. L., T. S. Leu, T. T. Chang, and A. L. Shiau. 1999. 'Hepatitis C virus core protein fused to hepatitis B virus core antigen for serological diagnosis of both hepatitis C and hepatitis B infections by ELISA', *J Med Virol*, 57: 104-10.

Xu, M., M. Molento, W. Blackhall, P. Ribeiro, R. Beech, and R. Prichard. 1998. 'Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog', *Molecular and Biochemical Parasitology*, 91: 327-35.

Yameogo, L. 2008. 'Special intervention zones', *Ann Trop Med Parasitol*, 102 Suppl 1: 23-4.

Yibulayin, A., X. H. Li, Y. D. Qin, X. Y. Jia, Q. Z. Zhang, and Y. B. Li. 2018. 'Biological characteristics of 18F-FDG PET/CT imaging of cerebral alveolar echinococcosis', *Medicine (Baltimore)*, 97: e11801.

Yoshida, C. F., C. D. Rouzere, R. M. Nogueira, E. Lampe, M. A. Travassos-da-Rosa, B. O. Vanderborght, and H. G. Schatzmayr. 1992. 'Human antibodies to dengue and yellow fever do not react in diagnostic assays for hepatitis C virus', *Braz J Med Biol Res*, 25: 1131-5.

Yoshida, H., and C. A. Hunter. 2015. 'The immunobiology of interleukin-27', *Annu Rev Immunol*, 33: 417-43.

Zhang, D., R. Yang, S. Wang, and Z. Dong. 2014. 'Paclitaxel: new uses for an old drug', *Drug Des Devel Ther*, 8: 279-84.

Zlotnik, A., and O. Yoshie. 2000. 'Chemokines: a new classification system and their role in immunity', *Immunity*, 12: 121-7.

Publications

Onchocerca volvulus infection and serological prevalence, ocular onchocerciasis and parasite transmission in northern and central Togo after decades of *Simulium damnosum* s.l. vector control and mass drug administration of ivermectin

Kossi Komlan[1,a], Patrick S. Vossberg[2,a], Richard G. Gantin[1,2], Tchalim Solim[3], Francois Korbmacher[2], Méba Banla[3], Koffi Padjoudoum[4], Potchoziou Karabou[4], Carsten Köhler[2], Peter T. Soboslay[1,2]

[1]Onchocerciasis Reference Laboratory, National Institute of Hygiene, Sokodé, Togo
[2]Institute for Tropical Medicine, University of Tübingen, University Clinics, Tübingen, Germany
[3]Centre Hospitalier Universitaire Campus, Université de Lomé, Lomé, Togo
[4]National Onchocerciasis Control Program, Kara, Togo
[a]Equal contribution as first author

PLoS Negl Trop Dis. 2018 Mar 1;12(3):e0006312

Abstract

Background

Mass drug administration (MDA) of ivermectin has become the main intervention to control onchocerciasis or “river blindness”. In Togo, after many years of MDA, *Onchocerca volvulus* infection has declined dramatically, and elimination appears achievable, but in certain river basins the current situation remains unknown. We have conducted parasitological, serological, ophthalmological, and entomological assessments in northern and central Togo within the river basins of Ôti, Kéran and Mô.

Methodology/Principal Findings

Examinations were completed in 1,455 participants from 11 onchocerciasis sentinel villages, and *O. volvulus* transmission by *Simulium damnosum* sensu lato (s.l.) was evaluated. In children (aged 1-10 years), the prevalence of microfilariae (Mf) was 2.3% and in adults it ranged from 5.1 to 13.3%. Positive IgG4 responses to *O. volvulus* adult (crude) worm antigen (OvAg) and the recombinant Ov16 antigen were in all-ages 48.7% and 34.4%, and 29.1% and 14.9% in children, respectively. In the river basin villages of Kéran, Mô and Ôti, the IgG4 seroprevalences to OvAg in children were 51.7%, 23.5% and 12.7%, respectively, and to the Ov16 antigen 33.3% (Kéran) and 5.2% (Ôti). Onchocerciasis ocular lesions (punctate keratitis, evolving iridocyclitis and chorioretinitis) were observed in children and young adults. *O. volvulus*-specific DNA (Ov150) was detected by poolscreen in vector samples collected from Tchitchira/Kéran (22.8%), Bouzalo/Mô (11.3%), Baghan/Mô (2.9%) and Pancerys/Ôti (4.9%); prevalences of *O. volvulus* infection in *S. damnosum* s.l. were, respectively, 1%, 0.5%, 0.1% and 0.2%.

Conclusions/Significance:

In the northern and central river basins in Togo, interruption of *O. volvulus* transmission has not yet been attained. Patent *O. volvulus* infections, positive antibody responses, progressive ocular onchocerciasis were diagnosed, and parasite transmission by *S. damnosum* s.l. occurred close to the survey locations. Future interventions may require approaches selectively targeted to non-complying endemic populations, to the seasonality of parasite transmission and national onchocerciasis control programs should harmonize cross-border MDA as a coordinated intervention.

Author Summary

Mass drug administration (MDA) with ivermectin has become the main tool in the efforts to control and eliminate onchocerciasis (“river blindness”). In some areas, and

after many years of MDA, levels of *Onchocerca volvulus* infection (the causative parasite) have declined greatly, and elimination appears achievable. In certain river basins of northern and central Togo, the present epidemiological situation remains unknown. The guidelines of the World Health Organization recommend that before ivermectin MDA can be stopped, interruption of *O. volvulus* transmission must be demonstrated. To this end, parasitological, serological, ophthalmological, and entomological assessments were conducted in the Ôti, Kéran and Mô river basins. *O. volvulus* infections and positive antibody responses were found in children aged ≤10 years and adults. Progressive ocular onchocerciasis was diagnosed, and parasite transmission by *Simulium damnosum* s.l. (the disease vector) occurred close to the survey locations. Thus, *O. volvulus* transmission continues in northern and central Togo, and future interventions may require approaches selectively adapted to seasonal migration of non-complying endemic populations in and out of the river basins, as well as seasonal transmission by the vectors. National control programmes should harmonize cross-border MDA as a coordinated intervention.

Introduction

In large parts of Africa, onchocerciasis has been controlled as a public health problem by the Onchocerciasis Control Programme in West Africa (OCP) and the African Programme for Onchocerciasis Control (APOC) by mass drug administration (MDA) of ivermectin, and this intervention has been applied for more than two decades. In a vast part of the initial control areas of the OCP, *Onchocerca volvulus* infection prevalence and intensity levels have greatly declined [1, 2], and currently, the elimination of onchocerciasis appears achievable in certain endemic regions [3-7]. In Togo, the northern territories had been part of the initial OCP anti-vectorial intervention areas since 1976, whereas the central regions were included into the vector control programme in 1987, and in both areas, blackfly vector control measures were supplemented since 1988 by MDA with ivermectin. When MDA with ivermectin started, this was implemented mainly by mobile teams and the initial coverage was not very satisfactory [2]. During some years of the early 1990's, aerial larvicide application was also suspended in several river basins. Regular epidemiological surveys conducted by the National Onchocerciasis Control Programme (NOCP) have shown that after nearly three decades of MDA in most of the onchocerciasis hyperendemic districts, the *O. volvulus* microfilarial prevalence has diminished below 5% in all age groups and below 1% in children aged less than 10 years, suggesting that considerable progress has

been made towards the elimination of onchocerciasis according to the operational prevalence thresholds proposed in the Conceptual Framework for Elimination of Onchocerciasis by APOC [3, 8]. Parasite transmission has never been interrupted completely in central and northern Togo and Benin; the Ôti, Kéran and Mô river basins were “special intervention zones” (SIZ) where vector control and intensified ivermectin distribution needed to be continued for years after OCP closure in 2002 [9]. The interventions in the post-OCP period included continued aerial larvicide application for five additional years (2003-2007) and biannual ivermectin mass treatment was implemented until the end of 2012 [9, 10]. Despite evidence of approaching elimination in certain regions of Togo, the current situation remains to be assessed by epidemiological and entomological surveys for detection of infection in human and vector population samples according to the recent World Health Organization (WHO) guidelines [11]. The WHO guidelines suggest, firstly, that entomological evaluations by Ov-150 PCR poolscreen be conducted to demonstrate interrupted transmission of *O. volvulus* larvae by female blackfly vectors, and secondly, that serological evaluations by Ov-16 enzyme linked immunosorbent assay (ELISA) be carried out to determine the presence of IgG4 antibodies to the *O. volvulus*-specific Ov-16 antigen in children [11]. The use of skin snip microscopy in parallel with Ov-16 serology is a conditional recommendation, and it may be used in transition during the phase of monitoring and evaluation. The assessment of ocular manifestations in populations where ocular onchocerciasis was present at baseline is considered to be of low priority [11]. In the present work, parasitological, serological, ophthalmological and entomological evaluations were conducted in onchocerciasis sentinel villages in central and northern Togo to assess the current epidemiological situation and to determine whether transmission has been interrupted and ivermectin MDA can be stopped.

Materials and methods

Ethics statement and approval

The protocol of the study was reviewed and approved by the Togolese Bioethics Committee for Research in Health (Comité de Bioéthique pour la Recherche en Santé; CBRS, Document #013/2015/CBRS/3.Septembre 2015), and study authorization and approval were granted by the Ministry of Health of Togo (Authorization Document #338/2015/MSPS/CAB/SG). All specimens (skin snips and blood samples) used in this study were collected from study participants who provided written informed consent.

The aims of the work, risks, procedures of examination and follow up were explained thoroughly to the respective village population, the village authorities and honorable community members, notably the village chief council. Consent from each study participant was documented and confirmed by signature, and consent for study participation by those younger than 18 years of age was given verbally by each participant (with written consent and approval for their participation always being obtained from their parents or accompanying responsible adults/guardians). For correct and complete understanding, explanations were always given in the local language. Before each follow-up survey, approval was obtained from the appropriate regional (Direction Régional de la Santé de la Population) and district-level (Direction Préfectural de la Santé) health authorities.

Regular epidemiological surveys conducted by the OCP and NOCP

Regular epidemiological surveys were conducted in Togo by the OCP and the NOCP, which assessed *O. volvulus* microfilarial prevalence and intensity, as well as treatment coverage and compliance to ivermectin MDA within the programme area. Such surveys were performed since 1976 during the early rainy season, and around 200 participants were recruited and examined in each selected sentinel village. All sentinel villages are located within less than 3 km of distance to rivers with known breeding sites for the blackfly vector *Simulium damnosum* sensu lato (s.l.).

In Togo, vector control and epidemiological surveys started in 1976 within the OCP-Phase-III-Eastern Extension in the northern river basins of Ôti, Koumoungou and Kara. In 1988, control measures and epidemiological surveys began for sentinel villages of the OCP-Southern Extension in the river basins of Mô and Mono, and at the same time, also in southern Togo in the river basins of Amou, Anie and Mono. The total number of sentinel villages in Togo included in the epidemiological surveys was 363, and the endemic populations were repeatedly examined over time. Figure 1 illustrates the temporal trends in microfilarial prevalence from 1976 to 2014.

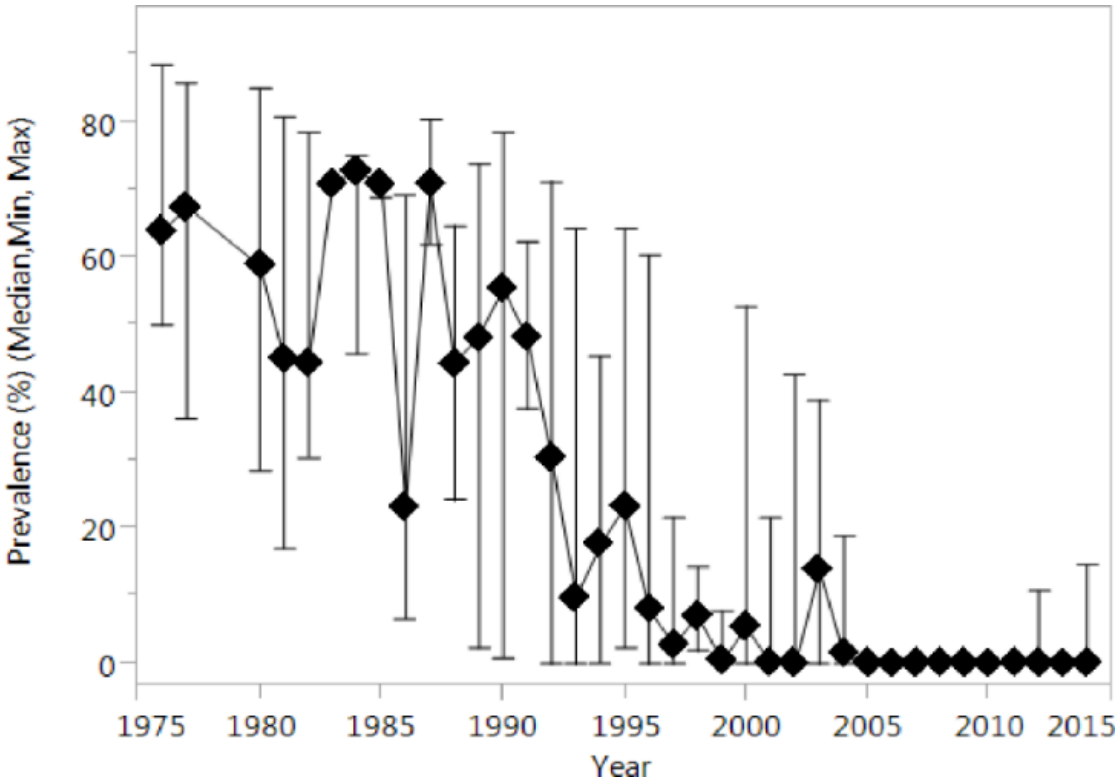


Fig 1. Prevalence of microfilariae (Mf) of *Onchocerca volvulus* in sentinel villages (n=363) of the National Onchocerciasis Control Programme (NOCP) in Togo.

Regular epidemiological surveys (n=957) were conducted by the NOCP over a 37-year period (1976-2014) in onchocerciasis endemic villages, and from each participant (n=193,742) a skin biopsy was taken from the left and right iliac crest and the emerging Mf were counted after snip incubation. The graph shows the microfilarial prevalence (median, minimum, maximum, in %) as detected during the annual surveys. Anti-vectorial interventions were applied since 1976, whereas the central regions were incorporated into the Programme in 1987. Since 1988, vector control measures were supplemented by MDA with ivermectin. Initially MDA was applied mainly by mobile teams; during some years of the early 1990's, aerial larvicide application was suspended in several river basins. In the northern territories (SIZ) vector control and intensified ivermectin distribution was continued after OCP's closure in 2002. Special interventions in the post-OCP period included continued aerial larvicide application for five additional years (2003-2007) and biannual ivermectin MDA until the end of 2012.

Survey sites

For this study, the parasitological, serological and ophthalmological surveys were performed in the central and northern regions of Togo (Régions Savanes and Kara), where the total populations according to the latest census, conducted in 2010, were 776,710 and 721,504 individuals, respectively. In these regions, 11 villages were selected by the NOCP for an annual survey. Figure 2 shows the selected villages and their location in Togo. Three villages are located in the Région Savanes in the Ôti river basin, i.e. Pancérys, Boutchakou and Koukoumbou. Four villages within the Kara

Region are situated along the river Kara, i.e. Goulbi, Tchitchira, Koukoumbou Solla and Kpantiiyagou. Four additional villages from the Région Kara are located in the Mô river basin, i.e. Bawlesi, Mô-Village, Katcha-Konkomba and Saboundi. All sentinel villages are located within less than 1 km of distance to the rivers Ôti, Kara or Mô with known breeding sites for the blackfly vectors.

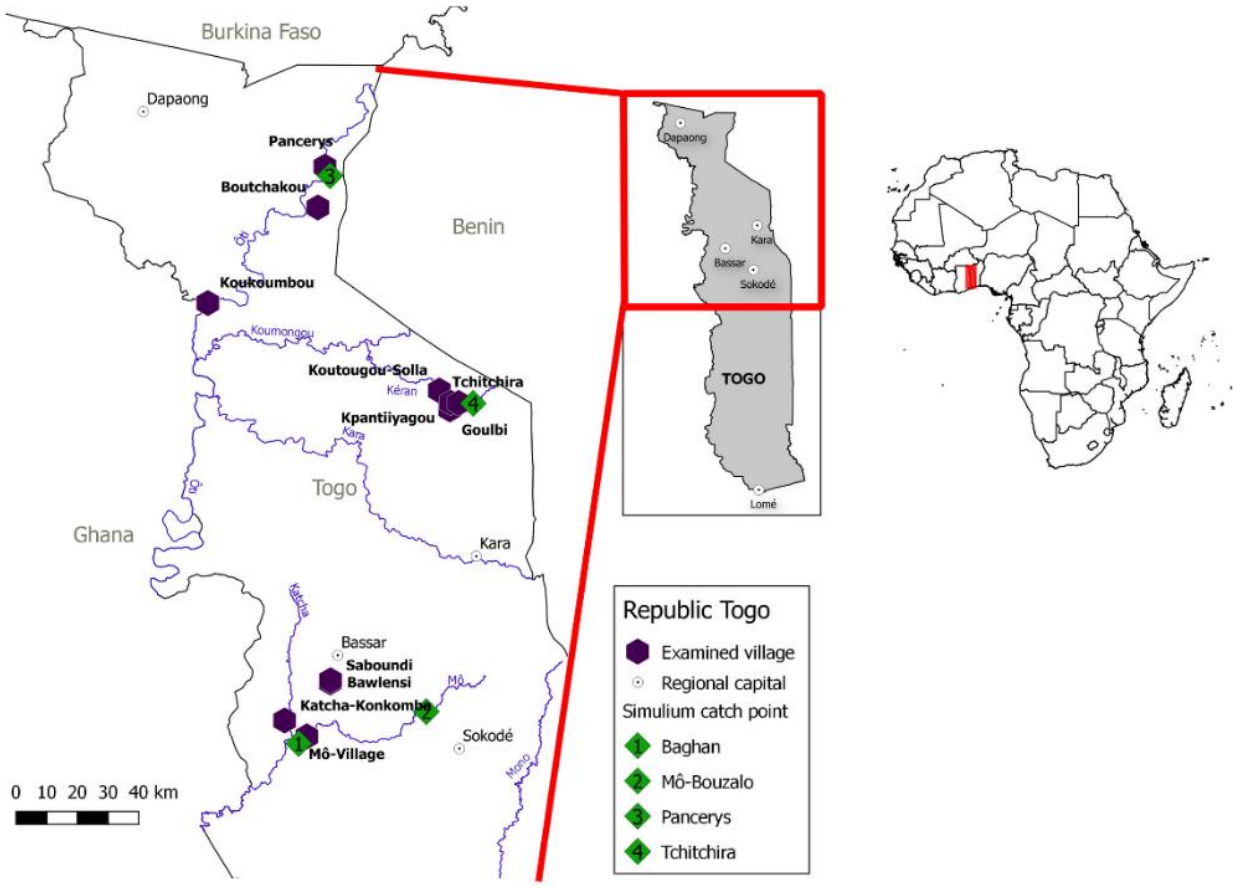


Fig 2. Locations of the villages surveyed and the Simulium collection sites in the three river basins (Ôti, Kéran, Mô) in Savanes, Kara and Central Regions in Togo.

For an explanation of the control measures and their timings in the northern and central areas of Togo, including SIZ, see legend of Fig 1.

Skin biopsy collection

Before ivermectin MDA (delivered by community-directed drug distributors, CDDs), participants gave their informed consent for the collection of skin biopsies to detect *O. volvulus* microfilariae (Mf). Participation and examination were conducted by family and followed the status: heads of family (parents), children, brothers, uncles, aunts, and grandparents. From each participant, a skin biopsy was taken from each the left

and right iliac crest (for a total of two snips) with a sterile 2-mm Holth corneo-scleral punch biopsy tool. Immediately, skin snips were placed on glass slides and incubated with physiological saline solution for 30 minutes. Each biopsy was microscopically examined for emerging *O. volvulus* Mf and their number counted. After this first examination, the two biopsies were transferred separately into a round-bottom well of a 96-well plate containing saline solution, and after a 24-hour incubation, biopsies were re-examined as before. The use of two incubation steps for skin biopsies is the standard procedure applied by the NOCP, and this approach makes it possible to detect *O. volvulus* Mf which may emerge slowly from skin. For each participant, village of residence, family affiliation, age, sex, number of ivermectin treatment rounds received and microfilarial counts in skin biopsies were recorded.

Blood sample collection

Blood drops were collected from fingertips pricked with a sterile lancet on Whatman 903 Protein Saver Cards. The cards were air dried, sealed in plastic bags and stored at 4°C until further use. As per skin biopsies and for each participant, village of residence, family affiliation, age, sex, number of ivermectin treatment rounds received and microfilarial counts in skin biopsies were recorded.

Ophthalmological examinations

All participants gave their informed consent for having an ocular evaluation and were examined by experienced ophthalmologists for ocular onchocerciasis lesions and additionally present ocular pathology. The ophthalmology examinations were performed by MB and TS and ocular pathologies, their grades of evolution and extent were classified as described previously [12]. All participants acknowledged having received ivermectin annually for several years through community directed treatment with ivermectin (CDTI). In the surveyed villages, therapeutic ivermectin coverage of the eligible population had been ≥80% during the past 10 years (Supporting Information S1 Table). Before ophthalmological examination, each participant was asked whether he or she had taken ivermectin annually for several years, and all confirmed having received treatment through CDTI. The anterior eye segment was examined by slit lamp (Haag Streit 900) after participants were asked to sit with their heads bent between their knees for at least two minutes. This position promotes the migration of Mf within the anterior chamber of the eye to be seen and counted. The examination of the posterior segment was done with an ophthalmoscope after pupil dilation with 1% tropicamide and 10% epinephrine hydrochloride. Next to individual

data (age, sex, occupation, village, number of ivermectin treatments), the microfilarial load in the anterior eye segment, punctate and sclerosing keratitis and iridocyclitis were recorded. Onchocerciasis cases with punctate keratitis were grouped according to the presence of dead Mf in the cornea (DMFC) or living Mf in the anterior chamber (MFAC) and further classified as low (presence of 1-10 Mf), moderate (11-20 Mf) or high (>20 Mf). Ocular lesions of the posterior segment were coded as evolving or advanced according to the classification adopted by WHO/OCP [13]. The ocular examinations included the testing of visual acuity eye by eye with an illiterate E chart (SNELEN) placed 6 meters away from the patient's seat, and visual acuity was graded according to WHO/OCP criteria; those with a visual acuity on one or both eyes of <1/20 (3/60 or unable to count fingers at 3 meters) were considered blind; those with impaired vision had a visual acuity on one or both eyes of <3/10 and ≥1/20 (3/60 or unable to count fingers at 3 meters), and those with good vision had a visual acuity equal to or greater than 3/10 (6/18).

Serological tests

For the OvAg-IgG4 ELISA, an adult worm antigen extract from male and female *Onchocerca volvulus* was used [14,15]; for the Ov16-IgG4 ELISA, the recombinant *O. volvulus*-specific antigen Ov16 was applied to measure serological IgG4 responses. The dry blood spot (DBS) samples collected as described above were stored refrigerated at 4°C until use. For the ELISA tests, 6-mm wide circles were punched out of the DBS cards and eluted in 200µl of phosphate buffered saline (PBS) containing 0.05% Tween20 and 5% bovine serum albumin for 2 days at 4°C in deep 96-well polystyrol plates (NUNC 278605). Microtiter plates (Costar 3690, half area) were coated with OvAg (conc. 5µg/ml) or Ov16Ag (conc. 5µg/ml) in PBS pH 7.4 overnight, after which the coating antigen solutions were discarded, and the plates were blocked with PBS-Tween20 containing 5% foetal bovine serum at room temperature for 1.5 hours. Thereafter, plates were washed with PBS-Tween20 (Sigma P3563), eluted blood samples were added without dilution and the plates were incubated at 37°C for 2 hours. After using PBS-Tween20 (Sigma P3563) for washing, an anti-human IgG4 horseradish peroxidase conjugated monoclonal antibody (Thermo Fisher Scientific, no. A10654) was added (dilution 1:500) for 1.5 hours. Plates were washed as above and tetramethylbenzidine (TMB) substrate (Thermo Scientific 34021) was added. Plates were then incubated at room temperature for 15 min and the reaction was stopped with

50 µl of 0.5M sulfuric acid (ROTH, K027.1). Optical densities (ODs) were measured at 450nm with a microplate reader (EL311, BioTex Instruments).

***Simulium damnosum s.l.* collection**

The collection of *S. damnosum* s.l. was conducted at specific catch points at river sites by trained fly catchers in proximity to sentinel villages in the Ôti river basin (village Pancérys/ Savanes Region), Kara (village Tchitchira/Kara Region), Mô (village Baghan/Kara Region and Bouzalo/Central Region) during the rainy season on five consecutive days in late August and beginning of September 2015. Collections took place from 7am to 6pm alternating the fly catchers every two hours. The blackflies caught daily were first frozen, then suspended in 70% alcohol, and 25 individual *S. damnosum* s.l. were pooled into a single tube in ethanol and stored below -20°C until DNA extraction and real-time-PCR (rtPCR). In addition, repeated weekly collections of *S. damnosum* s.l. were continued at the river Mô site, in proximity to the village Bouzalo (Region Centrale) until August 2017. The sampling procedure was the same as above, and this long-term 2015-2017 collection was used to determine the annual biting rate (ABR) for the year 2016, which was calculated by multiplying the average number of blackflies caught daily by the number of days per week in the month to add up to 12 months.

***O. volvulus*-specific Ov150-real-time-PCR**

From each fly catch location, the daily pooled *S. damnosum* s.l. flies were processed using the Qiagen DNA Mini Kit (Qiagen, Hilden, Germany). Whole blackflies were used for rtPCR analyses. The pools were first freeze-thawed three times in liquid nitrogen, then ground with a mini grinder in a 1.5ml micro-centrifuge tube and digested with proteinase K overnight at 56°C. The eluted DNA concentration for each sample was determined by absorbance at 260 nm and DNA was stored at -20 °C before PCR analysis. The DNA concentrations extracted from fly pools ranged from 540 ng/µl to 1200 ng/µl. Real-time PCR primers and probe used were as follows: OvFWD 5'-TGT GGA AAT TCA CCT AAA TAT G-3', OvREV 5'-AAT AAC TGA TGA CCT ATG ACC-3', OvProbe 5'-FAM-TAG GAC CCA ATT CGA ATG TAT GTA CCC-TAM-3' (Eurofins, Genomics). Primers and TaqMan probe sequences were designed to amplify a fragment of *O. volvulus* repeat DNA (Ov-150 bp, GenBank accession number: J04659.1). Taqman Universal PCR Master Mix (Applied Biosystems, P/N 4304437) and nuclease-free water were used with all reactions with the following concentrations and volumes: 2.5 µl of 20 µM OvFWD, 2.5 µl of 20 µM OvREV primer, 1.5 µl of 9.2 µM

OvProbe, 27.5 µl of 2×Master Mix, 50 ng of template DNA from extracted *S. damnosum* s.l. pools, or 1 ng of genomic DNA isolated from adult *O. volvulus*, and nuclease-free water was added up to a final volume of 55 µl. Reactions (2 × 25 µl per well) were run with the following cycling conditions: 50 °C for 2 min, 95 °C for 10 min, (95 °C for 15 s, 49 °C for 30 s, 60 °C for 2 min) × 40 cycles. The Applied Biosystems 7300 Real Time PCR System (96-well format) SDS version 1.4 software was used for *S. damnosum* s.l. pools collection in 2015 and for blackfly pools from 2016 the Corbett Rotor Gene RG-300, version 6, software was applied. Duplicate blackfly pool DNA samples with a cycle threshold (Ct) value of less than 30 were considered to be positive for *O. volvulus* DNA.

Data analysis

Data were entered in Microsoft Excel and analyses were conducted with the statistical software SAS JMP 11.1.1. For Mf prevalence values, the 95% confidence intervals (95% CI, Wilson score interval) were calculated. The sensitivity of the *O. volvulus*-specific IgG4 ELISA was determined with a contingency analysis. For explorative data analyses, the two-sample Wilcoxon test was applied to evaluate differences between groups. The Chi-square test was used to test differences between examined males and females (e.g. participation rates). Fisher's exact test (two-sided) was applied to compare Mf-prevalence and the ELISA IgG4-OvAg and Ov-16 positive responses between the river basins (Kéran, Ôti, Mô), and the number of Ov-150 DNA positive *Simulium damnosum* s.l. pools from Ôti/Pancery, Kéran/Baghan, Kéran/Tchitichira and Mô/Bouzalo. One-sided Fisher's exact test was used to evaluate differences in the prevalence of ocular pathologies in patients from the Ôti, Kéran and Mô river basins. Correlations between ophthalmological variables as well as between these and age were explored with Spearman correlation coefficient. For multiple testing, the application of the Bonferroni Holm adjustment (11 villages, 3 river basins, 7 age groups, microfilarial prevalences, IgG4 responses) resulted in an alpha level of 0.0023. For multiple comparisons, and to avoid type I errors, differences between groups were analyzed by the Tukey-Kramer Test.

Results

Onchocerca volvulus microfilarial prevalence in OCP sentinel villages during the past decades

The onchocerciasis control measures continuously applied from 1976 until 2002 consisted of aerial application of larvicidal compounds into the simuliid vector breeding sites, and from 1990 onwards, annual MDA of ivermectin was introduced, continuing until the present. In 1976, in most locations the prevalence of *O. volvulus* infection exceeded 50%, and 20 years later, Mf positivity in the survey populations decreased to below 20% (median) (Fig. 1). The *O. volvulus* microfilarial prevalence in onchocerciasis sentinel villages located in the major river basins of Ôti, Kéran, Kara, Mô, Koumoungou, Anie and Mono declined markedly (Fig. 1), and until the year 2014, the median prevalence of *O. volvulus* infections dropped below 5%, but in several locations the Mf-positivity exceeded this level in the river basins of Ôti, Kéran and Mô.

Participants' characteristics

In 2015, a total of 1,455 individuals from 11 NOCP sentinel villages gave their informed consent for participation. Of these, the proportions (and numbers) of individuals originating from each river basin were: 22.3% (n=324) in Ôti; 37.0% (n=539) in Kéran, and 40.7% (n=592) in Mô. Table 1 summarizes the numbers examined by age and sex and the proportion of positives for skin Mf. Information on age is missing from 41 of the 1,455 participants, so data in Table 1 are reported for a total 1,414 individuals. Of these, 819 were females and 595 males. The median age in females and males was 30 and 29 years, respectively. Until the age of 15 years, girls and boys were similarly represented in the survey, but participation in examination (and treatment) of men aged 16 to 40 years decreased significantly (Table 1). The Chi-square test was applied to compare differences between female and male survey participation within age groups, indicating a statistically significant difference with greater participation of females (16-20y: $p=0.0007$; age groups 21-25y, 26-30y and 31-35y: for each $p<0.0001$; 36-40y: $p=0.04$). In the age groups above 40 years, differences in participation between the sexes were not significant (Table 1).

Table 1. *Onchocerca volvulus* microfilarial prevalence (% Mf-positive) by age and sex groups in the survey participants from NOCP sentinel villages in central and northern Togo. The number of examined participants, the Mf-positive status and the percentage of Mf-positive individuals are shown. From n=41 study participants the age is missing. The 95% confidence intervals (95% CI, Wilson score interval) of the prevalence values are indicated in square brackets. (* significant differences between female and male survey participation)

Age group	Examined (female/male)	% in population	Mf-positive (female/male)	% Mf-positive [95% CI]
5-10y	172 (76/96)	12.2	4(3/1)	2.3 [0;5.8]
11-15y	212 (106/106)	15.0	4(3/1)	1.9 [0;5.0]
16-20y	95 (64/31)*	6.7	1(1/0)	1.1 [0;5.7]
21-25y	118 (86/32)*	8.3	6(4/2)	5.1 [0.8;9.3]
26-30y	195 (137/58)*	13.8	16(7/9)	8.2 [4.9;11.5]
31-35y	135 (90/45)*	9.5	13(7/6)	9.6 [5.7;13.6]
36-40y	105 (63/42)*	7.4	14(8/6)	13.3 [8.8;17.8]
41-45y	89 (44/45)	6.4	7(3/4)	7.9 [3.0;12.7]
46-50y	90 (53/37)	6.4	6(3/3)	6.7 [1.8;11.5]
51-55y	62 (30/32)	4.4	3(0/2)	3.2 [0;9.0]
56-60y	61 (32/29)	4.3	4(3/1)	6.6 [0.7;12.4]
61-80y	80 (38/42)	5.6	6(4/2)	7.5 [1.6;13.4]
	1,414 (819/595)*	100	83 (46/37)	5.9 [4.6;7.1]

Onchocerca volvulus microfilarial prevalence

A total of 1,455 individuals were examined by skin biopsy for *O. volvulus* Mf with 83 positives; the overall Mf prevalence in the survey participants was 5.7% [4.5;6.9] (Table 2). In the Ôti, Kéran and Mô river basins, the ranges of Mf prevalence were 0.8-5.3%, 7.7-13.6% and 0-8.6%, respectively. The districts in northern and central Togo, where the surveyed 11 villages are located, formed part of the SIZ, and here MDA attained ≥80% (median) treatment coverage of the eligible population from 2005 until 2015 (Supporting Information S1 Table).

IgG4 responses to *O. volvulus* (crude) adult worm antigen (OvAg) and to Ov16 antigen

The sensitivities of the OvAg- and Ov16-specific IgG4-ELISAs to detect Mf-positive participants were, respectively, 89.2% and 71.4% (Table 3). The all-ages IgG4 seroprevalence values for OvAg and Ov16 were, respectively, 49.4% and 34.4% (Table 2). Age-specific serological IgG4 responses to the OvAg and Ov16 antigens are shown in Figure 3. OvAg and Ov16 sero-prevalence increases with age (OvAg: Spearman ρ=0.346, Ov16: Spearman ρ=0.299, both $p<0.0001$) (Fig 3) and it attains a maximum level around the age of 50 years. During the first two decades of age, the mean OvAg- and Ov16-specific IgG4 reactivity was low in most cases; from 16 years onwards, an enhanced responsiveness was observed, and from 20 years and older the mean participants' serologic IgG4 responses to OvAg and Ov16 continued to rise steadily until the fifth decade of age (Fig 3). In children of 5-10 years, 29.1% and 14.9% showed a positive IgG4 serological response to OvAg and Ov16 (Fig 3). In the Kéran, Mô and Ôti river basins, IgG4 sero-prevalence values in children (5-10 years) to OvAg were 51.7%, 23.5% and 12.7%, respectively, and to Ov16 the values were 33.3% in Kéran and 5.2% in Ôti (Table 2).

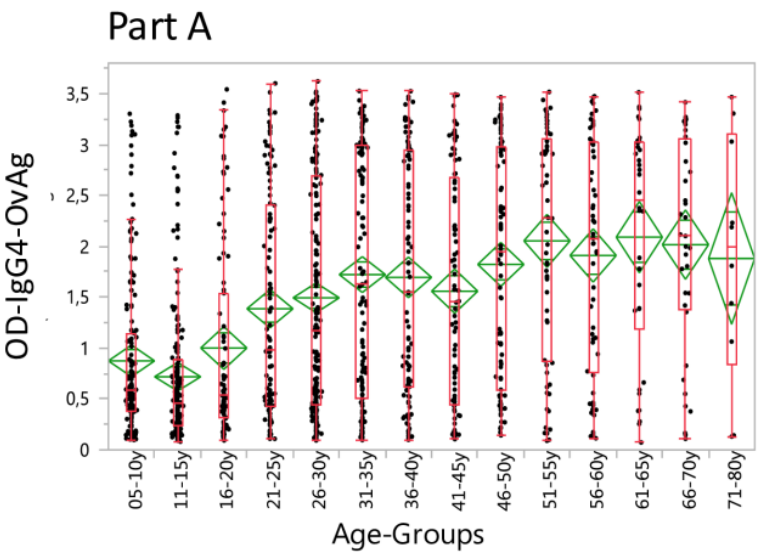
Table 2. The *Onchocerca volvulus* microfilarial (Mf) prevalence in all age participants, the IgG4 positive responses specific for *Onchocerca volvulus* adult worm antigens (OvAg) and for the *O. volvulus*-specific antigen Ov16 in all-ages participants and in children ≤10 years in NOCP sentinel villages in central and northern Togo by river basins. The total number of participants and of children ≤10 years examined and their positive IgG4 responses to OvAg and Ov16 are indicated. Numbers in square brackets indicate the 95% confidence intervals.

*** Fisher's exact Test (two-sided) with p<0.0001 compared to the other river basins; && Fisher's exact Test (two-sided) with p<0.001 compared to the river basin of Kéran; & Fisher's exact Test (two-sided) with p<0.003 for Kéran river basin compared to the river basin of Mô; n.d.: not done

River Basin	Village	*Onchocerca volvulus* infection			IgG4-OvAg ELISA				IgG4-Ov16 ELISA			
		examined all ages (n)	Mf-positive (n)	% Mf positive	positive (n)	% all ages positive	children (5-10y)	children positive	% children positive	examined all ages (n)	positive (n)	% all ages positive	children (5-10y)	children positive	% children positive
Ôti	Pancérys	140	4	2.9 [0;6.6]	34	24.3 [17.1;31.4]	32	3	9.4 [0;24.2]	92	10	10.9 [4.4;17.4]	25	1	4.0 [0;12.3]
Ôti	Boutchakou	127	1	0.8 [0;4.8]	32	25.2 [17.5;32.6]	17	3	17.6 [0;39.9]	92	15	16.3 [8.6;24.0]	15	1	6.7 [0;21.0]
Ôti	Koukoumbou	57	3	5.3 [0;11.2]	5	8.8 [1.2;16.3]	14	2	14.3 [0;36.6]	70	9	12.9 [4.8;20.9]	17	1	5.9 [0;18.4]
Ôti River Basin		**324**	**8**	**2.5 [0;4.9]**	71	21.9*** [17.4;26.4]	63	8	12.7&& [2.1;23.3]	254	34	13.4&& [9.2;17.6]	57	3	5.3&& [0;11.2]
Kéran	Gouibi	131	15	11.5 [7.5;15.4]	89	67.9 [59.8;76.0]	6	3	50 [15.8;84.1]	92	42	45.7 [35.3;56.0]	1	0	0
Kéran	Tchitchira	146	15	10.3 [6.6;14.0]	101	69.2 [61.6;76.7]	4	2	50 [8.2;91.8]	92	42	45.7 [35.3;56.0]	1	0	0
Kéran	Koutougou Solla	81	11	13.6 [8.6;18.5]	53	65.4 [54.9;76.0]	9	7	77.8 [49.9;105.6]	79	45	56.0 [45.8;68.1]	6	4	66.7 [12.4;120.6]
Kéran	Kpantiyagou	181	14	7.7 [4.4;11.1]	116	64.1 [57.0;71.1]	39	18	46.2 [32.7;59.5]	92	46	50.0 [39.6;60.4]	22	6	27.3 [7.1;47.5]
Kéran River Basin		539	**55*** **	**10.2*** ** [8.3;12.1]**	359	66.6*** [62.6;70.6]	58	30	51.7& [40.7;62.8]	355	175	49.3 [44.1;54.5]	30	10	33.3 [15.4;51.2]
Mô	Bawlensi	148	0	0 [0;3.7]	46	31.1 [23.5;38.6]	11	2	18.2 [0;43.4]	n.d.			n.d.		
Mô	Mô-Village	151	13*	8.6 [4.9;12.2]	91	60.3 [52.4;68.2]	11	4	36.4 [11.1;61.6]	22	9	40.9 [18.6;63.2]	n.d.		
Mô	Katcha-Konkomba	188	5	2.7 [0;5.9]	74	39.4 [32.3;46.4]	20	2	10.0 [0;28.7]	n.d.			n.d.		
Mô	Saboundi	105	2	1.9 [0;6.3]	67	63.8 [54.5;73.2]	9	4	44.4 [16.6;.72.3]	13	1	7.7 [0;24.4]	n.d.		
Mô River Basin		592	**20**	**3.4 [1.5;5.2]**	278	47.0*** [42.9;51.0]	51	12	23.5 [11.7;35.3]	35	10	28.6 [12.8;44.3]	n.d.		
TOTAL		**1455**	**83**	**5.7 [4.5;6.9]**	**708**	**48.7 [46.1;51.2]**	**172**	**50**	**29.1 [22.;35.9]**	**644**	**219**	**34.0 [30.3;37.7]**	**87**	**13**	**14.9 [7.3;22.6]**

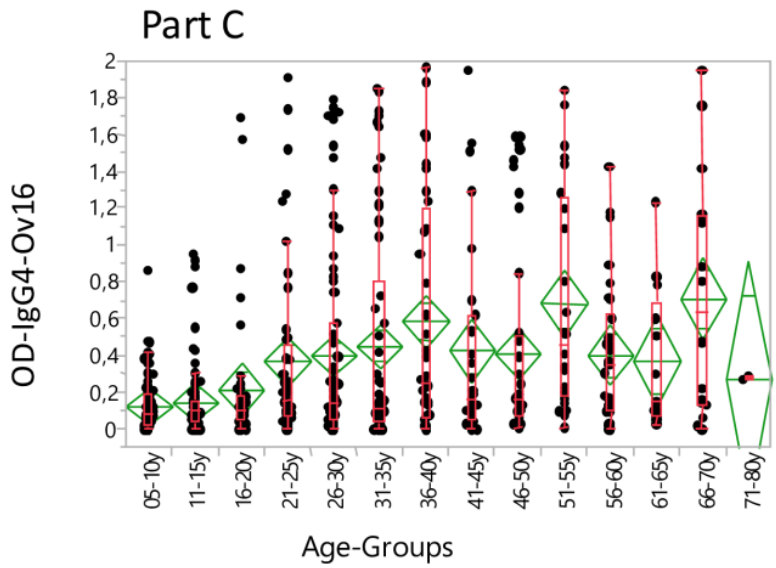
Table 3. The sensitivity of the IgG4 ELISAs based on *Onchocerca volvulus* adult worm antigen (OvAg) (left panel) and on the *O. volvulus*-specific recombinant antigen Ov16 (right panel) for the detection of patent *O. volvulus* infection (Mf positivity). The contingency table indicates, in the upper left and right panels, the percentage of false-negative results in relation to Mf positive test results. In the lower left and right panels, the percentage of correct positive results in relation to Mf positivity is highlighted for the OvAg-IgG4 and Ov16-IgG4 ELISAs. The cutoff for IgG4-OvAg and IgG4-Ov16 positive responses was set at the upper limit of the 95% confidence interval of the mean optical density (OD) in *O. volvulus* microfilariae (Mf) negative 5-10 year old children.

OvAg-IgG4					Ov16-IgG4			
	Ov-Mf-neg	Ov-Mf-pos				Ov-Mf-neg	Ov-Mf-pos	
OvAg-IgG4-neg	738	9	747		Ov16-IgG4-NEG	403	22	425
	53.8	**10.8**				71.1	**28.6**	
OvAg-IgG4-pos	634	74	708		Ov16-IgG4-POS	164	55	219
	46.2	**89.2**				28.9	**71.4**	
	1372	83	1455	TOTAL		567	77	644
	94.3	5.7		%		88.0	12.0	



Part B

Age Groups	Participant (n)	IgG-OvAg POS (n)	% IgG4 OvAg Positive Responses (Mean [95% upper and lower CI])	Tukey Kramer Test
05-10y	172	50	29.1 [22.0;36.1]	C D
11-15y	212	48	22.6 [16.3;29.0]	D
16-20y	95	31	32.6 [23.2.;42.1]	B C D
21-25y	118	60	50.8 [42.4; 59.3]	A B
26-30y	195	101	51.8 [45.2;58.4]	A B
31-35y	135	80	59.3 [51.3;67.2]	A
36-40y	105	62	59.0 [50.1;68.0]	A
41-45y	89	54	60.47 [50.9;70.4]	A
46-50y	90	60	66. 7 [57.0;76.4]	A
51-55y	62	46	74.2 [62.5;85.9]	A
56-60y	61	44	72.1 [60.3;83.9]	A
61-65y	34	26	76.4 [60.7;92.3]	A
66-70y	36	28	77.8 [62.4;93.1]	A
71-80y	10	8	80.0 [50.9;109.1]	A B C
	1414	**698**	**49.4 [46.1;51.2]**	



Part D

Age Groups	Participants (n)	IgG-Ov16 POS (n)	% IgG4 Ov16 Positive Responses (Mean [95% upper and lower CI])	Tukey Kramer Test
05-10y	87	13	14.9 [5.4;24.5]	C
11-15y	76	12	15.8 [5.6;26.0]	C
16-20y	41	7	17.1 [3.1;31.0]	B C
21-25y	44	18	40.9 [27.5;54.4]	A B C
26-30y	75	31	41.3 [31.0;51.6]	A B
31-35y	58	22	37.9 [26.2;49.6]	A B C
36-40y	40	20	50.0 [35.9;64.1]	A B
41-45y	27	11	40.7 [23.6;57.9]	A B C
46-50y	43	15	34.9 [21.3;48.5]	A B C
51-55y	24	15	62.5 [44.3;80.7]	A
56-60y	30	17	56.7 [40.4;73.0]	A
61-65y	13	6	46.2 [21.4;70.9]	A B C
66-70y	16	9	56.3 [34.0;78.6]	A B C
71-80y	2	2	100.0 [36.9;163.1]	A B C
	576	**198**	**34.4 [30.3;37.7]**	

Fig 3. *Onchocerca volvulus* adult worm (OvAg) and recombinant Ov16 antigen-specific IgG4 reactivity (optical densities; OD) in participants and positive and negative IgG4 responses in age groups. In A and C the data on antigen-specific IgG4 reactivity are shown as mean optical densities (ODs) with 95% confidence intervals for the means (diamonds). The data presented in box plots show the median OD per age group with the 25% and 75% quartiles and the 1.5x of the interquartile range. In B and D the antigen-specific-IgG4 positive and negative responses in age groups are indicated (in %).

O. volvulus-specific DNA (Ov150) in *Simulium* spp. black flies collected

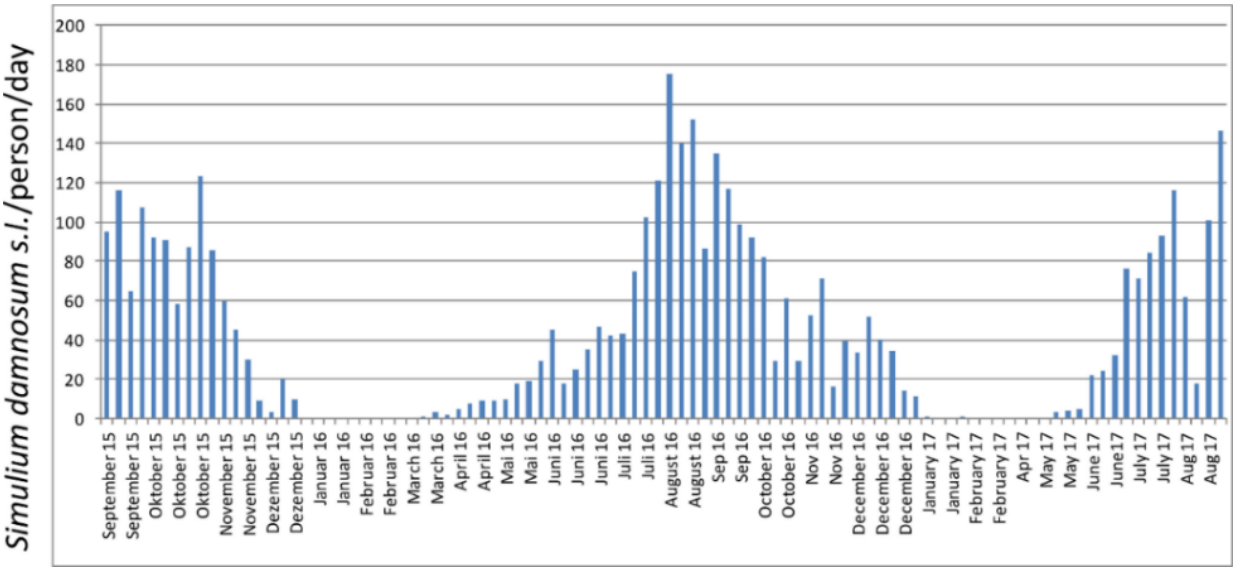
In total, 5,575 blackflies were grouped in 223 pools (25 flies each). Eight out of 35 pools from Tchitchira were positive for Ov150-DNA (22.8%) and the calculated prevalence of *O. volvulus* infection in *S. damnosum* s.l. was 1% [16]. In Baghan, three positive pools were detected (2.9%) and the calculated infecton prevalence was 0.1%. In Mô, five positive *S. damnosum* s.l. pools were identified (11.3%) with a 0.5% prevalence of *O. volvulus* in blackflies (Table 4). At Pancéry, in the Ôti river basin, two *O. volvulus*-positive pools (4.9%) were found, with a 0.2% prevalence of *O. volvulus* in black flies. *O. volvulus*-DNA-positive pools from Mô were found from early August 2015 until late October 2015, suggesting that transmission of *O. volvulus* occurred during the rainy season. An annual biting rate (ABR, Jan 2016-Dec 2016) was calculated as 15,519 bites/person/year at Mô-Bouzalo (Figure 4).

Table 4. Ov150 rt-PCR (Poolscreen) testing of *Simulium damnosum* s.l. black flies collected at sentinel catch points in northern and central Togo in 2015. Each pool consists of 25 blackflies and is listed with the total number of tested flies by catch point, and the number of Ov150-positive pools. The prevalence (in % with 95% confidence intervals) of *O. volvulus* in *S. damnosum* s.l. is calculated according to Katholi et al. 1995 [16]. Fisher's exact test was applied to evaluate differences in infection rates between pools.

Ov150 rt-PCR		
Locality (no. flies tested)	Ov-150 positive pools	Prevalence of *O. volvulus* in *Simulium damnosum* s.l.
Ôti/Pancery (41x25=1,025)	2	0.2% [0.03; 1.3]
Kéran/Tchitchira (35x25=875)	8*	1.0% [0.9; 2.1]
Mô/Baghan (103x25=2,575)	3	0.1% [0.03; 0.5]
Mô/Bouzalo (44x25=1,100)	5	0.5% [0.2; 1.3]

p<0.05, difference between Ôti/Pancery, Kéran/Baghan and Mô/Bouzalo (Fisher's exact test).

Fig 4. Collection of *Simulium damnosum* s.l. at the catch point at the Mô river (village of Bouzalo) caught per person (one day per week). The annual biting rate (ABR) from January 2016 until December 2016 was calculated by multiplying the number of blackflies caught daily by the number of days per week for each month to add up to 12 months. Collections were conducted from 7am to 6pm with alternating fly catchers every two hours as described in Materials and Methods.



Ocular pathology

The main ocular pathologies in the examined village populations, reported for the right eye, were papillitis (19.5%), cataract (17.6%), chorioretinitis (9.8%), conjunctivitis (7.8%), tropical limbo-cojunctivitis (LCET) (6.3%), iridocyclitis (4.6%), sclerosing keratitis (3.9%) and blindness of either eye (7.4%) (Table 5). Of note were punctate keratitis lesions with 1-10 Mf of *O. volvulus* in the cornea present in children (aged 12-15 years) and adults (28-52 years) and sclerosing keratitis in adults (Table 5, Fig 5). In four cases, alive or dead *O. volvulus* Mf were detected in the anterior chamber of the eye. Iridocyclitis in evolution (n=7) was found in youngsters and adults; retinal lesions (chorioretinis) were present in younger adults and older ages (Fig 5) with n=19 being in evolution and n=41 at an advanced stage. Evolving cataract (all causes) was diagnosed in a few children and mainly in older ages. Cataracts caused by *O. volvulus* infection and blindness caused by onchocerciasis were observed in individuals aged above 50 years (Fig 5). Cataract, blindness, chorioretinitis, iridocyclitis, punctate and sclerosing keratitis were observed similarly in female and male participants, while LCET, papillitis, trichiasis and low vision were more often diagnosed in females (Table 5). Cataract (Spearman's rank correlation: ρ=0.71; p=0.005), blindness (ρ=0.75; p=0.002), sclerosing keratitis (ρ=0.78; p=0.001), iridocyclitis (ρ=0.71; p=0.005) and low

visual acuity (ρ=0.56; p=0.04) correlated positively with age disclosing the age-related decline in visual functions (Table 5). LCET and normal vision were negatively correlated with participants' age (Table 5). Several manifestations (right eye), notably cataract, sclerosing keratitis and iridocyclitis, were diagnosed less often (p<0.001, one-sided Fisher exact Test) in patients from villages situated in the Mô river basin (Table 5). Of note were the positive correlations of cataract with sclerosing keratitis (Spearman's rank correlation: (ρ=0.874; p<0.0001), cataract with iridocyclitis (ρ=0.716; p=0.0004), with blindness (ρ=0.765; p=0.0014) and with low visual acuity (ρ=0.753; p=0.0019) (Table 6). Strongly associated (ρ=0.8134, p=0.0004) were sclerosing keratitis with iridocyclitis, both pathologies of the anterior segment of the eye, and similarly, vascular retinopathy and druzen correlated positively (ρ=0.7723, p=0.0012) (Table 6), both are posterior eye segment lesions.

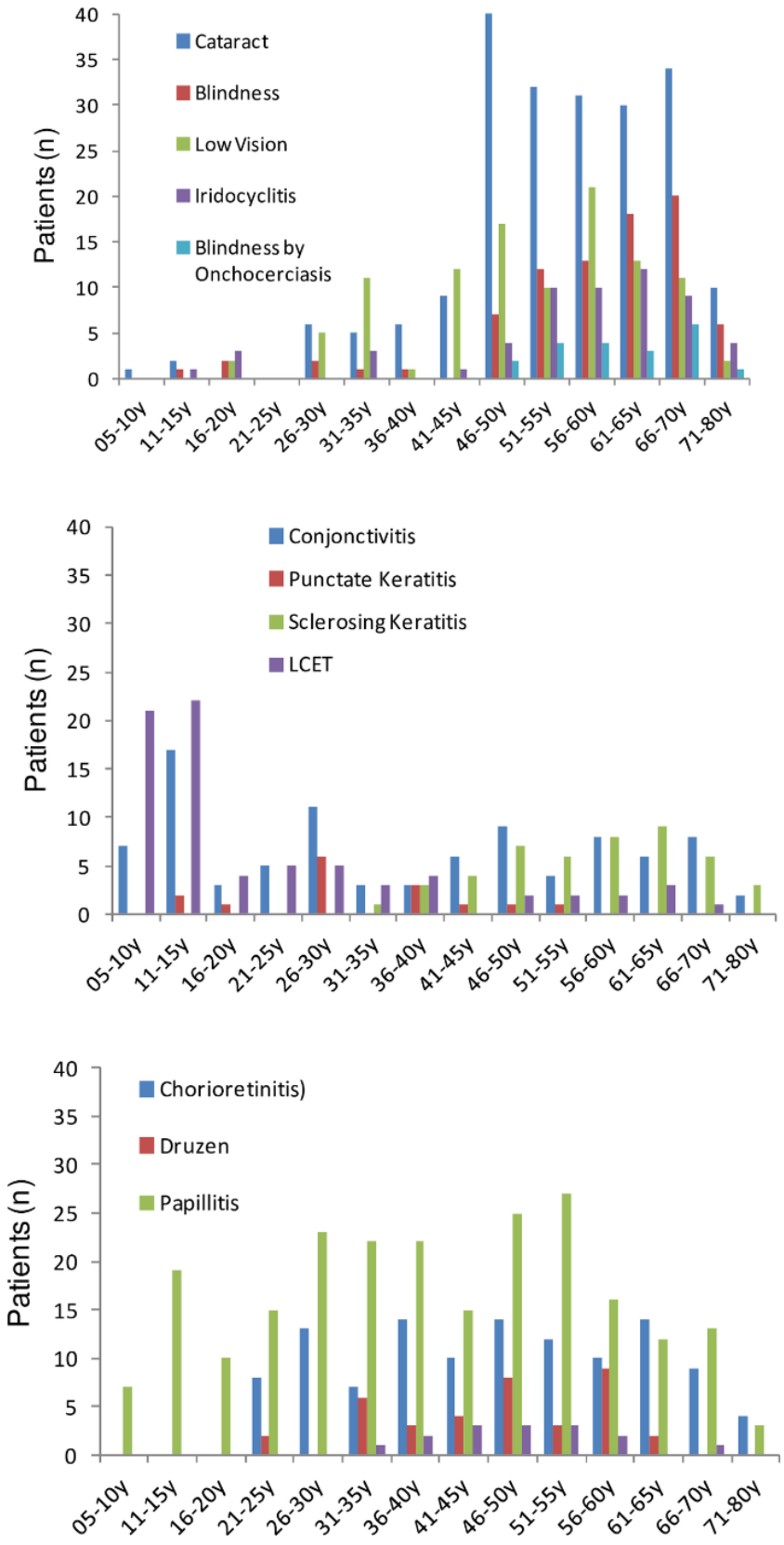


Fig 5. Ocular pathologies and visual acuity in study participants (n=1,172) according to age. The ocular pathologies, their grades of evolution and extent were classified as described previously [12].

Table 5. Ocular pathologies in study participants (n=1,183) from onchocerciasis endemic villages situated in the river basins of Kéran, Mô and Ôti in northern and central Togo. The village populations have been treated annually via CDTI. The ocular pathologies, their grades of evolution and extent were classified as described previously [12] and are indicated for the right and left eye (RLE), the left eye (LE) and the right eye (RE). Chi-Square tests were applied to compare ocular pathologies of the right eye (RE) between female and male patients. One-sided Fisher exact test was used to evaluate differences in the prevalence of ocular pathologies in patients from the Ôti, Kéran and Mô river basins, and significant differences are indicated by $p<0.05$. Spearman's rank correlation analyses of ocular pathologies of the right eye (RE) and age were conducted; the correlation coefficient ρ and the significant associations ($p<0.05$) are shown.

Ocular Pathology	RLE	RE	LE	% RE	% LE	RE pathology in female/male (p-value)	RE pathology in correlation (ρ) with age (p-value)	RE Pathology in Kéran(K), Mô(M), Ôti(O)[&] (p-value)
Cataract	405	206	199	17.6	16.9	n.s	ρ=0.7087; p=0.0045	K>M: p=0.0009; K>O: p=0.015; [K:111;M:84;O:11]
Blindness	168	84	84	7.2	7.2	n.s.	ρ=0.7488; p=0.0021	n.s.: [37;43;4]
Chorioretinitis	232	115	117	9.8	9.9	n.s.	n.s.	K>O: p=0.014; M>O: p<0.0015; [45;68;2]
Conjunctivitis	183	92	91	7.8	7.7	n.s.	n.s	M>K: p=0.03; O>K: p=0.03; [29;52;11]
Druzen	78	37	41	3.1	3.4	p=0.03; (25/12)	n.s.	K>M: p<0.007; K>O: p=0.01; [25;12;0]
Iridocyclitis	112	57	55	4.8	4.6	n.s.	ρ=0.7298; p=0.0045	K>M: p<0.0003; [38;16;3]
Keratitis (not onchocerciasis)	28	14	14	1.1	1.1	n.s.	n.s.	n.s.
Punctate keratitis	26	15	11	1.3	0.9	n.s.	n.s.	n.s.
Sclerosing keratitis	93	47	46	4.0	3.9	n.s.	ρ=0.7806; p=0.001	K>M: p<0.0001; [34;10;3]
LCET*	148	74	74	6.3	6.3	p=0.02; (27/47)	ρ= -0.7168; p=0.0039	n.s.
Papillitis	454	229	225	19.5	19.1	p=0.04 (130/99)	n.s.	O>K: p=0.03; O>M: p<0.09; [99;103;27]
Vascular retinopathy	30	15	15	1.2	1.2	n.s.	n.s.	n.s.
Trichiasis	26	12	13	1.0	1.1	p=0.004; (11/1)	n.s.	n.s.
Low vision (code WHO)**	193	105	88	4.7	4.0	p<0,000; (78/27)	ρ=0.5583; p=0.038	n.s.
Normal vision (code WHO)	2028	1011	1017	45.5	45.8	p=0.0062; (548/461)	ρ= -0.8263; p=0.0003	n.s.

RLE = Right and Left Eye; LE = Left Eye; RE = Right Eye; n.s. = not significant; *LCET = Tropical Endemic Limbo-Conjunctivitis; ** The criteria for low and normal visual acuity according to WHO (n=2,221 examinations) are indicated in Materials and Methods. [&] The number of cases with the respective ocular pathology in the river basins of Kéran, Mô and Ôti are indicated in square brackets. n.s. (p>0.05): not significant

Table 6. Spearman's rank correlation analyses of ocular pathologies and visual acuity of the right eye (RE) in the survey participants and significant associations (p<0.05)

RE Variable 1	RE Variable 2	Correlation Coefficient ρ	p value
Cataract	Sclerosing keratitis	0.874	<.0001
Cataract	Iridocyclitis	0.7159	0.0004
Cataract	Blindness	0.7653	0.0014
Cataract	Low vision	0.7528	0.0019
Cataract	LCET*	-0.7222	0.0035
LCET	Sclerosing keratitis	-0.7001	0.0053
Blindness	Iridocyclitis	0.8568	<.0001
Blindness	Sclerosing keratitis	0.7191	0.0038
Chorioretinitis	Sclerosing keratitis	0.6485	0.0121
Chorioretinitis	LCET	-0.6221	0.0175
Chorioretinitis	Papillitis	0.5787	0.0301
Vision Low	Sclerosing keratitis	0.8163	0.0004
Vision Low	Druzen	0.7841	0.0009
Vision Low	Iridocyclitis	0.7068	0.0047
Vision Low	Vascular retinopathy	0.6132	0.0197
Vision Low	Trichiasis	0.5822	0.0289
Vision Normal	Sclerosing keratitis	-0.7447	0.0022
Vision Normal	Iridocyclitis	-0.7415	0.0024
Druzen	Vascular retinopathy	0.7723	0.0012
Iridocyclitis	sclerosing keratitis	0.8135	0.0004

***LCET = Tropical Endemic Limbo-Conjunctivitis**

Discussion

The annual mass distribution of ivermectin during the past 25 years has greatly reduced *O. volvulus* infection prevalence in Togo. In the surveyed populations, the overall *O. volvulus* microfilarial prevalence has decreased below 5%. While this is strong evidence that elimination of onchocerciasis is a realistic outcome, in several locations in northern and central Togo, Mf-positivity ranged above 5% and 10%, respectively. The present surveys were conducted within the Ôti, Kéran and Mô river basins, in locations where patent *O. volvulus* infections still persisted in children aged ≤10 years and also in adults; further, progressive ocular pathology was diagnosed, and transmission of *O. volvulus* by *S. damnosum* s.l. occurred close to the studied locations.

Recently, the focus of onchocerciasis programmes has changed from control to elimination in Africa [17], and indeed, in several areas of the former OCP in Mali and Senegal [5, 18], and in foci of the Onchocerciasis Elimination Program for the Americas (OEPA) (e.g., Guatemala [7, 19] and Mexico [20]), onchocerciasis has been reported to have been eliminated. Parasite transmission has been interrupted in northern Venezuela [21] and also in western Uganda [22]. The present observations in northern and central Togo suggest that despite the long-term repeated MDA with ivermectin, elimination of onchocerciasis and interruption of parasite transmission has not yet been attained. The required duration of MDA with ivermectin for onchocerciasis elimination is under scrutiny. The ONCHOSIM and EPIONCHO models predicted that the provisional operational thresholds for treatment interruption and commencement of surveillance (pOTTIS) can be reached by annual treatments (coverage 80%) in locations with a mesoendemic Mf prevalence within 14-17 years (ONCHOSIM and EPIONCHO) [23]. The operational threshold for treatment interruption has been achieved when the Mf prevalence reaches <1.4 % in the population aged ≥5 years (in this modelling comparison, children under 5 were excluded [23]). The predicted duration of MDA (coverage 80%) for hyperendemic locations was 17 years (ONCHOSIM) or >25 years (EPIONCHO). Such predictions, however, may not be applicable all throughout the endemic areas in Africa [24]. The differing parasite transmission intensities and vector species with differing abilities to transmit *O. volvulus* [25], the pre-control Mf prevalence [26], the persisting transmission despite long-term MDA [27, 28], and the proportion of systematic non-compliers to treatment [29] may influence the overall success in achieving the elimination goals. Modelling the

elimination of river blindness using long-term data from Mali and Senegal foci has adequately shown the epidemiological trends during mass treatment; resurgence of patent *O. volvulus* infection, with low microfilarial prevalence, was also predicted (EPIONCHO) in areas with high pre-intervention endemicities and intense vector biting rates [30]. In Togo, the median pre-MDA Mf prevalence was at mesoendemic levels, and in some survey sites hyperendemic onchocerciasis was found with a prevalence of around 80% (Fig 1). As such, the predicted duration of MDA of 14-17 years until the pOTTIS are reached may not suffice, and in hyper-endemic locations, 25 years of MDA may not lead to elimination. It must be noted that reaching the pOTTIS is not equivalent to reaching the transmission breakpoints below which parasite populations cannot persist [23]. Current control strategies will require prolonged continuation and comprehensive operational MDA approaches which extend and account for the various factors mentioned above.

In Cameroon, and after more than 15 years of CDTI, onchocerciasis has remained mesoendemic in surveyed communities [31], and in some rainforest river basins, several communities had a microfilarial prevalence above 40% despite over a decade of CDTI [32].

In the present study in Togo, we complemented skin biopsy surveys with sensitive and specific serological, ophthalmological and entomological assessments. For the serological ELISA-based evaluations, an *O. volvulus* adult worm antigen extract (OvAg) and the Ov16 antigen were applied, with an all-ages sensitivity of 89% and 71%, respectively. The seroprevalence values in children and adults reflect the extent by which the endemic population is still *O. volvulus* positive, and further, we could distinctly identify those locations and river basins where both children and adults remain still exposed to *O. volvulus*. Previously, we have applied the Ov16 IgG4 ELISA as a marker of active infection in all ages with a sensitivity of 60% [33], and such an assay would miss many Mf-positive cases and underestimate the actual *O. volvulus* infection prevalence, notably in adult populations. *O. volvulus* Ov16-based ELISA is recommended for testing children aged <10 years in order to detect continuing parasite transmission, but those most exposed to *O. volvulus* infection are agricultural field workers at river sites, and those often are women above primary school age.

Human migration in and out of the river basins may limit treatment coverage; particularly, males aged 15 to 40 years were absent when examination and treatment were conducted (Table 1). These age groups may represent a parasite reservoir which

should selectively be approached to improve therapeutic coverage with ivermectin. The reasons given by families for the absence of male members was travel and temporary work away from the villages, but the return of those absent men for agricultural activities was asserted. All surveyed villages are in close location to the Benin and Ghana borders and migration across these is common (Fig 2). Similarly, in the West Region of Cameroon, the major issue for ivermectin non-compliance was absence, firstly as a result of seasonal migration, and secondly because of fear of severe adverse events (as some areas are co-endemic with loiasis); in this area the majority of systematic non-compliers were female [34].

Our ophthalmological assessments identified ocular pathologies caused by active *O. volvulus* infections; the observed evolving onchocerciasis ocular lesions revealed that parasite transmission is ongoing where “river blindness” was formerly severely present. The occurrence of young individuals with punctate keratitis, with evolving iridocyclitis and chorioretinitis, indicates recent parasite exposure, whereas sclerosing keratitis and blindness in older age groups indicate prevalent cases of disease due to *O. volvulus* infection acquired in the past (these lesions do not respond to ivermectin treatment). In the rural communities surveyed, all-cause cataract was the main cause of visual impairment; further ocular pathologies like conjunctivitis, papillitis and non-onchocercal keratitis contribute to the low vision in the examined populations. The fewer cases of chorioretinitis, iridocylitis, punctate and sclerosing keratitis in the Ôti river basin may indicate that onchocerciasis-induced ocular pathologies have regressed, and such favourable evolution should be confirmed by larger surveys. The Ôti river basin was part of the initial OCP vector control programme since 1976, and ivermectin MDA has been applied for almost three decades. The present results support previous observations that annual ivermectin treatments eliminate and prevent the migration of *O. volvulus* Mf into the anterior eye chamber and cornea, with punctate keratitis resolving completely and early-stage sclerosing keratitits and iridocyclitis regressing, whereas advanced lesions of the anterior and posterior eye segment remain progressive [12, 13, 35]. Annual ivermectin treatments may prevent the emergence of ocular pathology in those populations still exposed to *O. volvulus* infection, but the present ophthalmological surveys support that the interruption of *O. volvulus* transmission for the purpose of stopping MDA has not yet been attained.

In fact, parasite transmission is ongoing in the Kéran and Mô river basins and also along the Ôti, but in the latter, the number of blackflies collected was low and time-

limited; further studies are planned which will extend entomological collections over several months. The geography of the river basins in northern and central Togo is extensive; the Ôti flows from Benin crossing Togo east to west; the Kéran joins with the Koumoungou and Ôti, and the Mô starts in central Togo and continues west into the Volta river basin in Ghana. Parasite transmission has never been interrupted completely in central and northern Togo and Benin; the Ôti, Kéran and Mô river basins were SIZ where vector control and intensified ivermectin distribution needed to be continued for years after OCP closure in 2002 [9, 10]. Special interventions in the post-OCP period included continued aerial larvicide application for five additional years (2003-2007) and biannual ivermectin mass treatment (which attained a treatment coverage >80%) until the end of 2012 [9, 10]. In the Mô river basin, a high vector density was documented in 2015 and 2016, with an ABR of 15,519 bites/person/year of *S. damnosum* s.l. (Fig 5). This value was similar to that observed before launching the OCP [36], and this intense biting may favor parasite transmission. The positive rtPCR results confirm ongoing transmission of *O. volvulus*. Because whole blackflies were used (rather than just fly heads) our positive results may indicate transmission from humans to vectors as well as transmission from vectors to humans. The latter requires the presence of infective-stage larvae (L3) in the head of the vector. We have confirmation for parasite–vector contact in our analysis of body pools from northern and central regions in Togo, and in the next collections, *S. damnosum* s.l. head pools will be tested to gain an accurate estimate of the prevalence of flies carrying L3 larvae.

Persistent *O. volvulus* transmission in the study river basins can be attributed to geographical conditions which allow for trans-border migration of vectors from east to west and vice versa, notably during the rainy seasons, when *S. damnosum* s.l. flies may migrate across larger distances as previously observed [37, 38]. In the savannah areas of north-central Togo the savannah members of the *Simulium damnosum sensu lato* species complex prevail, notably *S. squamosum* in the Mô valley, *S. sirbanum* along the Kara and Ôti, and *S. soubrense* and *S. sanctipauli* group in eastern areas [39]. In the analysis presented here, *O. volvulus* DNA was detected in simuliids collected during the rainy season in the Kéran, Ôti and Mô river basins, tributaries to the Volta river basin. Thus, *O. volvulus* transmission control efforts should expand into cross-border collaborations [40] and ivermectin MDA should be coordinated and applied to populations that live at national frontiers, as well as be well-timed when main parasite transmission occurs.

Conclusions

The present surveys have shown that the northern and central regions in Togo are gradually approaching the elimination of onchocerciasis [7, 8]; however, the geographical and demographic conditions in the Ôti, Kéran and Mô river basins will require continuous, comprehensively intensified and well-adapted interventions which should reach beyond the operationally standardized MDA. In formerly hyperendemic areas in northern Togo that formed part of the SIZ, biannual MDA attained >80% treatment coverage until 2015, yet many foci remain positive for onchocerciasis and parasite transmission continues. Here, the future interventional strategy may selectively adapt to the particular characteristics of the endemic populations, notably, to the seasonal migrations in and out of the river basins, to the age and gender profiles of the non-complying groups, and to the seasonal patterns of parasite transmission by the local *S. damnosum* s.l. vector species. Moreover, national control programmes should harmonize cross-border MDA strategies as a coordinated control measure.

References

1. Sékétéli A, Adeoye G, Eyamba A, Nnoruka E, Drameh P, Amazigo UV, et al. The achievements and challenges of the African Programme for Onchocerciasis Control (APOC). Ann Trop Med Parasitol 96 Suppl 1: S15-28.
2. Boatin B, (2008) The Onchocerciasis Control Programme in West Africa (OCP). Ann Trop Med Parasitol 102 Suppl 1: 13-7. https://doi.10.1179/136485908X337427.
3. Thylefors B, Alleman M (2006) Towards the elimination of onchocerciasis. Ann Trop Med Parasitol 100: 733-46.
4. Basáñez MG, Pion SDS, Churcher TS, Breitling LP, Little MP, Boussinesq M, et al. (2006) River blindness: a success story under threat? PLoS Med. 3: e371.
5. Diawara L, Traoré MO, Badji A, Bissan Y, Doumbia K, Goita SF, et al. (2009) Feasibility of onchocerciasis elimination with ivermectin treatment in endemic foci in Africa: first evidence from studies in Mali and Senegal. PLoS Negl Trop Dis. 3:e497.
6. Cupp EW, Sauerbrey M, Richards F (2011) Elimination of human onchocerciasis: history of progress and current feasibility using ivermectin (Mectizan((R))) monotherapy. Acta Trop. 120 Suppl 1: S100–S108. https://doi.10.1016/j.actatropica.2010.08.009

7. World Health Organization (WHO) (2016) Progress towards eliminating onchocerciasis in the WHO Region of the Americas: verification of elimination of transmission in Guatemala.Wkly Epidemiol Rec. 91: 501-505.

8. African Programme for Onchocerciasis Control (APOC) (2010) Operational Framework of Onchocerciasis Elimination with Ivermectin Treatment. World Health Organization, Geneva, Switzerland, WHO/APOC/MG/10.1. http://www.who.int/apoc/oncho elimination report english.pdf.

9. Kale O, Grunewald J, Koulischer G, Massougbodji A, Sachndeva P (2002) Onchocerciasis Control Programme External Evaluation. External Evaluation Team (EET). Onchocerciasis Control Programme, Ouagadougou, Burkina Faso.

10. Yaméogo L (2008) Special intervention zones. Ann Trop Med Parasitol. 102 Suppl 1: 23-24. https//doi.10.1179/136485908X337445.

11. World Health Organization (2016) Guidelines for Stopping Mass Drug Administration and Verifying Elimination of Human Onchocerciasis: Criteria and Procedures.WHO; Geneva.

12. Banla M, Tchalim S, Karabou PK, Gantin RG, Agba AI, Kére-Banla A, et al. (2014) Sustainable control of onchocerciasis: ocular pathology in onchocerciasis patients treated annually with ivermectin for 23 years: a cohort study. PLoS One. 9:e98411. https://doi. 10.1371/journal.pone.0098411.

13. Lariviere M, Vingtain P, Aziz M, Beauvais B, Weimann D, Derouin F, et al. (1985) Double blind study of ivermectin and diethylcarbamazine in african onchocerciasis patients with ocular involvement. Lancet 1: 174–177.

14. Mai CS, Hamm DM, Banla M, Agossou A, Schulz-Key H, Heuschkel C, et al. (2007) *Onchocerca volvulus*-specific antibody and cytokine responses in onchocerciasis patients after 16 years of repeated ivermectin therapy. Clin Exp Immunol 147: 504-412.

15. Lechner CJ, Gantin RG, Seeger T, Sarnecka A, Portillo J, Schulz-Key H, et l. (2012) Chemokines and cytokines in patients with an occult Onchocerca volvulus infection. Microbes Infect 14: 438-446.

16. Katholi CR, Toé L, Merriweather A, Unnasch TR. (1995) Determining the prevalence of *Onchocerca volvulus* infection in vector populations by polymerase chain reaction screening of pools of black flies. J Infect Dis 172: 1414-1417.

17. Evans DS, Unnasch TR, Richards FO (2015) Onchocerciasis and lymphatic filariasis elimination in Africa: it's about time. Lancet 385: 2151-2152.

18. Traore MO, Sarr MD, Badji A, Bissan Y, Diawara L, Doumbia K, et al. (2012) Proof-of-principle of onchocerciasis elimination with ivermectin treatment in endemic foci in Africa: final results of a study in Mali and Senegal. PLoS Negl Trop Dis 6:e1825.

19. Kazura JW (2015) More progress in eliminating transmission of *Onchocerca volvulus* and *Wuchereria bancrofti* in the Americas: A portent of global eradication. Am J Trop Med Hyg 93:1128-1129. doi: 10.4269/ajtmh.15-0688.

20. Rodríguez-Pérez MA, Fernández-Santos NA, Orozco-Algarra ME, Rodríguez-Atanacio JA, Domínguez-Vázquez A, Rodríguez-Morales KB, et al. (2015) Elimination of onchocerciasis from Mexico. PLoS Negl Trop Dis 9:e0003922.

21. Convit J, Schuler H, Borges R, Olivero V, Domínguez-Vázquez A, Frontado H, et al. (2013) Interruption of *Onchocerca volvulus* transmission in Northern Venezuela. Parasit Vectors. 6:289. doi: 10.1186/1756-3305-6-289.

22. Lakwo T, Garms R, Wamani J, Tukahebwa EM, Byamukama E, Onapa AW, et al. (2017) Interruption of the transmission of *Onchocerca volvulus* in the Kashoya-Kitomi focus, western Uganda by long-term ivermectin treatment and elimination of the vector *Simulium neavei* by larviciding. Acta Trop 167: 128-136.

23. Stolk WA, Walker M, Coffeng LE, Basáñez MG, de Vlas SJ (2015) Required duration of mass ivermectin treatment for onchocerciasis elimination in Africa: a comparative modelling analysis. Parasit Vectors. 8: 552. doi: 10.1186/s13071-015-1159-9.

24. Turner HC, Walker M, Churcher TS, Basáñez MG (2014) Modelling the impact of ivermectin on River Blindness and its burden of morbidity and mortality in African Savannah: EpiOncho projections. Parasit Vectors 7: 241. doi: 10.1186/1756-3305-7-241.

25. Cheke RA (2017) Factors affecting onchocerciasis transmission: lessons for infection control. Expert Rev Anti Infect Ther 3:1-10. doi: 10.1080/14787210.2017.1286980.

26. O'Hanlon SJ, Slater HC, Cheke RA, Boatin BA, Coffeng LE, Pion SD, et al. (2016) Model-based geostatistical mapping of the prevalence of *Onchocerca volvulus* in West Africa. PLoS Negl Trop Dis 10:e0004328. doi: 10.1371/journal.pntd.0004328.

27. Lamberton PHL, Cheke RA, Winskill P, Tirados I, Walker M, Osei-Atweneboana MY, et al. (2015) Onchocerciasis transmission in Ghana: persistence under different control strategies and the role of the simuliid vectors. PLoS Negl Trop Dis. 9: e0003688. doi: 10.1371/journal.pntd.0003688.

28. Wilson NO, Badara Ly A, Cama VA, Cantey PT, Cohn D, Diawara L, et al. (2016) Evaluation of lymphatic filariasis and onchocerciasis in three Senegalese districts treated for onchocerciasis with ivermectin. PLoS Negl Trop Dis 10:e0005198. doi: 10.1371/journal.pntd.0005198.

29. Turner HC, Walker M, Churcher TS, Osei-Atweneboana MY, Biritwum NK, Hopkins A, et al. (2014) Reaching the London Declaration on neglected tropical diseases goals for onchocerciasis: an economic evaluation of increasing the frequency of ivermectin treatment in Africa. Clin Infect Dis. 59:923-32. doi: 10.1093/cid/ciu467.

30. Walker M, Stolk WA, Dixon MA, Bottomley C, Diawara L, Traoré MO, et al. Modelling the elimination of river blindness using long-term epidemiological and programmatic data from Mali and Senegal. Epidemics 18:4-15. doi: 10.1016/j.epidem.2017.02.005.

31. Kamga GR, Dissak-Delon FN, Nana-Djeunga HC, Biholong BD, Ghogomu SM, et al. (2016) Still mesoendemic onchocerciasis in two Cameroonian community-directed treatment with ivermectin projects despite more than 15 years of mass treatment. Parasit Vectors 9: 581.

32. Wanji S, Kengne-Ouafo JA, Esum ME, Chounna PW, Tendongfor N, Adzemye F, et al. (2015) Situational analysis of parasitological and entomological indices of onchocerciasis transmission in three drainage basins of the rain forest of South West Cameroon after a decade of ivermectin treatment. Parasit Vectors 8:202. doi: 10.1186/s13071-015-0817-2.

33. Golden A, Faulx D, Kalnoky M, Stevens E, Yokobe L, Peck R, et al. (2016) Analysis of age-dependent trends in Ov16 IgG4 seroprevalence to onchocerciasis. Parasit Vectors 9: 338.

34. Senyonjo L, Oye J, Bakajika D, Biholong B, Tekle A, Boakye D, et al. (2016) Factors Associated with Ivermectin Non-Compliance and Its Potential Role in Sustaining Onchocerca volvulus Transmission in the West Region of Cameroon. PLoS Negl Trop Dis 10: e0004905. doi: 10.1371/journal.pntd.0004905.

35. Abiose A (1998) Onchocercal eye disease and the impact of Mectizan treatment. Ann Trop Med Parasitol. 92 Suppl 1: S11-S22.

36. Onchocerciasis Control Programme in West Africa (2002) Joint Program Committee. Country-Specific Onchocerciasis Control Issues. JPC23/INF/DOC.1; Ouagadougou, 4-6 December 2002.

37. Baker RH, Guillet P, Sékétéli A, Poudiougo P, Boakye D, Wilson MD, et al. (1990) Progress in controlling the reinvasion of windborne vectors into the western area of the Onchocerciasis Control Programme in West Africa. Philos Trans R Soc Lond B Biol Sci. 328:731-47, discussion 747-50.

38. Garms R, Cheke RA, Vajime C, Sowah SA (1982) The occurrence and movements of different members of the *Simulium damnosum* complex in Togo and Benin. Zeit Ange Zool 69: 219–236.

39. Boakye DA, Back C, Fiasorgbor GK, Sib AP, Coulibaly Y. (1998) Sibling species distributions of the *Simulium damnosum* complex in the west African Onchocerciasis Control Programme area during the decade 1984-93, following intensive larviciding since 1974. Med Vet Entomol. 12: 345-358.

40. Gustavsen K, Sodahlon Y, Bush S. (2016) Cross-border collaboration for neglected tropical disease efforts-Lessons learned from onchocerciasis control and elimination in the Mano River Union (West Africa). Global Health. 12: 44. doi: 10.1186/s12992-016-0185-5.

Identification of *Onchocerca volvulus*-specific peptide antigens for serodiagnosis of onchocerciasis

Patrick S. Voßberg[1*], Stefan Stevanović[2], Christian Hotz[2],
Richard G. Gantin[3], Kossi Komlan[3],Peter T. Soboslay[1], Carsten Köhler[1]

[1]Institute for Tropical Medicine, University of Tübingen (UKT), Wilhelmstr. 27, 72074 Tübingen, Germany.
[2]Interfaculty Institute for Cell Biology, Department of Immunology, University of Tübingen, Auf der Morgenstelle 15, 72076 Tübingen, Germany.
[3] National Institute of Hygiene, Onchocerciasis Reference Laboratory, Sokodé, Togo.

Submitted for Publication to
Parasitology Research

Abstract

Background: Onchocerciasis is caused by the filarial nematode *Onchocerca volvulus*. The detection of patent *O. volvulus* infection with skin biopsies has become increasingly challenging because mass drug administration of ivermectin will significantly reduce microfilaria density in the skin. For monitoring the progression towards the elimination of onchocerciasis sensitive diagnostic tools are necessary and peptide-based diagnostic tests are successfully applied for several infectious diseases.

Methods: From *O. volvulus* granuloma tissues, parasite-specific peptide antigens were immunoprecipitated, analyzed by mass spectrometry and synthesized peptides were evaluated for antibody recognition and serodiagnosis of onchocerciasis. Using the SYFPEITHI database for MHC ligands and epitope prediction, *O. volvulus*-specific peptides motifs were identified and analyzed by serological methods. Overlapping peptides spanning the entire Ov33 protein were applied to identify antibody-reactive epitopes.

Results: We identified 6 peptides specific for *O. volvulus* in granuloma tissues from onchocerciasis patients. These peptides were parts of Ov33, P-Glycoprotein, onchocystatin and *Wolbachia*. The peptide antibody recognition was mainly mediated by IgG4. The SYFPEITHI algorithm identified 9 O. volvulus-specific peptide motifs, predicted for the most frequent HLA-DRB1*1-16 haplotypes in the study population. These peptide antigens were mainly recognized by IgG3. Antibody responses to overlapping peptides of Ov33 were evaluated in *O. volvulus* microfilaria-positive patients, onchocerciasis-free and helminth mono-infected participants. We identified 4 *O. volvulus*-specific peptides with a diagnostic performance of 100% sensitivity and above 80% specificity.

Conclusion: Our results disclose the potential of peptide antigens for sensitive and specific serologic diagnosis of patent *O. volvulus* infection and recommend their application for immune-epidemiological surveillance.

Background

Onchocerciasis, or ”river blindness“ is caused by the nematode Onchocerca volvulus which is transmitted to human by blood-feeding black flies of the *Simulium damnosus s.l.* complex (Diptera: Simuliidae). An estimated 37 million humans are infected (WHO 2016b), more than 99% of patients live in 31 sub-Saharan African countries (Coffeng et al. 2014; WHO 2015) and 0.96 million disability-adjusted life-years (DALYs) were lost to onchocerciases in 2016 (DALYs and Collaborators 2017). Over decades, the “gold standard” for diagnosis of patent *O. volvulus* infection was the microscopic detection of microfilaria (Mf) in skin biopsies (Bottomley et al. 2016; WHO 2016a). With to the mass treatment of ivermectin since 1988, the microfilaria density in the skin of onchocerciasis patients has been reduced dramatically and sensitive tests are needed to be employed during different stages of the onchocerciasis elimination programs (Vlaminck et al. 2015). Today, the WHO recommends the IgG4 antibody test based on the Ov16 antigen (WHO 2016a). The anti-Ov6 IgG4 antibody ELISA showed a sensitivity of 97% in children, and of 60% over all age groups (Golden et al. 2016). Peptide-based diagnostic tests have been developed for virus, bacteria and parasite infections, e.g. Hepatitis C (Yoshida et al. 1992) *Chlamydia trachomatis* (Morre et al. 2002) and also cystic echinococcosis (Petrone et al. 2017). Peptides are easy to produce at low cost and offer the advantage of high specificity Their chemical structure can be modified (Alcaro et al. 2003; Gauna et al. 2015; Gomara and Haro 2007; Noya et al. 2003; Wu et al. 1999), they can be engineered to design putative multi-epitope vaccine candidates e.g. agains *Theileria* parasites (Kar and Srivastava 2018) or leishmaniasis (De Brito et al. 2018) and multi-peptide-based methods are an attractive alternative in Chagas disease diagnosis (Mucci et al. 2017).

In this study, we isolated from *O. volvulus* granuloma tissues parasite-specific peptides presented on MHC-molecules of antigen-presenting cells. Macrophages, dendritic cells and B cells and play a major role in initiating immune responses (Leddon and Sant 2010). We focused on MHC class 2 and HLA-DRB1 alleles frequently found in West African populations (Goeury et al. 2018). In addition, the SYFPEITHI database for MHC ligands and epitope prediction (Rammensee et al. 1999) was used to identify *O. volvulus*-specific peptides. Several peptides specific for *O. volvulus* and its endosymbiont *Wolbachia sp.* (Stern and Santambrogio 2016) were isolated and the serodiagnostic evaluation revealed their suitability for sensitive detection of patent *O. volvulus* infection.

Methods

Serum sample collection and ethical approval.

A total of 147 serum samples have been tested for their antibody reactivity against synthetic *O. volvulus*-specific peptides. 106 serum samples from onchocerciasis patients were received from a double-blind placebo-controlled dose-finding study of ivermectin for treatment of onchocerciasis (Lechner et al. 2012; Mai et al. 2007). Authorization was granted by the Ministry of Health in Togo and ethical approval for study and sample collection was granted by the Comité de Bioethique pour la Recherche en Santé (CBRS), no.04070/2007/MS/CAB/DGS, no.0129/2011/MS/CAB/DGS/DPLET/CBRS, no.013/2015/ CBRS and no.338/2015/MS/CAB/DGS/DPLET/CBRS. Patients were apparently healthy males and non-pregnant women from the regions Centrale and Kara in Togo, West Africa, with a body weight over 30 kg and without any known drug intolerances or multiple allergies (Table 1). Participants were informed about the examinations in their local language and gave their informed consent. The surveys included physical, parasitological and ophthalmological examinations. Skin biopsies were taken from the left and right iliac crest by corneoscleral punch (Holth- or Walser-type) and microfilaria densities were calculated as Mf/mg skin. Onchocercomata (nodules) and microfilaria of *O. volvulus* in skin biopsies were present in all patients (Table 1). All onchocerciasis patients were treated with ivermectin (Lechner et al. 2012). In addition, 41 serum samples from O. volvulus infection-free European travelers to tropical countries were applied as controls.

Table1 Characteristics of serum donors

Onchocerciasis patients		
O. volvulus Microfilaria positive (n)		106
Mean age (range)		31.6 (12-60)
Mean *O. volvulus* microfilaria/mg skin (range)		61.2 (0.44-345.6)
Parasite infections (in stool samples)	*Amoeba* (number)	7
	Amoeba + *Ancylostoma duodenale*	15
	Amoeba + *A. duodenale* + *Schistosoma haematobium*	1
	Amoeba + *A. duodenale* + *Trichomonas*	1
	A. duodenale	53
	A. duodenale+ *Ascaris*	3
	A. duodenale + *Giardia lamblia*	1
	A. duodenale + *Schistosoma mansoni*	1
	A. duodenale + *Strongyloides stercoralis*	2
	Trichomonas	1
	Negative stool samples	9
European Controls		
Number (n)		41
Mean age		unknown
Parasite infections		infection free

For assessing the diagnostic performance of the peptides, in total 68 serum samples from Togolese patients from the central region with single parasite infections were used as negative control group (Table 2). Due to the absence of patients with mono-infections of *Mansonella perstans*, *Loa loa* and *Wuchereria bancrofti*, these helminth infections were not analyzed in the present study. As positive controls, 15 dry blood samples from *O. volvulus* microfilaria-positive patients who have repeatedly received annually ivermectin for several years were collected during the annual mass drug administration (Komlan et al. 2018).

Table 2 Characteristics of serum donors for sensitivity determination

Onchocerciasis-free participants with a single helminths or protozoa infection (n)	*Ankylostoma duodenalis*	9
	Entamoeba histolytica	34
	Schistosoma haematobium	24
	Schistosoma mansoni	1
Onchocerciasis patients (n)	with Ivermectin treatment	15

MHC class II haplotype HLA-DBR1 frequency.

HLA-typing by PCR amplification with sequence-specific primers (Dynal DR “low resolution”-SSP) (Olerup and Zetterquist 1992) was applied to analyze 30 blood samples originating from onchocerciasis patients and endemic controls from the Region Centrale of Togo.

Identification of O. volvulus-specific peptides bound by human MHC class II molecules. Onchocercomata (nodules) which contain antigen-presenting cells (macrophages, dendritic cells) were isolated from onchocerciasis patients. MHC class I and class II bound peptides were eluted from the MHC molecules by acid extraction and analyzed by HPLC separation connected to on-line tandem mass spectrometry (Kowalewski and Stevanovic 2013). Peptides eluted from MHC molecules were sequenced and annotated via SEQUEST with sequence databases of *O. volvulus* and *Wolbachia sp.*

Identification of peptide ligands by the SYFPEITHI algorithm

The SYFPEITHI algorithm (access via www.syfpeithi.de) was used to identify putative peptide ligands to the HLA alleles DRB1*15, DRB1*11, DRB*07 and DRB1*03. Those alleles were identified as the most frequent haplotypes in West African populations (Goeury et al. 2018; Meyer et al. 1994). Ligands to the HLA DRB1*13 allele were not possible to identify, because SYFPEITHI did not contain this predictive option.

Synthesis of peptides

MHC ligands identified by mass spectrometry as well as peptide ligands predicted by SYFPEITHI were synthesized. Peptide MHC ligands contained in the immunodominant *O. volvulus* antigen Ov33 were identified in onchocerciasis granulomas (nodules) and further studied by means of overlapping peptides (n = 24) spanning the whole Ov33 protein. Peptides were synthesized with 20 amino acids in length and an overlap of 10 amino acids. All peptides were eluted and synthesized at the Department of Immunology of the Interfaculty Institute for Cell Biology, University of Tübingen.

Enzyme-linked immunosorbent assay (ELISA).

Micro titer plates (Corning Costar 3690, half area) were coated with 1 µg/ml of the specific peptide in PBS pH 7.4 and incubated overnight at 4 °C. The next day, the coating solution was discarded, and plates were blocked with 50 µl Reagent Diluent (R&D Systems) at room temperature for 1.5 hours. Thereafter, plates were washed 3 times with PBS-Tween20® (Sigma P3563) and 50 µl serum samples were added. After incubation for 1.5 hours at room temperature, plates were again washed with PBS-Tween20® and 50 µl monoclonal mouse anti-human horseradish peroxidase conjugated Antibodies (life Technologies, total IgG, IgG1, IgG2, IgG3, IgG4) were added (dilution 1:500 in blocking solution). Plates were then incubated for 1.5 hours, then washed as above and 50 µl TMB substrate (Thermo Fisher Scientific 34021) was added. After 15 min incubation at room temperature, reaction was stopped with 50 µl of 0.5 M sulfuric acid (ROTH K027.1) and optical densities were measured at 450 nm with a microplate reader (EL311, BioTex Instruments). To obtain background-corrected optical density values, each peptide-specific signal was corrected using a blank control signal. In case of peptide-specific signals below the background, optical densities were adjusted to “0”.

Bioinformatics and statistical analysis.

BLAST searches were applied as available at the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov/). P-values were calculated by the Wilcoxon/Kruskal-Wallis-Test (Oneway Test, Chi-Square-Approximation) using SAS JMP 13.1 and differences were considered significant at $p < 0.05$. Confidence intervals were calculated by means of Oneway ANOVA. Cutoff values for positive or negative responses were set to the mean antibody responses (OD) plus standard deviation determined in onchocerciasis-free participants. The sensitivity of the peptide-specific ELISAs was calculated with a contingency analysis.

To evaluate the diagnostic performance of the peptides in the IgG3 ELISA, a receiver operating curve (ROC) analysis was performed (Fawcett 2006). Areas under the ROC Curves (AUCs), Standard Errors and 95% Confidence Intervals were calculated using IBM SPSS 25. The optimal cutoff value for each peptide was selected according to the highest Youden’s Index. (Youden 1950) Sensitivity and Specificity were calculated at the selected cutoff values.

Results

Parasite-specific peptides have the potential to improve the immune diagnosis of onchocerciasis. In this study, we describe the stepwise progress of identification and selection of peptides for antibody-based diagnosis of *O. volvulus* infection.

O. volvulus-specific peptides isolated from onchocercomata (nodules).

By means of HPLC, immune-precipitation and tandem mass spectrometry, several MHC-bound class I and II peptide ligands specific for *O. volvulus* were identified in granuloma tissues (nodules) from onchocerciasis patients. This attempt identified 6 MHC-bound *O. volvulus*-specific peptides on antigen-presenting cells from onchocercomata tissues and mass spectrum analysis with reference peptides confirmed their specificity. Two MHC-eluted peptides were identified as parts of the Ov33 antigen (UniProtKB-P21250; positions 54 - 64 and 55 - 65) and one peptide was from the *O. volvulus* P-Glycoprotein (UniProtKB-Q9U8G3; position 1265 - 1277). One peptide originated from onchocystatin (UniProtKB-P22085; position 152 - 162), one peptide derived from the heat shock protein 60 of *Wolbachia sp.* (HSP60) (UniProtKB-P91886; position 473 - 486) and one was identified as part of the NADH-quinone oxidoreductase subunit D of *Wolbachia sp.* (UniProtKB-Q73HJ8; position 199 - 207) (Table 3). After identification, all peptides were synthesized.

Table 3 Identified peptides from Onchocercomata tissues

PEPTIDE ID	SEQUENCE	ORIGIN	POSITION	UNIPROTKB
P1	TVGISKMSI	*Wolbachia* NADH-quinone oxidoreductase subunit D	199-207	Q73HJ8
P2	LRELTTEEQREL	Ov33	55-65	P21250
P3	MRVEILGTKEV	Onchocystatin	152-162	P22085
P4	FLRELTTEEQR	Ov33	54-64	P21250
P5	IKQNNKELIYNVEA	*Wolbachia* HSP60	473-486	P91886
P6	GKYADLIRKQDLS	P-Glycoprotein	1265-1277	Q9U8G3

Peptide-specific IgG1 and IgG3 responses.

The potential of MHC peptide ligands for specific serodiagnosis of patent *O. volvulus* infection was evaluated by means of IgG subclasses-specific ELISA.

IgG1 as well as IgG3 from onchocerciasis patients responded strongly to P1 (*Wolbachia* peptide), while almost no reactivity was detected in controls ($p < 0.0001$).

The sensitivity for both IgG1 and IgG3 to detect patent *O. volvulus* infection was 86%. Both IgG1 and IgG3 in onchocerciasis patients showed significant responses to P2 and P4 (Ov33 peptides) as compared to infection-free controls (both $p < 0.0001$) and sensitivity ranged from 59.4% (P2) to 70.3% (P4) with IgG1, and from 86.3% (P4) to 87.5% (P2) with IgG3. The IgG1 responses to the onchocystatin peptide (P3) were higher in onchocerciasis patients than in controls ($p < 0.0001$) (mean PAT OD = 0.4 vs. OD CTRL = 0.06) and sensitivity was 71.9% (IgG1) and 72.5% (IgG3). The IgG1 and IgG3 reactivity in onchocerciasis patients to the *Wolbachia* HSP60 peptide (P5) was sensitive with 62.5% (IgG1) and 80% (IgG3), and significantly higher in patients ($p < 0.0001$) (Fig. 1). As such, the MHC eluted *Wolbachia*, Ov33, P-Glycoprotein and onchocystatin peptides performed with limited IgG1- and IgG3-mediated sensitivity, not exceeding 90%.

IgG4 responses to the *O. volvulus*-specific peptides.

In order to improve detection of patent *O. volvulus* infection, further bioinformatic and biochemical approaches oriented towards IgG4-mediated peptide recognition were applied. We developed an IgG4-based peptide ELISA and evaluated its sensitivity. In onchocerciasis patients, the IgG4 response to P1 (*Wolbachia* peptide) was significantly stronger that in controls ($p < 0.0032$) and the sensitivity was 87.5% (Table 3). The IgG4 reactivity to the Ov33-peptides (P2, P4) was significantly higher in onchocerciasis patients than in controls ($p < 0.006$) and sensitivity was 83.3% (P2), respectively 87.5% (P4). The onchocystatin peptide (P3) was bound strongly by IgG4 in onchocerciasis patients ($p < 0.0018$) and sensitivity was 95.8%. The *Wolbachia*-specific HSP60 (P5) IgG4 recognition differed significantly in patients and controls ($p < 0.0016$), and sensitivity for patent *O. volvulus* infection was 91.7%. The P-Glycoprotein (P6) peptide-specific IgG4 responses were 87.5% sensitive while *O. volvulus*-negative controls showed no reactivity ($p < 0.0041$) (Fig. 1).

MHC class II haplotype HLA-DBR1 frequency.

To identify naturally presented *O. volvulus*-specific peptide motifs ligated to MHC complexes, the phenotypic distribution of HLA haplotypes in the endemic study population must be determined. The HLA-DRB1*01-*16 haplotypes' distribution was analyzed in 30 onchocerciasis patients and endemic controls from the central region in Togo. The most frequent HLA alleles found were: DRB1*13 (frequency 0.27), DRB1*11 (0.17), DRB1*08 (0.14), DRB1*03 (0.11), DRB1*15 (0.10), DRB1*07 (0.07). The alleles DRB1*01, DRB1*16, DRB1*04, DRB1*09, DRB1*14 had frequencies

below 5%. As such, the HLA allele distribution in the onchocerciasis endemic population and computer-based algorithms (SYFPEITHI) were applied to predict peptide antigens ligated to the MHC molecules.

Prediction of putative peptide ligands by SYFPEITHI.

Several Ov33 and *Wolbachia* peptides were found in onchocercomata tissues. The SYFPEITHI algorithm identified and predicted 5 peptide ligands with a high binding score to HLA-DRB1* alleles (Table 4) within the immunodominant Ov33 protein (UniProtKB-P21250). The peptide S1 is located at the N-terminus (position 17 - 31), and 4 peptides (S2-S5) near the C-terminus of the Ov33 molecule. The computer algorithm (SYFPEITHI) identified within the Wolbachia Surface Protein (WSP) (UniProtKB-Q0RAI4) 4 HLA-DBR1* ligands. The peptides S6 and S7 are situated at the N-terminus (positions 8 - 22 and 15 - 29) and peptides S8 and S9 at central position of the WSP (positions 110 - 124, 145 - 159) (Table 4).

Table 4 Characteristics of SYFPEITHI-predicted peptides from the Ov33 and Wolbachia Surface Protein.

Protein	UniProtKB	Peptide ID	Peptide sequence	position on protein	Predicted HLA
Ov33	P21250	S1	AGVVKRYNKRFAGFN	17-31	DRB1*1501
		S2	VTQLKTFDAKMTAYQ	157-171	DRB1*1501
		S3	DAKMTAYQKYLSSTI	164-178	DRB1*1501
		S4	MTAYQKYLSSTIQKQ	167-181	DRB1*0701
		S5	FNLFADTRTEATSQA	193-207	DRB1*0701
Wolbachia Surface Protein	Q0RAI4	S6	YKKFFSATALVTLLS	8-22	DBR1*1501
		S7	TALVTLLSLSNAAFS	15-29	DBR1*1101
		S8	VIPYVGIGVGAAYLS	145-159	DBR1*1101
		S9	RDTFETAPAPAIADN	110-124	DBR1*0701

Prominent IgG3 responses to the SYFPEITHI predicted Ov33 peptides.

All S1-S5 Ov33 peptides were recognized by IgG3 ($p < 0.0001$) from onchocerciasis patients but not by infection-free controls (Fig. 2). The IgG3 responses to each of the 5 Ov33 peptides were higher in onchocerciasis patients (mean OD = 0.54) than in controls (mean OD = 0.002) and sensitivity was 80% to 97.5%. The IgG4 reactivity to the S1-S5 Ov33 peptides was weak and did not differ between patients and controls (data not shown). To the WSP peptide antigens, onchocerciasis patients' IgG3 responded the strongest, reactivity was significant higher ($p < 0.0001$) than in *O.*

volvulus infection-free controls (Fig. 3) and sensitivity ranged from 72.5% to 87.5%. IgG3 reactivity to WSP peptides were much lower than to the Ov33 peptides. As such, the elution of peptide ligands from MHC complexes as well as the SYFPEITHI algorithm identified Ov33 as an immunodominant antigen.

Antibody isotype profiling reveals strong IgG3 responses.

For further identification of immuno-reactive epitopes, the entire Ov33 protein was synthesized in 24 overlapping peptides, each with 20 amino acids of length. Serological responses to the Ov33-specific 24 peptides were low for IgG2 and IgG4 and similar in onchocerciasis patients and controls. Moderate IgG1 (Table 5, see IgG1 responsiveness) but higher IgG3 responses (Table 5, see IgG3) were observed with clear differences between patients and controls. IgG1 responses to the Ov33 peptides were significantly higher in patients than in controls, but OD values remained low (max. mean OD = 0.9). IgG1 responded 100% sensitive to 5 Ov33-specific peptides (PO14, PO16, PO17, PO23 and PO24) and with over 90% sensitivity to 3 Ov33 peptides (PO5, PO13 and PO15) (Table 5). For IgG3, sensitivity was 100% for 11 overlapping Ov33 peptides (Table 5), and in onchocerciasis patients, IgG3 reactivity exceeded significantly those in controls ($p<0.02$). Next, the peptides with 100% sensitivity for IgG3 were analyzed by means of receiver operating characteristics (ROC) for the calculation of the diagnostic test performance.

Table 5 Overlapping Ov33 peptides. IgG1 and IgG3 antibody responses with a sensitivity above 90% for accurate detection of patent O. volvulus infection to the overlapping peptides of the Ov33 *O. volvulus*-specific antigen are listed. The peptides' amino acid positions in Ov33, the mean antibody reactivity (optical densities (OD)) in onchocerciasis patients (PAT) and controls (CTRL), the OD values' statistical difference between PAT and CTRL, and the sensitivities for correct and false detection (in %) of patent *O. volvulus* infection are indicated

Anti-body response	*Peptide ID*	*Peptide amino acid position*	*Peptide amino acid sequence*	*Mean OD PAT (95% lower/ upper CI)*	*Mean OD CTRL (95% lower/ upper CI)*	*OD Ratio Pat/ CTRL*	*p-value*	*Sensitivity*
IgG1	PO5	041-060	CVVVDNKLFANSFFLRELTT	0.67[0.53;0.81]	0.32[0.09;0.56]	2,09	0,0041	90,9
	PO13	121-140	FCSAGDTTQYYFDGCMVQND	0.41[0.29;0.53]	0.13[-0.08;0.33]	3,15	0,0041	90,9
	PO14	131-150	YFDGCMVQNDKIYVGRAYVR	0.71[0.57;0.84]	0.38[0.15;0.60]	1,87	0,0041	100,0
	PO15	141-160	KIYVGRAYVRDLTPDEVTQL	0.62[0.42;0.81]	0.15[-0.18;0.47]	4,13	0,0041	90,9
	PO16	149-163	VRDLTPDEVTQLKTF	0.26[0.14;0.38]	0.05[-0.14;0.24]	5,20	0,0041	100,0
	PO17	151-170	DLTPDEVTQLKTFDAKMTAY	0.52[0.41;0.64]	0.17[-0,016;0.36]	3,06	0,0040	100,0
	PO23	211-230	ATAGATTTQAPVEAPEPPHF	0.26[0.14;0.38]	0.05[-0.15;0.25]	5,20	0,0041	100,0
	PO24	221-235	PVEAPEPPHFCVAIY	0.31[0.17;0.45]	0.06[-0.17;0.30]	5,17	0,0041	100,0
IgG3	PO1	001-020	MKILFCLLLLAITALEAGVV	0.67[0.54;0.81]	0.17(-0.05;0.40]	3,94	0,0041	90,9
	PO5	041-060	CVVVDNKLFANSFFLRELTT	0.89[0.72;1.05]	0.23[-0.03;0.49]	3,87	0,0041	100,0
	PO7	061-080	EEQRELAQYIEDSNRYKEEV	0.79[0.62;0.97]	0.19[-0.10;0.48]	4,16	0,0041	100,0
	PO8	071-090	EDSNRYKEEVKESLEERRKG	0.85[0.68;1.03]	0.21[-0.08;0.50]	4,05	0,0041	100,0
	PO9	081-100	KESLEERRKGWQLARDGKED	0.87[0.71;1.03]	0.24[-0.03;0.50]	3,63	0,0040	90,9
	PO11	101-120	SKVLSALAEKKLPKPPKKPS	0.97[0.81;1.14]	0.36[0,09;0.63]	2,69	0,0041	90,9
	PO13	121-140	FCSAGDTTQYYFDGCMVQND	0.61[0.44;0.78]	0.07[-0.21;0.35]	8,71	0,0041	100,0
	PO15	141-160	KIYVGRAYVRDLTPDEVTQL	0.79[0.58;1.01]	0.11[-0.24;0.47]	7,18	0,0041	100,0
	PO16	149-163	VRDLTPDEVTQLKTF	0.52[0.34;0.70]	0.02[-0.28;0.32]	26,00	0,0041	100,0
	PO17	151-170	DLTPDEVTQLKTFDAKMTAY	0.70[0.52;0.89]	0.13[-0.18;0.44]	5,38	0,0041	100,0
	PO18	161-180	KTFDAKMTAYQKYLSSTIQK	0.72[0.55;0.89]	0.15[-0.13;0.42]	4,80	0,0041	100,0
	PO20	181-200	QVDSLFGEKSNLFNLFNLFADTR	0.56[0.34;0.77]	0.05[-0.30;0.40]	11,20	0,0040	100,0
	PO22	201-220	TEATSQASDDATAGATTTQA	0.36[0.24;0.47]	0.04[-0.16;0.23]	9,00	0,0040	100,0
	PO24	221-235	PVEAPEPPHFCVAIY	0.48[0.33;0.64]	0.04[-0.21;0.30]	12,00	0,0041	100,0

*Sensitivity Threshold is mean + standard deviation of the cumulative absorbance units of European serum samples. Correct POS= serum sample from onchocerciasis patients, that were detected right-positive. Correct NEG= Serum samples from controls, that were not detected as positive

Evaluation of the diagnostic performance.

To assess the performance of 11 Ov33-specific peptides, showing a 100% sensitivity, an IgG3 ELISA was applied to evaluate serological cross-reactivity with other helminth infections. Sera from *O. volvulus* Mf-negative participants (Table 2, n=68) with intestinal and intravascular helminths as well as intestinal protozoan mono-infections

were evaluated for cross-reactive responsiveness. In addition, dry blood samples from Mf-positive onchocerciasis patients (n=15) were tested, those patients have received repeatedly over several years ivermectin treatment during the annual mass drug administrations. ROC Curves analysis revealed moderate to good diagnostic performances with Areas Under the Curve (AUC) ranging from 0.66 to 0.96 (Table 6). The maximum Youden's Index for each of the Ov33 peptides ranged from 0.32 to 0.94 with specificity from 38% to 94% and sensitivity from 73% to 100% (Table 6). Notably, the peptide PO 13 (Table 5) performed as a good candidate for diagnostic purposes with a sensitivity of 100% and specificity of 94% with a cutoff of OD=0.6.

Table 6 ROC Curve analysis of the Ov33 peptides

PEPTIDE ID	AUC	ASYMPTOTIC 95% CI FOR ROC AUC		MAX YOUDEN'S INDEX	CUTOFF	SENSITIVITY (%)	SPECIFIC (%)
		Lower Bound	Upper Bound				
PO5	0.94	0.89	0.99	0.82	0.35	100	82
PO7	0.90	0.82	0.97	0.73	0.38	87	87
PO8	0.87	0.78	0.96	0.65	0.36	80	85
PO13	0.96	0.92	1.00	0.94	0.60	100	94
PO15	0.79	0.66	0.91	0.56	0.33	80	76
PO16	0.95	0.90	1.00	0.85	0.39	100	85
PO17	0.87	0.80	0.95	0.70	0.34	87	84
PO18	0.66	0.52	0.80	0.32	0.21	93	38
PO20	0.82	0.71	0.94	0.56	0.36	73	82
PO22	0.89	0.80	0.98	0.74	0.39	80	94
PO24	0.95	0.91	0.99	0.82	0.36	100	82

Discussion

In this study, we describe the identification of *O. volvulus*-specific peptide antigens with biochemical, biostatistical and immunological methods, and the evaluation of their diagnostic potential for accurate detection of patent *O. volvulus* infection.

Peptides identified on MHC molecules from onchocercomata tissues.

Using HPLC, immune-precipitation and tandem mass spectrometry, we identified *O. volvulus*-specific MHC-ligands from onchocercomata tissues and the synthesized peptides were found to be antibody reactive. The isolated peptides were parts of the

immunodominant Ov33, P-Glycoprotein, onchocystatin and the *Wolbachia* endosymbiont of *O. volvulus*. Those antigens were previously applied as recombinant antigens for serological diagnosis or vaccination with varied success. In our hands, the *O. volvulus* Ov33 peptide antigens elicited significant IgG3 and IgG4 responses with sensitivities above 83%. Differences in the IgG isotypes' reactivity between the Ov33 peptides could be attributed to minor alterations in the amino acid sequence influencing the antibody binding affinity (Yuan et al. 2012). Ov33 was suggested as an early marker for *O. volvulus* infection and was antibody-reactive in more than 95% of onchocerciasis patient, irrespective of the parasite strain, origin of the patient or the clinical status (Berdoulay et al. 2004; Frank et al. 1998; Hamlin et al. 2012; Hong et al. 1996; Moss et al. 2011). The 33-kDa protein of *O. volvulus* (Ov33) is located in the reproductive organs and muscles of female worms and is released from the adult worms which then will stimulate IgG responses (Lucius et al. 1986; Lucius et al. 1988; Lustigman et al. 1992). Previous studies have shown, that Ov33 protein-reactive antibodies can perform as a highly specific immunodiagnostic test, showing a sensitivity above 97% mainly mediated by IgG4 (Lucius et al. 1992; Nde et al. 2002; Ogunrinade et al. 1993; Tawill et al. 1995). Further *O. volvulus*-specific peptides eluted from MHC molecules originated from the endosymbiotic bacteria *Wolbachia sp.* One peptide was part of the heat shock protein 60 (HSP60) and the second was part of the membrane-bound NADH-quinone oxidoreductase subunit D of the respiratory chain (Yagi 1991). The HSP60 suppresses T cell activation (Shiny et al. 2011) and it evokes IgG1 responses in filarial patients (Suba et al. 2007). The MHC-eluted *Wolbachia* HSP60 peptide was reactive with IgG1 and IgG3 in onchocerciasis patients with moderate sensitivity, and IgG4 responses to the NADH subunit were sensitive above 85%, and those peptides were not evaluated further. We isolated peptides being part of the P-Glycoprotein, which is a well-studied member of the ABC-transporter superfamily (Lespine et al. 2007) and another peptide contained in onchocystatin, which is a cysteine proteinase inhibitor located in larval and adult stages of *O. volvulus* (Lustigman et al. 1992). Both peptides were recognized with 87.5% (P-Glycoprotein) and above 95% (Onchocystatin) by IgG4 from onchocerciasis patients. The prominent IgG4 isotype reactivity could be due to the fact, that the MHC-eluted peptides were located on antigen-presenting cells in onchocercomata tissues. The preferential recognition of those peptides by IgG4 suggests a continuous antigen presentation, which may favor an isotype switch and recognition of linear epitopes by IgG4 (Collins

and Jackson 2013; Kurniawan et al. 1993). In contrast to IgG1 and IgG3, IgG4 interacts poorly with FcγRII and FcγRIII and complement and is considered a “non-inflammatory” immunoglobin isotype, associated with chronic persistence of helminth parasites (Davies et al. 2014). P-Glycoproteins have been associated with resistance to ivermectin (Lespine et al. 2012) and onchocystatin with protective antibody responses that increase with age (Cho-Ngwa et al. 2010; Collins and Jackson 2013). Both molecules are of importance for parasite resistance and persistence and whether the identification and application of such specific peptides may interfere with the parasite-host interplay should find further study. The observed lower sensitivity with IgG1 and IgG3 for the MHC-eluted peptides P1-P6 may be due to our random selection of controls. Controls were returned travelers from the tropics, who may have become exposed and infected with *O. volvulus* and *Wolbachia*. Those serum samples were not excluded from analysis.

Our approach to isolate *O. volvulus*-specific peptide antigens ligated on MHC molecules of antigen-presenting cells has to our knowledge not yet been done; mass spectrometry-based identification of parasite-specific MHC ligands can detect and select those antigens most relevant for immunogenicity and immuno-diagnosis, and peptide-based diagnostic approaches have the advantages of unlimited availability of antigens and moderate costs of production.

Peptide antigen identification on HLA haplotypes using SYFPEITHI algorithm.

We extended our approach to identify peptide antigens based on the HLA allele distribution in the endemic study population. The most frequent HLA-DRB1 alleles were DRB1*13, DRB1*11, DRB1*08, DRB1*03, DRB1*15, DRB1*07, and this distribution has previously been confirmed (Goeury et al. 2018; Meyer et al. 1994). Onchocerciasis patients were classified according to clinical and laboratory findings in generalized or localized onchocerciasis and putative immunes, and distinct haplotypes were found significantly more often with generalized than localized onchocerciasis or putative immunes (Meyer et al. 1994). Population analysis from sub-Saharan Africa confirmed the high occurrence of DRB1*13:04 suggesting a genetic sweep between the DRB1*13 allele and protection to *O. volvulus* (Goeury et al. 2018). The computer-based SYFPEITHI algorithm identified several HLA-DRB1 peptide ligands (Table 4) from the immunodominant *O. volvulus* antigen Ov33. In contrast to the peptides isolated from MHC molecules in onchocercomata, which were largely recognized by IgG4, the most reactive antibody isotype was IgG3 with sensitivities between 80% and

97.5%. IgG3 may bind to Ov33 and activate the complement, but the activation will arrest before the formation of the terminal complexes (Garred et al. 1989; Meri et al. 2002). While 4 of the SYFPEITHI-predicted Ov33 peptides are in the center of the molecules, the MHC-eluted Ov33 peptides are located N-terminal and presentation of peptide antigens situated at the N-terminus may occur preferentially.

The SYFPEITHI algorithm was also applied to predict MHC peptide ligands from the Wolbachia Surface Protein (WSP). *Wolbachia* are intracellular endosymbiotic bacteria in *Wuchereria bancrofti*, *Brugia malayi* and *O. volvulus* (Kozek and Marroquin 1977; Taylor and Hoerauf 1999) and found in all developmental stages most abundant in adult filarial worms. The SYFPEITHI algorithm identified 4 ligands to HLA-DRB1*01-*16 haplotypes and the IgG3 isotype responded strongest, however with moderate sensitivity (>70%). In *B. malayi*- and *W. bancrofti* filariasis patients, WSP was recognized by total IgG, IgG1 and IgG4, respectively (Punkosdy et al. 2003; Shiny et al. 2009) and the observed differences in the IgG isotype reactivity could be due to the use of full length recombinant WSP, while in our works, we applied linear short length peptides.

Overlapping peptides of Ov33.

Aiming to improve sensitivity we analyzed the IgG isotype responses to 24 overlapping peptides, each 20 amino acids in length, spanning the entire Ov33 molecule. Serological responses to the peptides were mainly mediated by IgG3 and 11 Ov33-specific peptides were 100% sensitive. This isotype reactivity contrasted with responses against other *O. volvulus* antigens where IgG4 was the dominant antibody (Lagatie et al. 2017) and further improved total IgG ELISA attained 100% sensitivity with linear epitopes (Lagatie et al. 2018b). The lack of IgG4 responsiveness may be due to the short linear structure of peptides without glycosylation, not processed and not exposed by antigen-presenting cells (Lagatie et al. 2017; Lagatie et al. 2018b). With helminth infections, IgG4 responses may emerge with repetitive antigen stimulation in the presence of persistently active immune modulating molecules required for isotype switching to IgG4 (Maizels and Yazdanbakhsh 2003; van Riet et al. 2007).

Diagnostic performance of peptides.

The significant reduction of skin-dwelling microfilaria of *O. volvulus* following repeated ivermectin mass drug administration has made it difficult to diagnose patent *O. volvulus* infection, as skin biopsies were the “gold standard” procedure. New diagnostic tools

have recently been developed and evaluated, and the Ov16-IgG4-ELISA is recommended for epidemiological surveillance of onchocerciasis (Golden et al. 2016; Richards et al. 2018). Peptide-based diagnostic tests have found use for various infectious diseases with good performances (Alcaro et al. 2003; Gauna et al. 2015; Gomara and Haro 2007; Morre et al. 2002; Noya et al. 2003; Shen et al. 2009; Wu et al. 1999). Linear epitopes, peptide cocktails and recombinant proteins, specific for *O. volvulus*, have been evaluated for their diagnostic potential with considerable success (Gonzalez-Moa et al. 2018; Lagatie et al. 2017; Lagatie et al. 2018a; Lagatie et al. 2018b; Nde et al. 2002; Shey et al. 2018). For monitoring the progress towards elimination of onchocerciasis, and with constantly decreasing microfilaria densities in ivermectin-treated patients, the detection of active or expiring *O. volvulus* infection has become increasingly challenging.

In our works, we have isolated and evaluated several *O. volvulus*-specific peptide antigens which were processed and exposed as MHC-ligands on antigen-presenting cells, contained in onchocerciasis granulomas. Those *O. volvulus*-specific peptides originated from the immunodominant Ov33 protein, P-Glycoprotein, onchocystatin and the Wolbachia endosymbiont, and such peptide repertoire presented on MHC disclosed the breadth of immune recognition. The peptides isolated from MHC and selected for evaluation were serologic reactive with high sensitivity, notably by IgG4. The ROC analysis identified Ov33-derived peptides with IgG3 sensitivities at 100% and specificities above 80%, and multi-peptide-based applications may improve serological diagnosis of low level and expiring *O. volvulus* infection.

Acknowledgments. We thank the laboratory and technical staff from the peptide production facility at the Interfaculty Institute for Cell Biology, Department of Immunology, University Tübingen, the laboratory staff at the Centre Hospitalier Regional (CHR) de Sokodé/Togo. We are thankful for the successful collaboration with professional support from the National Onchocerciasis Control Program (NOCP), Kara/Togo.

Compliance with Ethical Standards.

Conflicts of Interest. The authors declare that they have no conflict of interest.

Ethical approval. Authorization was granted by the Ministry of Health in Togo and ethical approval for study and sample collections was granted by the Comité de Bioethique pour la Récherche en Santé(CBRS), no.04070/2007/MS/CAB/DGS,

no.0129/2011/MS/CAB/DGS/DPLET/CBRS, no.013/2015 /CBRS and no.338/2015/MS/CAB/DGS/DPLET/CBRS).

Informed consent. Informed consent was obtained from all individual participants included in the study

Financial Support This study was supported by the research program of the Federal Ministry of Education and Research Germany (BMBF grant 01KA1008) and the Commission of the European Community FP7 Project EPIAF (Grant Acronym E-PIAF #242131).

References

Alcaro MC, Peroni E, Rovero P, Papini AM (2003) Synthetic peptides in the diagnosis of HIV infection. Curr Protein Pept Sci 4(4):285-90

Berdoulay P, et al. (2004) Comparison of serological tests for the detection of natural heartworm infection in cats. J Am Anim Hosp Assoc 40(5):376-84 doi:10.5326/0400376

Bottomley C, et al. (2016) Modelling Neglected Tropical Diseases diagnostics: the sensitivity of skin snips for Onchocerca volvulus in near elimination and surveillance settings. Parasit Vectors 9(1):343 doi:10.1186/s13071-016-1605-3

Cho-Ngwa F, Liu J, Lustigman S (2010) The Onchocerca volvulus cysteine proteinase inhibitor, Ov-CPI-2, is a target of protective antibody response that increases with age. PLoS Negl Trop Dis 4(8):e800 doi:10.1371/journal.pntd.0000800

Coffeng LE, et al. (2014) African programme for onchocerciasis control 1995-2015: updated health impact estimates based on new disability weights. PLoS Negl Trop Dis 8(6):e2759 doi:10.1371/journal.pntd.0002759

Collins AM, Jackson KJ (2013) A Temporal Model of Human IgE and IgG Antibody Function. Front Immunol 4:235 doi:10.3389/fimmu.2013.00235

DALYs GBD, Collaborators H (2017) Global, regional, and national disability-adjusted life-years (DALYs) for 333 diseases and injuries and healthy life expectancy (HALE) for 195 countries and territories, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet 390(10100):1260-1344 doi:10.1016/S0140-6736(17)32130-X

Davies AM, et al. (2014) Structural determinants of unique properties of human IgG4-Fc. J Mol Biol 426(3):630-44 doi:10.1016/j.jmb.2013.10.039

De Brito RCF, et al. (2018) Peptide Vaccines for Leishmaniasis. Front Immunol 9:1043 doi:10.3389/fimmu.2018.01043

Fawcett T (2006) An introduction to ROC analysis. Pattern Recogn Lett 27(8):861-874 doi:10.1016/j.patrec.2005.10.010

Frank GR, Mondesire RR, Brandt KS, Wisnewski N (1998) Antibody to the Dirofilaria immitis aspartyl protease inhibitor homologue is a diagnostic marker for feline heartworm infections. J Parasitol 84(6):1231-6

Garred P, Michaelsen TE, Aase A (1989) The IgG subclass pattern of complement activation depends on epitope density and antibody and complement concentration. Scand J Immunol 30(3):379-82

Gauna A, et al. (2015) Synthetic peptides for the immunodiagnosis of hepatitis A virus infection. J Immunol Methods 427:1-5 doi:10.1016/j.jim.2015.08.013

Goeury T, et al. (2018) Deciphering the fine nucleotide diversity of full HLA class I and class II genes in a well-documented population from sub-Saharan Africa. HLA 91(1):36-51 doi:10.1111/tan.13180

Golden A, et al. (2016) Analysis of age-dependent trends in Ov16 IgG4 seroprevalence to onchocerciasis. Parasit Vectors 9(1):338 doi:10.1186/s13071-016-1623-1

Gomara MJ, Haro I (2007) Synthetic peptides for the immunodiagnosis of human diseases. Curr Med Chem 14(5):531-46

Gonzalez-Moa MJ, Van Dorst B, Lagatie O, Verheyen A, Stuyver L, Biamonte MA (2018) Proof-of-Concept Rapid Diagnostic Test for Onchocerciasis: Exploring Peptide Biomarkers and the Use of Gold Nanoshells as Reporter Nanoparticles. ACS Infect Dis 4(6):912-917 doi:10.1021/acsinfecdis.8b00031

Hamlin KL, et al. (2012) Longitudinal monitoring of the development of antifilarial antibodies and acquisition of Wuchereria bancrofti in a highly endemic area of Haiti. PLoS Negl Trop Dis 6(12):e1941 doi:10.1371/journal.pntd.0001941

Hong XQ, Santiago Mejia J, Kumar S, Perler FB, Carlow CK (1996) Cloning and expression of DiT33 from Dirofilaria immitis: a specific and early marker of heartworm infection. Parasitology 112 (Pt 3):331-8

Kar PP, Srivastava A (2018) Immuno-informatics Analysis to Identify Novel Vaccine Candidates and Design of a Multi-Epitope Based Vaccine Candidate Against Theileria parasites. Front Immunol 9:2213 doi:10.3389/fimmu.2018.02213

Komlan K, et al. (2018) Onchocerca volvulus infection and serological prevalence, ocular onchocerciasis and parasite transmission in northern and central Togo after

decades of Simulium damnosum s.l. vector control and mass drug administration of ivermectin. PLoS Negl Trop Dis 12(3):e0006312 doi:10.1371/journal.pntd.0006312

Kowalewski DJ, Stevanovic S (2013) Biochemical large-scale identification of MHC class I ligands. Methods Mol Biol 960:145-157 doi:10.1007/978-1-62703-218-6_12

Kozek WJ, Marroquin HF (1977) Intracytoplasmic bacteria in Onchocerca volvulus. Am J Trop Med Hyg 26(4):663-78

Kurniawan A, et al. (1993) Differential expression of IgE and IgG4 specific antibody responses in asymptomatic and chronic human filariasis. J Immunol 150(9):3941-50

Lagatie O, Van Dorst B, Stuyver LJ (2017) Identification of three immunodominant motifs with atypical isotype profile scattered over the Onchocerca volvulus proteome. PLoS Negl Trop Dis 11(1):e0005330 doi:10.1371/journal.pntd.0005330

Lagatie O, et al. (2018a) Evaluation of the Diagnostic Performance of Onchocerca volvulus Linear Epitopes in a Peptide Enzyme-Linked Immunosorbent Assay. Am J Trop Med Hyg 98(3):779-785 doi:10.4269/ajtmh.17-0756

Lagatie O, et al. (2018b) Evaluation of the Diagnostic Performance of Onchocerca volvulus Linear Epitopes in a Peptide Enzyme-Linked Immunosorbent Assay. Am J Trop Med Hyg doi:10.4269/ajtmh.17-0756

Lechner CJ, et al. (2012) Chemokines and cytokines in patients with an occult Onchocerca volvulus infection. Microbes Infect 14(5):438-46 doi:10.1016/j.micinf.2011.12.002

Leddon SA, Sant AJ (2010) Generation of MHC class II-peptide ligands for CD4 T-cell allorecognition of MHC class II molecules. Curr Opin Organ Transplant 15(4):505-11 doi:10.1097/MOT.0b013e32833bfc5c

Lespine A, et al. (2007) Interaction of macrocyclic lactones with P-glycoprotein: structure-affinity relationship. Eur J Pharm Sci 30(1):84-94 doi:10.1016/j.ejps.2006.10.004

Lespine A, Menez C, Bourguinat C, Prichard RK (2012) P-glycoproteins and other multidrug resistance transporters in the pharmacology of anthelmintics: Prospects for reversing transport-dependent anthelmintic resistance. Int J Parasitol Drugs Drug Resist 2:58-75 doi:10.1016/j.ijpddr.2011.10.001

Lucius R, Buttner DW, Kirsten C, Diesfeld HJ (1986) A study on antigen recognition by onchocerciasis patients with different clinical forms of disease. Parasitology 92 (Pt 3):569-80

Lucius R, Erondu N, Kern A, Donelson JE (1988) Molecular cloning of an immunodominant antigen of Onchocerca volvulus. J Exp Med 168(3):1199-204

Lucius R, et al. (1992) Specific and sensitive IgG4 immunodiagnosis of onchocerciasis with a recombinant 33 kD Onchocerca volvulus protein (Ov33). Trop Med Parasitol 43(3):139-45

Lustigman S, Brotman B, Huima T, Prince AM, McKerrow JH (1992) Molecular cloning and characterization of onchocystatin, a cysteine proteinase inhibitor of Onchocerca volvulus. J Biol Chem 267(24):17339-46

Mai CS, et al. (2007) Onchocerca volvulus-specific antibody and cytokine responses in onchocerciasis patients after 16 years of repeated ivermectin therapy. Clin Exp Immunol 147(3):504-12 doi:10.1111/j.1365-2249.2006.03312.x

Maizels RM, Yazdanbakhsh M (2003) Immune regulation by helminth parasites: cellular and molecular mechanisms. Nat Rev Immunol 3(9):733-44 doi:10.1038/nri1183

Meri T, Jokiranta TS, Hellwage J, Bialonski A, Zipfel PF, Meri S (2002) Onchocerca volvulus microfilariae avoid complement attack by direct binding of factor H. J Infect Dis 185(12):1786-93 doi:10.1086/340649

Meyer CG, et al. (1994) HLA-D alleles associated with generalized disease, localized disease, and putative immunity in Onchocerca volvulus infection. Proc Natl Acad Sci U S A 91(16):7515-9

Morre SA, et al. (2002) Comparison of three commercially available peptide-based immunoglobulin G (IgG) and IgA assays to microimmunofluorescence assay for detection of Chlamydia trachomatis antibodies. J Clin Microbiol 40(2):584-7

Moss DM, et al. (2011) Multiplex bead assay for serum samples from children in Haiti enrolled in a drug study for the treatment of lymphatic filariasis. Am J Trop Med Hyg 85(2):229-37 doi:10.4269/ajtmh.2011.11-0029

Mucci J, et al. (2017) Next-generation ELISA diagnostic assay for Chagas Disease based on the combination of short peptidic epitopes. PLoS Negl Trop Dis 11(10):e0005972 doi:10.1371/journal.pntd.0005972

Nde PN, Pogonka T, Bradley JE, Titanji VP, Lucius R (2002) Sensitive and specific serodiagnosis of onchocerciasis with recombinant hybrid proteins. Am J Trop Med Hyg 66(5):566-71

Noya O, Patarroyo ME, Guzman F, Alarcon de Noya B (2003) Immunodiagnosis of parasitic diseases with synthetic peptides. Curr Protein Pept Sci 4(4):299-308

Ogunrinade AF, Chandrashekar R, Eberhard ML, Weil GJ (1993) Preliminary evaluation of recombinant Onchocerca volvulus antigens for serodiagnosis of onchocerciasis. J Clin Microbiol 31(7):1741-5

Olerup O, Zetterquist H (1992) HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. Tissue Antigens 39(5):225-35

Petrone L, et al. (2017) A T-cell diagnostic test for cystic echinococcosis based on Antigen B peptides. Parasite Immunol 39(12) doi:10.1111/pim.12499

Punkosdy GA, Addiss DG, Lammie PJ (2003) Characterization of antibody responses to Wolbachia surface protein in humans with lymphatic filariasis. Infect Immun 71(9):5104-14

Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S (1999) SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics 50(3-4):213-9

Richards FO, et al. (2018) Operational Performance of the Onchocerca volvulus "OEPA" Ov16 ELISA Serological Assay in Mapping, Guiding Decisions to Stop Mass Drug Administration, and Posttreatment Surveillance Surveys. Am J Trop Med Hyg 99(3):749-752 doi:10.4269/ajtmh.18-0341

Shen G, Behera D, Bhalla M, Nadas A, Laal S (2009) Peptide-based antibody detection for tuberculosis diagnosis. Clin Vaccine Immunol 16(1):49-54 doi:10.1128/CVI.00334-08

Shey RA, et al. (2018) Prediction and validation of the structural features of Ov58GPCR, an immunogenic determinant of Onchocerca volvulus. PLoS One 13(9):e0202915 doi:10.1371/journal.pone.0202915

Shiny C, Krushna NS, Archana B, Farzana B, Narayanan RB (2009) Serum antibody responses to Wolbachia surface protein in patients with human lymphatic filariasis. Microbiol Immunol 53(12):685-93 doi:10.1111/j.1348-0421.2009.00172.x

Shiny C, Krushna NS, Babu S, Elango S, Manokaran G, Narayanan RB (2011) Recombinant Wolbachia heat shock protein 60 (HSP60) mediated immune responses in patients with lymphatic filariasis. Microbes Infect 13(14-15):1221-31 doi:10.1016/j.micinf.2011.07.004

Stern LJ, Santambrogio L (2016) The melting pot of the MHC II peptidome. Curr Opin Immunol 40:70-7 doi:10.1016/j.coi.2016.03.004

Suba N, Shiny C, Taylor MJ, Narayanan RB (2007) Brugia malayi Wolbachia hsp60 IgG antibody and isotype reactivity in different clinical groups infected or exposed to human bancroftian lymphatic filariasis. Exp Parasitol 116(3):291-5 doi:10.1016/j.exppara.2006.12.006

Tawill SA, Kipp W, Lucius R, Gallin M, Erttmann KD, Buttner DW (1995) Immunodiagnostic studies on Onchocerca volvulus and Mansonella perstans infections using a recombinant 33 kDa O. volvulus protein (Ov33). Trans R Soc Trop Med Hyg 89(1):51-4

Taylor MJ, Hoerauf A (1999) Wolbachia bacteria of filarial nematodes. Parasitol Today 15(11):437-42

van Riet E, Hartgers FC, Yazdanbakhsh M (2007) Chronic helminth infections induce immunomodulation: consequences and mechanisms. Immunobiology 212(6):475-90 doi:10.1016/j.imbio.2007.03.009

Vlaminck J, Fischer PU, Weil GJ (2015) Diagnostic Tools for Onchocerciasis Elimination Programs. Trends Parasitol 31(11):571-582 doi:10.1016/j.pt.2015.06.007

WHO (2015) Investing to overcome the global impact of neglected tropical diseases : Third WHO report on neglected tropical diseases. World Health Organization, Geneva

WHO (2016a) Guidelines for Stopping Mass Drug Administration and Verifying Elimination of Human Onchocerciasis: Criteria and Procedures. World Health Organization, Geneva

WHO (2016b) Progress report on the elimination of human onchocerciasis, 2015-2016. Wkly Epidemiol Rec 91(43):505-14

Wu CL, Leu TS, Chang TT, Shiau AL (1999) Hepatitis C virus core protein fused to hepatitis B virus core antigen for serological diagnosis of both hepatitis C and hepatitis B infections by ELISA. J Med Virol 57(2):104-10

Yagi T (1991) Bacterial NADH-quinone oxidoreductases. J Bioenerg Biomembr 23(2):211-25

Yoshida CF, et al. (1992) Human antibodies to dengue and yellow fever do not react in diagnostic assays for hepatitis C virus. Braz J Med Biol Res 25(11):1131-5

Youden WJ (1950) Index for rating diagnostic tests. Cancer 3(1):32-5

Yuan HC, et al. (2012) Mapping of IgE and IgG4 antibody-binding epitopes in Cyn d 1, the major allergen of Bermuda grass pollen. Int Arch Allergy Immunol 157(2):125-35 doi:10.1159/000327544

Inflammatory and regulatory CCL and CXCL chemokine and cytokine cellular responses in patients with patent *Mansonella perstans* filariasis

Wangala B[§], Gantin RG[§,$], Voßberg PS[§,$], Vovor A[&], Poutouli WP[#], Komlan K[§], Banla M[§,€], Köhler C[$,1], Soboslay PT[§,$,1 *]

[§] National Institute of Hygiene, Onchocerciasis Reference Laboratory, Sokodé, Togo

[$] Institute for Tropical Medicine, University Clinics of Tübingen, Tübingen, Germany

[&] Centre Hospitalier Universitaire Sylvanus Olympio, Laboratory for Hematology, Université de Lomé, Togo

[#] Faculté de Sciences, Université de Lomé, Lomé, Togo

[€] Centre Hospitalier Universitaire Campus, Université de Lomé, Togo

[1] Both senior authors contributed equally to this work.

Clin Exp Immunol. 2019 Apr;196(1):111-122. doi: 10.1111/cei.13251

ABSTRACT

Mansonella perstans (Mp) filariasis is present in large populations in sub-Saharan Africa and to which extent patent Mp infection modulates the expression of immunity in patients, notably their cellular cytokine and chemokine response profile, remains not well known. We studied in mansonelliasis patients (Mp-PAT) and mansonelliasis-free controls (CTRL) the spontaneous and the inducible cellular production of chemokines CXCL9(MIG), CXCL10(IP10), CCL24(Eotaxin-2), CCL22(MDC), CCL13(MCP4), CCL18(PARC), CCL17(TARC) and IL-27. Freshly isolated peripheral mononuclear blood cells (PBMC) were stimulated with helminth, protozoan and bacterial antigens (Ag) and mitogen(PHA). PBMC from Mp-PAT produced spontaneously (without Ag stimulation) significantly higher levels of Eotaxin-2, IL-27, IL-8, MCP4 and MDC than cells from CTRL while IP10 was lower in Mp-PAT. Helminth antigens activated IL-27 and MCP4 only in CTRL, while AscarisAg, OnchocercaAg, SchistosomaAg, EntamoebaAg, StreptococcusAg, MycobacteriaAg and PHA stimulated MIG release in CTRL and Mp-PAT. Notably, EntamoebaAg and PHA strongly depressed (P<0.0001) Eotaxin2(CCL24) production in both study groups. Multiple regression analyses disclosed in Mp-PAT and CTRL dissimilar cellular chemokine and cytokine production levels being higher in MpPAT for CCL24, IL-27, IL-8, MCP4, MDC and PARC (for all P<0.0001), at baseline (P <0.0001), in response to EhAg (P<0.0001), OvAg (P=0.005), PHA (P<0.0001) and PPD (P<0.0001) stimulation. In Mp-PAT with hookworm co-infection, the cellular chemokine production of CXCL10 (IP-10) was diminished. In summary, the chemokine and cytokine responses in MpPAT were in general not depressed, PBMC from Mp-PAT produced spontaneously and selectively inducible inflammatory and regulatory chemokines and cytokines at higher levels than CTRL and such diverse and distinctive reactivity supports that patent *M. perstans* infection will not polarize innate and adaptive cellular immune responsiveness in patients.

INTRODUCTION

Mansonelliasis is caused by four species of nematodes belonging to the genus *Mansonella, i.e. M. perstans*, *M. streptocerca*, *M. ozzardi*, and *M. rodhaini*, three of which are endemic in Africa [1]. *M. perstans* is considered to be the most frequent in sub-Saharan Africa; more than 100 million people may be infected [1,2]. The transmission of *M. perstans* is through the bite of blood-feeding *Culicoides* midges (Diptera: Ceratopogonidae). Adult *M. perstans* filariae are described in the connective

tissue of the serous body cavities and the unsheathed microfilariae (Mf) circulate in the peripheral blood [2]. The longevity of adult *M. perstans* in humans is unknown, but the Mf may persist for several months [2,3]. *M. perstans* is considered to be of little pathogenicity, and although often asymptomatic, infections may cause eosinophilia, subcutaneous swellings, aches, pains and skin rashes in a considerable proportion of patients [2]. The lack of specificity of symptoms might be explained by a thoroughly modulated immune response which has develop and adapted to chronic infection and repeated re-infection in an endemic environment, but symptoms may also be caused by co-infection with other filariae [2,3].

The control of lymphatic filariasis is based on annual mass drug administration (MDA) of ivermectin together with albendazole, which will clear blood circulating microfilaria, and such intervention may also interrupt parasite transmission [4]. Mansonelliasis is often coendemic with onchocerciasis [5,6] and lymphatic filariasis [7,8,9], and the Onchocerciasis Control Programs in Africa (OCP, 1974–2002; APOC, 1995–2015) have largely controlled onchocerciasis disease burden through the MDA of ivermectin [10,11]. With the implementation MDA of ivermectin for decades large populations became permanently negative for microfilaria (Mf) of *O. volvulus*, but ivermectin alone will not clear Mf of *M. perstans* and chronic mansonelliasis will persist [2,5,7,12].

Ivermectin treatment will influence the parasite-host equilibrium and change the immune response profile in patients. In ivermectin-treated onchocerciasis patients, Th1-type cytokines will re-activate while regulatory and Th2-type-promoting cytokines and chemokines lessened [13,14,15]. Such changes may reflect decreasing eosinophil granulocyte activation against Mf [15,16], and in parallel, lower Plasmodium-specific Th17 immune responses [17]. De-worming will alleviate the helminth-induced cellular hyporesponsiveness, repeated anti-helminth treatment resulted in significant increases in proinflammatory cytokine responses to *Plasmodium falciparum* antigens and mitogen with a significant decline in the expression of the inhibitory molecule CTLA-4 on $CD4^+$ T cells of treated individuals [18]. To which extent persistent *M. perstans* infection may influence or bias cellular reactivity and the immune response profile in patients remains not well known. We studied the *in vitro* cellular responsiveness of mononuclear peripheral blood cells from mansonelliasis patients to helminth, protozoan and bacterial antigen stimulation and observed that significant pro-inflammatory chemokines and cytokines were produced.

MATERIALS AND METHODS

Location of study, participants and examinations

This study was conducted in central Togo in West Africa, within the previously vectorcontrolled area of the former Onchocerciasis Control Program (OCP) where annually repeated mass drug administration (MDA) by community directed treatment with ivermectin (CDTI) is being applied since 1989. The mansonelliasis patients and endemic controls were from the Prefecture Tchaoudjo in the Central Region of Togo and permanent residents in rural villages. This investigation was authorized by the Ministry of Health in Togo (No.0407/2007MS/CAB/DGS; No.0060/2013/MS/CAB/DGS) and the Comité de Bioéthique pour la Recherche en Santé (No.013/2015/CBRS). All participants gave their written informed consent, and for correct and complete understanding explanations were always given in the local language. At the time of sampling, participants were healthy male and female (non-pregnant) individuals that permanently resided in the villages of Bouzalo (N09°06.134'; E001°02.588') and Sagbadai (N09°03.819'; E001°04.473'). All participants received annually by means of mass drug administration through the community-directed treatment with ivermectin (CDTI; 150 ug/kg) a single dose distributed by the National Onchocerciasis Control Program (NOCP) in Togo. From all participants venous blood samples (18 ml), skin biopsies and stool and urine samples were collected, fresh stools were examined by microscopy for helminth and protozoa infections and then the samples examined by the Kato–Katz methodology (helm-TEST; Labmaster, Belo Horizonte, MG, Brazil). From each participant 10 ml of urine were centrifuged and the sediment examined under a microscope for eggs of *Schistosoma haematobium*.

Microscopy examination for blood-dwelling *Mansonella perstans* microfilariae

Microfilariae of *M. perstans* were detected after Biocoll-Gradient-Centrifugation (Biochrom, Berlin, Germany) of 20 ml of whole blood samples (diluted 1:2 in RPMI) in the peripheral blood mononuclear cells (PBMC) fractions and in the polymorph nuclear cell pellets (PMNC)[19]. The PMNC pellets were re-suspended in PBS (part 1), mixed with an equal volume of 5% Dextran 500 in PBS (part 2) and an equal volume of PBS (part 3) was added. Such re-suspended PMNC were let sediment at 1g for 1 hour at room temperature (RT), thereafter the supernatant collected, centrifuged at 800g for 15 min at RT and the pellets examined by microscopy for the presence of microfilaria of *M. perstans.* The isolated PBMC were dispensed in equal volumes into 24-well cell

culture plates, and after overnight incubation plate wells were examined under an inverted microscope for the presence of dwelling microfilariae of *M. perstans*.

Real-time polymerase chain reaction (PCR) for the detection of *M. perstans* DNA in whole blood samples

For DNA extraction, whole venous blood samples (200 µl) were collected into microcentrifuge tubes and processed using the Qiagen DNA Investigator kit (Qiagen, Hilden, Germany) according to the recommended manufacturer's protocol. After overnight proteinase K digestion at 56°C, three DNA elutions of 50 µl each were performed. The eluted DNA concentrations were determined and samples stored at -20°C before rt-PCR analysis. Extracted blood DNA concentrations ranged from 4 ng/µl to 166 ng/µl. As previously applied [6], for the detection *of M. perstans* DNA the real-time quantitative PCR (qPCR) was carried out with the PCR cycler rotor gene RG 3000 (Corbett Research/Corbett Life Science, QIAGEN NV, Netherlands). The primer and probe sequences selection was carried out using the online software Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). The applied qPCR reaction was to detect the *Mansonella perstans* 18S and 5.8S ribosomal RNA gene, internal transcribed spacer 1 (GenBank: KJ631373). The qPCR primer pairs, probes and test conditions used for the detection of *M. perstans* (Mp) were: Mp-primer-fwd 5'-CTGCGGAAGGATCATTAA-3' (Tm 51.4°C); Mp-primer rev 5'-TGCATGTTGCTAAATAAAAGTG-3' (Tm 52.8 °C); Mp-probe 5'-FAM-CGAGCTTCCAAACAAATACATAATAAC-TAM-3' (Tm 58.9°C); The rtPCR conditions were 50°C/2 Min, 95°C/10 Min, [95°C/15 Sec, 53°C/1 Min] x 45 cycles.

Preparation of antigens

Schistosoma mansoni adult worms were isolated aseptically by perfusion from portal veins of infested mice; adult *Ascaris lumbricoides* were collected as expulsed worms from patients who received a 3-day treatment of mebendazole, and the collected worms were extensively washed in sterile PBS (phosphate-buffered saline, pH 7.6). *Entamoeba histolytica* trophozoites (axenic strain HB3) were a kind gift of Dr. B. Walderich (Tübingen, Germany). Adult filarial worms of *O. volvulus* were isolated from nodules (onchocercomata) as described by Schulz-Key et al. 1977 [20]. Adult *A. lumbricoides*, *S. mansoni* and *O. volvulus* were extensively washed in sterile PBS (Phosphate-buffered saline, pH 7.6), transferred into a Ten-Broek tissue grinder and then homogenized on ice. Similarly, *E. histolytica* trophozoites were grinded and homogenized. The homogenates were then sonicated twice (30% intensity, pulse 1 s)

for 10 min on ice, centrifuged at 16000 g for 30 min at 4 °C. The supernatants were sterile filtered (0.22 µm) and the protein concentration of each antigen was determined by the BCA method (Pierce). The limulus amebocyte lysate assay (E-Toxate Kit; Sigma Aldrich; ET0100) was used to detect endotoxin in the worm and protozoa antigen extracts. The endotoxin levels were at 0.25 EU/ml in the *Entamoeba histolytica* and *Schistosoma mansoni* extracts and at 1.25 EU/ml in the *Ascaris lumbricoides* and *Onchocerca volvulus* antigens. Purified Protein Derivative (PPD) from *Mycobacterium tuberculosis* was purchased from Behring (Marburg, Germany), and Streptolysin-O (SL-O) from *Streptococcus pyogenes* was obtained from DIFCO (Augsburg, Germany).

Isolation of PBMC, cell culture experiments and determination of cytokine production

Heparinized venous blood was collected from mansonelliasis patients and endemic controls, and PBMC were isolated and cell culture experiments were conducted as described previously [14,19]. Briefly, PBMC were adjusted to $1x10^7$/ml in RPMI supplemented with 25 mM HEPES buffer, 100 U/ml penicillin and 100 mg/ml streptomycin, 0·25 mg/ml amphotericin B. Freshly isolated PBMC were cultured at a concentration of $2.5x10^6$ PBMC/ml in RPMI (as above) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biochrom, Berlin, Germany) in the presence of either *Ascaris lumbricoides* adult worm extract (AscAg; 5 µg/ml), *Entamoeba histolytica* strain HM1 antigen (EhAg; 10 µg/ml), *Onchocerca volvulus* adult worm-derived antigen (OvAg, 35 □g/ml), *Mycobacterium tuberculosis* purified protein derivative (PPD, 100 □g/ml), phytohaemagglutinin (PHA) (1:100, Sigma, St.Louis, Missouri, USA), *Schistosoma mansoni* adult worm extract (SmAg; 10 µg/ml), or *Streptococcus pyogenes* derived Streptolysin-O (SL-O, 1:50; Difco, Augsburg, Germany) in 5% CO_2 at 37°C and saturated humidity. Cell culture supernatants were collected after 48 h and stored below –20°C until further use. Cytokine secretion by stimulated PBMC was quantified by sandwich ELISA using cytokine- and chemokine–specific monoclonal and polyclonal antibodies as recommended by the manufacturers. The detection limits of the cytokine and chemokine ELISAs (DuoSet, R&D Systems, Minneapolis, USA) were at 50pg/ml; all concentration values below that threshold were set to 0 pg/ml.

O. volvulus antigen-specific enzyme-linked immunosorbent assay (ELISA)

O. *volvulus* antigen-specific (OvAg) IgG4 isotype reactivity was determined by ELISA as described by Mai *et al.*[14,19]. Briefly, microtiter plates (Corning 3690; Costar, Assay Plate) were coated with *O.volvulus* adult worm extract (OvAg 5 g/ml) in PBS. Non-specific binding capacity was blocked at room temperature (RT) for 2 hours with PBS containing 5% fetal bovine serum (FBS). Samples and reference control sera were added to OvAg-coated wells and incubated for 2 hours at RT. After washing with PBS containing 0.05% Tween 20 (Sigma, P-3563, St. Louis, MO, USA), a horseradish peroxidase conjugated mouse anti-human IgG4 monoclonal antibody (Invitrogen; Eugene, Oregon, USA) at a dilution of 1:500 was added for 2 hours at RT. After washing as above specific binding was visualized by addition of TMB substrate, reactions were stopped by addition of 0.5M H_2SO_4 and the optical density was determined at 450nm.

Data analysis

JMP software (versions 11.1.1.; SAS Institute) was used for statistical analysis of data. Because of multiple comparisons, the level of significance was adjusted according to Bonferroni–Holm. For the cytokine and chemokine analyses, differences between groups were determined after logarithmic transformation to stabilize the variance of data (log [pg/ml + 1]). The application of the Bonferroni Holm adjustment resulted in an alpha level of *alpha*=0.003. The data from the patient and control group were compared using Wilcoxon's rank sum test (Mann–Whitney *U* test). Multiple regression analysis was applied to analyze the chemokine and cytokine production in mansonelliasis patients and controls with and without hookworm co-infection. The cytokine production was analyzed with the predictors: study groups, cytokine, chemokine, antigen stimulations, mitogen stimulation, patient number * study groups (i.e. the random factor is patients' number) and their corresponding interaction of degree 2. For post hoc testing and for comparison of the different groups with and without hookworm co-infection the Tukey Kramer Test was applied. For multivariate analysis, epidemiological (gender, age) and immune parameters (cytokines, chemokines, antigen and mitogen stimulations) were added as covariates and compared with one another. Comparisons were made between mansonelliasis patients and controls with and without hookworm co-infection.

RESULTS

Study groups and patients' characteristics

The demographic, hematological and parasitology data for the study groups are shown in Table 1. All participants (n=50) were treated annually with 150 □g/kg ivermectin since more than 15 years and were negative for microfilariae of *O. volvulus* at repeated skin biopsy examinations (Table 1). Antibody responses (IgG4) to *O. volvulus* antigen (OvAg) were similarly low in Mp-PAT and in mansonelliasis-free controls (Table 1). *Mansonella perstans* microfilariae and *M. perstans* DNA were diagnosed in 37 participants (Mp-PAT) and none in endemic controls (n=13; CTRL). Hookworm larvae were detected in stool samples from MpPAT (POS: n=13; NEG: n=24) and also in endemic controls (POS: n=3; NEG: n=10). Mp-PAT presented with lower lymphocyte but higher eosinophil granulocyte counts (P = 0·016) than CTRL.

Table 1. Demographics, hematological and parasitology data (median;[min;max]) of the *Mansonella perstans* microfilariae(Mf)-positive patients and *M. perstans* Mf-negative endemic controls, their leucocytes counts and blood cell differential. The spontaneous cellular production of chemokines and cytokine by PBMC from patients with patent *M. perstans* infection and mansonelliasis-free controls. The spontaneous production, i.e. without antigen or mitogen stimulation, was elevated in mansonelliasis patients (Mp-PATs) (Fig. 1 and Fig. 2, "1Baseline"). PBMC from Mp-PAT, as compared to CTRL, released spontaneously significantly higher amounts of CCL24/Eotaxin (*P* = 0.002), IL-27 (*P* = 0·002), IL-8 (*P* = 0·003), MCP4 (*P* = 0·009) and MDC (*P* = 0·028) (Fig. 1 and Fig. 2). In contrast, less IP-10/CXCL9 (*P* = 0·002) was secreted spontaneously by PBMC from Mp-PAT than by cells from CTRL (Fig.2, "1Baseline").

	Mansonelliasis	**Mansonelliasis-free**
	PATIENTS (n=37)	**Endemic CONTROLS (n=13)**
Age [min;max	49** [25; 75]	36 [21; 54]
Gender [F/M]	12/25	3/10
Hemoglobulin [g/dl]	16·0 [10·8; 23·7]	15·3 [11·5; 17·7]
Leucocytes [cells/ul]	5259 [3600; 8100]	5443 [4100; 8100]
Neutrophil Granulocytes	34·6% [27; 52]	35·8% [27; 52]
Eosinophil Granulocytes	1·6%* [0; 6]	0·8% [0; 2]
Basophil Granulocytes	0%	0%
Lymphocytes	61·8% [46; 84]	61·4% [48; 26]
Monocytes	2% [1; 4]	2% [2; 2]
M. perstans qPCR	Mp-Positive	Mp-Negative
ct-value median [min; max]	36·5 [29·6; 42·7]	[none]
O. volvulus Mf / skin biospy	0	0
IgG_4 OvAg-ELISA OD median [min; max]	0·232 [0; 0·52]	0·176 [0; 0·398]
P. falciparum qPCR	Pf-positive n=26	Pf-positive n=6
ct-value median [min; max]	33·4 [24·6; 38·6]	37·2 [34·5; 39·8]
Hookworm	positive n=13	positive n=3
eggs/g stool median [min;max]	792 [144; 9264]	552 [72; 4728]
E. histolytica / E. dispar (cysts)	positive n=12	positive n=4

Wilcoxon rank sum test: PAT vs CTRL * *P* = 0·016; ** *P* = 0·001

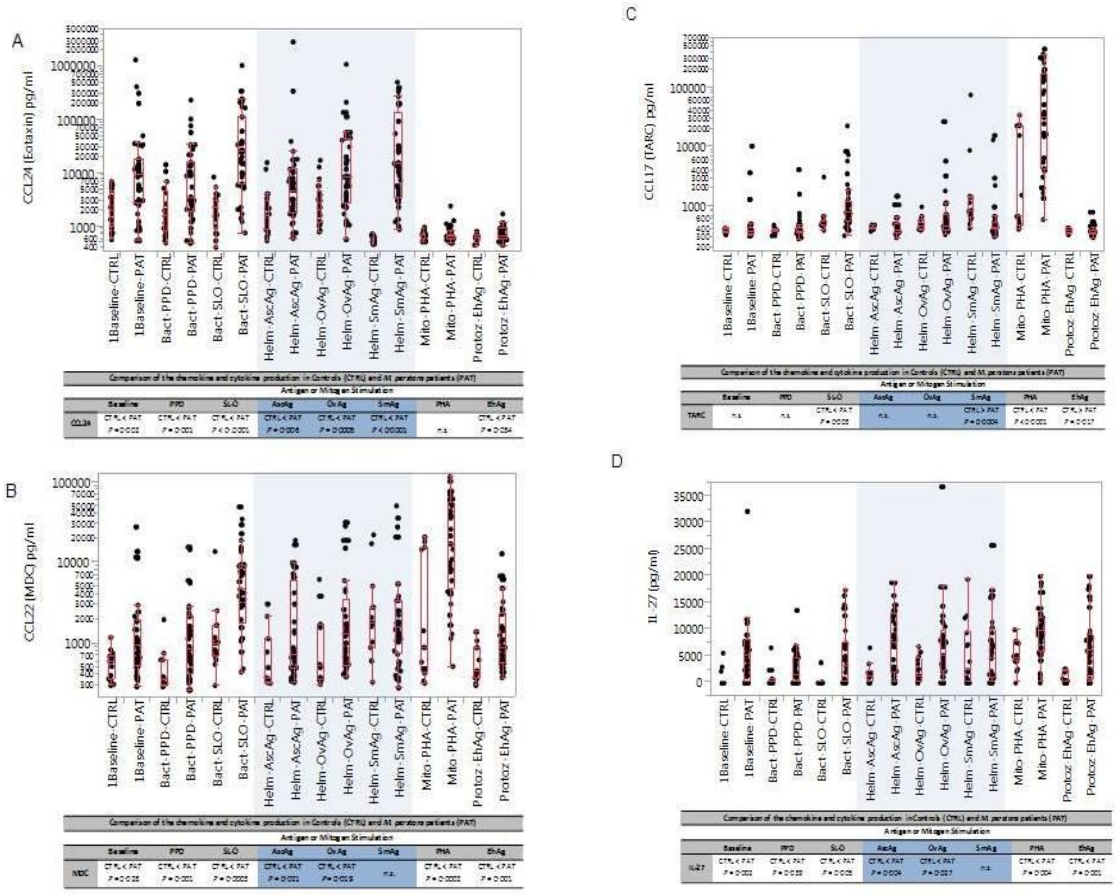


Fig. 1. The cellular production of chemokines Eotaxin (CCL24), MDC (CCL22), TARC (CCL17) and the cytokine IL-27 by PBMC from mansonnelliasis patients (PAT, n=37) and by PBMC from mansonelliasis-free controls (CTRL, n=13). Freshly isolated PBMC were either without stimulation with antigen or mitogen (Baseline) or PBMC were stimulated with helminth antigens from *Ascaris lumbricoides* adult worm extract (AscAg; 5 µg/ml), *Onchocerca volvulus* adult worm-antigen (OvAg, 35 □g/ml) or *Schistosoma mansoni* adult worm extract (SmAg; 10 □g/ml), or stimulated with *Entamoeba histolytica* strain HM1 antigen (EhAg; 10 µg/ml), *Mycobacterium tuberculosis* purified protein derivative (PPD, 100 □g/ml), the mitogen phytohaemagglutinin (PHA) (1: 100, Sigma) or *Streptococcus pyogenes* derived Streptolysin-O (SL-O, 1 : 50). Cell culture supernatants were collected after 48 hours and chemokine and cytokine secretion was quantified by specific ELISA (R&D Systems). The amounts of chemokines and cytokine released into cell culture supernatants are shown as box blots with the median and the 25% and 75% quartiles, the 1.5x of the interquantile range and with all outliers as individual points. The cellular production (pg/ml) of chemokines and cytokine (i.e. without baseline subtraction) in CTRL and PAT was compared using Wilcoxon's rank sum test, and significantly different production levels between CTRL and PAT groups are indicated with $P \leq 0{\cdot}05$. The level of significance was adjusted according to Bonferroni-Holm (*alpha* = 0.003). Significant differences between controls (CTRL) and patients (PAT) are indicated with *P* values and: CTRL > PAT = chemokine or cytokine production greater in controls than patients; CTRL < PAT = chemokine or cytokine production greater in patients than controls; n.s. = not significant

The cellular production of Th2-type chemokines CCL24(Eotaxin-2), CCL22(MDC) and CCL17(TARC)

The spontaneous cellular production of CCL24(Eotaxin-2) was significantly higher in Mp-PAT than in endemic controls (P =0.002) (Fig. 1, Part A). Stimulation *in vitro* of PBMC from MpPAT with helminth antigens (AscAg, OvAg, SmAg) did not induce a CCL24 production above baseline levels, but noteworthy, *Entamoeba histolytica* antigen (EhAg) and the mitogen (PHA) depressed CCL24 release in CTRLS and Mp-PAT drastically (P < 0·0001). The *Schistosoma mansoni* adult worm antigen (SmAg) depressed CCL24 in CTRLs (P < 0·0001) but had no such effect in PAT (Fig. 1, Part A). The chemokine CCL22(MDC) was inducible above baseline only in PBMC from CTRLs and PAT in response to the mitogen PHA and antigen SLO (Fig. 1, Part B). In mansonelliasis patients the MDC production in responses to PHA, PPD and SLO was above the levels measured in cell cultures supernatants from PAT (Fig. 1, Part B). The cellular production of CCL17/TARC (Fig. 1, Part C) was similar in Mp-PAT and CTRL; neither the helminth antigens (AscAg, OvAg), *Entamoeba histolytica* extract (EhAg) nor bacterial PPD and SLO activated a cellular production of CCL17/TARC above the spontaneous release (Fig. 1, Part C). Following PBMC stimulation with *Schistosoma mansoni* adult worm antigen (SmAg) more CCL17/TARC was produced in CTRL than in Mp-PAT (P = 0·0004) while PHA induced higher levels of CCL17/TARC (P < 0·0001) and CCL18/PARC (P < 0·0001) in Mp-PAT than in CTRL.

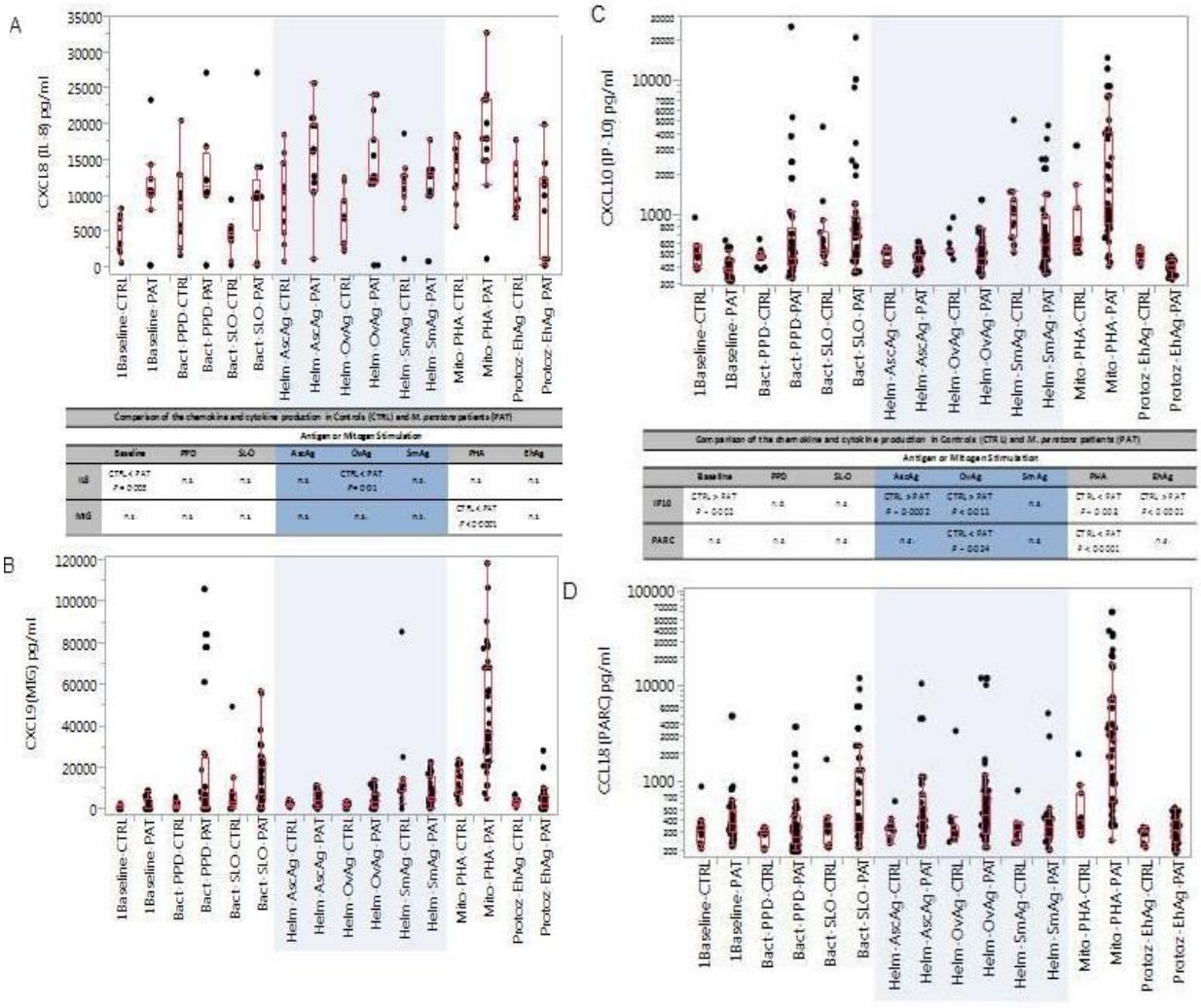
The cellular production of regulatory interleukin 27 (IL-27) and chemokine CCL18(PARC)

The spontaneous release of IL-27 was higher in Mp-patients than CTRL (Fig. 1). In CTRL, the A*scaris (AscAg)*, E*ntamoeba* (EhAg), bacterial Streptolysin O (SLO) and mitogen PHA activated PBMC responses remained below the IL-27 amounts released by cells from mansonnelliasis patients (Fig. 1, Part D). The cellular production of CCL18/PARC was similar in Mp-PAT and CTRL and neither the helminth antigens (AscAg, OvAg), *Entamoeba histolytica* extract (EhAg) nor bacterial PPD and SLO activated a cellular production of CCL18/PARC above the spontaneous release (Fig. 2, Part D).

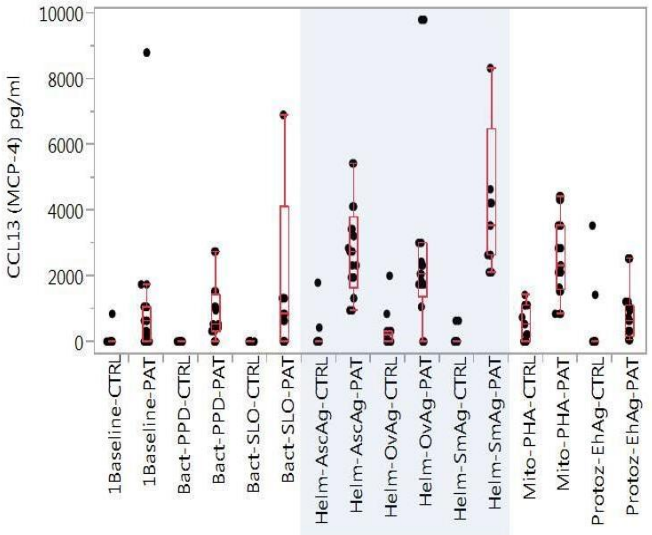
The cellular production of pro-inflammatory chemokines CXCL8(IL-8), CXCL9(MIG) and CXCL10(IP-10)

The amounts of CXCL8(IL-8) (Fig. 2, Part A) produced by PBMC in response to mitogen PHA, bacteria-derived SLO and PPD, extracts from *Entamoeba*-Ag and

helminth-specific *Ascaris*Ag and *Schistosoma*-Ag were higher in Mp-PAT than in CTRL but without significant differences. In Mp-PAT the cellular release of CXCL8/IL-8 in response to *Onchocerca volvulus*-specific antigen (OvAg: 13935 pg/ml) was more than twice as high as in CTRL (mean 6526 pg/ml)(Fig. 2, Part A). The production of CXCL9 (monokine inducible by interferon gamma; MIG) was inducible above baseline levels by helminth (AscAg, OvAg, SmAg), bacterial antigens (SLO, PPD) and the mitogen PHA (Fig.2, Part B). MIG responsiveness was similar in CTRL and Mp-PAT and only PHA induced in PAT higher MIG responses than in CTRL (Fig.2, Part B). The chemokine CXCL10 (interferon inducible protein 10, IP-10) (Fig. 2, Part C) was produced in higher amounts in CTRL than in Mp-PAT when PBMC were stimulated with helminth antigens AscAg (P<0.001), OvAg (P<0.01), SmAg (P<0.006), and also in response to the *Entamoeba histolytica* extract (EhAg; P<0.0001). When PBMC were activated with the mitogen PHA secretion of CXCL10 was higher in Mp-PATs than CTRLs (P<0.003), while the extracts from *Streptococcus pyogenes* (SLO) and *Mycobacterium tuberculosis* (PPD) activated similar IP10/CXCL10 amounts in both groups. Multiple regression analyses disclosed in Mp-PAT and CTRL dissimilar cellular chemokine and cytokine production levels being higher in Mp-PAT for CCL24, IL-27, IL-8, MCP4, MDC and PARC (for all P < 0·0001), at baseline (P < 0·0001), in response to EhAg (P < 0·0001), OvAg (P = 0·005), PHA (P < 0·0001) and PPD (P < 0·0001) stimulation.



E



Comparison of the chemokine and cytokine production in Controls (CTRL) and *M. perstans* patients (PAT)

	Antigen or Mitogen Stimulation							
	Baseline	PPD	SLO	AscAg	OvAg	SmAg	PHA	EhAg
MCP4	CTRL < PAT P = 0·009	CTRL < PAT P = 0·0004	CTRL < PAT P = 0·005	CTRL < PAT P = 0·0002	CTRL < PAT P = 0·003	CTRL < PAT P = 0·0001	CTRL < PAT P = 0·0004	CTRL < PAT P = 0·015

Fig. 2. The cellular production of the chemokines CXCL8(IL-8), MIG (CXCL9), IP10 (CXCL10), PARC (CCL18) and (CCL13) MCP4 by PBMC from mansonnelliasis patients (PAT, n=37) and by PBMC from infection-free endemic controls (CTRL, n=13). Freshly isolated PBMC were without antigen and without mitogen stimulation (Baseline). PBMC were stimulated with helminth antigens from *Ascaris lumbricoides* adult worm extract (AscAg; 5 µg/ml), *Onchocerca volvulus* adult worm-antigen (OvAg, 35 □g/ml) or *Schistosoma mansoni* adult worm extract (SmAg; 10 □g/ml), or with *Entamoeba histolytica* strain HM1 antigen (EhAg; 10 µg/ml), *Mycobacterium tuberculosis* purified protein derivative (PPD, 100 □g/ml), the mitogen phytohaemagglutinin (PHA) (1: 100, Sigma) or *Streptococcus pyogenes* derived Streptolysin-O (SL-O, 1 : 50). Cell culture supernatants were collected after 48 hours and chemokine and cytokine secretion was quantified by specific ELISA (R&D Systems). The amounts of chemokines and cytokine released into cell culture supernatants are shown as box blots with the median and the 25% and 75% quartiles, the 1.5x of the interquantile range and with all outliers as individual points. The cellular production (in pg/ml) of chemokines and cytokine (i.e. without baseline subtraction) in CTRL and PAT was compared using Wilcoxon's rank sum test, and significant different differences between CTRL and PAT groups are indicated with $P \leq 0{\cdot}05$. The level of significance was adjusted according to Bonferroni-Holm (*alpha* = 0.003). Significant differences between controls (CTRL) and patients (PAT) are indicated with *P* values and: CTRL > PAT = chemokine or cytokine production greater in controls than patients; CTRL < PAT = chemokine or cytokine production greater in patients than controls; n.s. = not significant; n.d. = not done

The cellular production of chemokines and IL-27 in mansonelliasis patients and *M. perstans* negative controls co-infected with hookworm

The cellular chemokine production of Eotaxin (CCL24), MCP4 (CCL13), MDC (CCL22), MIG (CXCL9), PARC (CCL18), TARC (CCL17), CXCL8(IL-8) and the cytokine IL-27 were similar in mansonelliasis patients and *M. perstans* negative controls co-infected with hookworm (Table 2). Solely the chemokine IP10 (CXCL19) was produced significantly less in hookworm-positive mansonelliasis patients (Hook+Fil+) when their PBMC were stimulated with antigen extracts from *A. lumbricoides* (Tukey-Kramer test; p=0·0163), *O. volvulus* (p=0·0415) and *E. histolytica* antigen ($P = 0{\cdot}0051$). The CXCL10(IP-10) production levels were also less in hookworm infected Mp-PAT in response to *S. mansoni* (not significant) (Table 2).

Table 2. The cellular production of the chemokine inducible by interferon gamma IP10 (CXCL10) by PBMC from mansonnelliasis (Mp+) patients co-infected with hookworm (Hook+) and by PBMC from mansonelliasis-free participants was compared. PBMC were without antigen (Baseline) or with mitogen or antigen stimulation. In stool samples from mansonnelliasis (Mp+) patients hookworm larvae (Hook+) were detected (Hook+Mp+: n=13) or not (HookMp+: n=24) and also in mansonelliasis-free (Mp-) participants (Hook+Mp-: n=3; Hook-Mp-: n=10). The mean cellular production in mansonelliasis patients without hookworm (HookMp+) and with hookworm co-infection (Hook+Mp+) and in hookworm infected and mansonelliasis free (Hook+Mp-) individuals and doubly negative (Hook-Mp-) controls were compared. The amounts of CXCL10 (IP-10) released into cell culture supernatants are shown as means (in pg/ml) with the 95% lower and 95% upper confidence intervals, and the chemokine CXCL10 (IP-10) production was compared using Tukey-Kramer test, and significant differences between study groups are indicated. Freshly isolated peripheral blood mononuclear cells PBMC (1x10^6/ml in 500□l) were stimulated with helminth (Helm) antigen extracts from *Ascaris lumbricoides* (AscAg; 5 µg/ml), *Onchocerca volvulus* (OvAg, 35 µg/ml), *Schistosoma mansoni* adult worm extract (SmAg; 10 µg/ml), or with protozoan (Protoz) antigen extract from *Entamoeba histolytica* strain HM1 antigen (EhAg; 10 µg/ml), or with bacteria (Bact) antigens from *Mycobacterium tuberculosis* purified protein derivative (PPD, 100 µg/ml) or *Streptococcus pyogenes* derived Streptolysin-O (SL-O, 1 : 50), or the mitogen (Mito) phytohaemagglutinin (PHA) (1: 100, Sigma). Cell culture supernatants were collected after 48 hours and chemokine and cytokine secretion was quantified by specific ELISA (R&D Systems).

Antigen or Mitogen Stimulation	CXCL10 (IP-10) pg/ml (mean [95% lower CI; 95% upper CI])				Tukey-Kramer test *P*-value
	Study Groups				
	Hook-Mp-	Hook+Mp-	Hook-Mp+	Hook+Mp+	
Baseline	454·5 [425; 484] A	450·6 [401; 500] AB	374·3 [279; 470] AB	378·6 [324; 434] B	*P* = 0·0184*
Bact-PPD	2142·8 [752; 3533] A	925·3 [0; 3138] A	626·7 [0; 500] A	414 [0; 2888] A	
Bact-SLO	1334·8 [304; 2366] A	1623·7 [5; 3243] A	552·7 [0; 3688] A	2312·6 [502; 4123] A	
Helm-AscAg	483 [464; 502] A	451·6 [420; 484] AB	406·6 [345; 468] AB	418·7 [382; 456] B	*P* = 0·0163
Helm-OvAg	566·4 [513; 620] A	543·7 [455; 632] AB	490·9 [319; 663] AB	413·3 [314; 513] B	*P* = 0·0415
Helm-SmAg	1229·7 [929; 1530] A	1006·6 [5535; 1478] A	541·7 [0; 1455] A	484·5 [0; 1012] A	
Mito-PHA	3183·8 [2170; 4197] A	2551·1 [896; 4206] A	2789 [0; 5993] A	884·5 [0; 2735] A	
Protoz-EhAg	446·5 [430; 463] A	433·9 [407; 461] AB	392 [340; 444] AB	387·5 [358; 418] B	*P* = 0·0051

* ·· Groups that are not linked by the same letter differ significantly.

Study Groups: Hook-Mp-: hookworm negative and *M. perstans* negative; Hook+Mp-: hookworm positive and *M. perstans* negative; Hook-Mp+: hookworm negative and *M. perstans* positive; Hook+Mp+: hookworm positive and *M. perstans* positive

DISCUSSION

The impact of ivermectin treatment on *Mansonella perstans*

The present study was conducted in central Togo where the mass drug administration (MDA) of ivermectin implemented by the Onchocerciasis Control Programs in Africa (OCP, 1974–2002; APOC, 1995–2015) will progressively eliminate onchocerciasis and may reduce *O. volvulus* parasite transmission [21,22,23,24], but the drug will not affect *M. perstans* and mansonelliasis will persist. The villages from where patients originated are situated in mosaic forest savanna and manonelliasis prevalence was recently detected with 7% in Sagbadai and 19% in Bouzalo [6], and annual MDA with ivermectin is implemented there since more than 2 decades [25]. Similarly observed in the Guinea and mosaic forest savanna zones in Cameroon, *M. perstans* has not changed after > 8 years of MDA treatments, but prevalence and infection intensities decrease in communities within the deciduous equatorial rainforest suggesting that ivermectin has a partial effect on *M. perstans* [26]. In savanna type western Burkina Faso, *M. perstans* did not respond over a 14-year period to bi-annual ivermectin treatments [7]. Both studies used thick blood smears for *M. perstans* diagnosis which may have missed low level changes of Mf counts in the savanna type ecological zones. To which extent persistent *M. perstans* infection, and repeated ivermectin treatments, may modulate, activate or counteract patients' cellular immune responsiveness remains unclear. In ivermectin-treated onchocerciasis patients the expression of immunity will change; with parasite clearance depressed cellular responsiveness of both type 1 and type 2 will re-activate to *O. volvulus* and to bystander antigens while parasite-specific antibody responses will lessen gradually [13,14,15,16,17,18]. Repeated treatments with ivermectin may have changed or even re-activated cellular responsiveness, as observed in onchocerciasis, but these changes may not suffice to eliminate patent *M. perstans* infection. In the present study we observed that with patent *M. perstans* infection, the spontaneous and antigen inducible cellular production levels of several type 1 and type 2 proinflammatory and regulatory chemokines and cytokines were significantly higher in Mp-PAT than in CTRL. Such mixed and non-polarized responses may account for the lack of grave immune-mediated pathology with mansonelliasis, and may represent a balance between a tolerable parasite load causing little vascular and lymphatic damage, and an immune adaptation which to some extent may limit parasite numbers without causing severe disease manifestation.

Th2-type chemokine cellular production of Eotaxin, TARC, MDC and CXCL8 (IL-8)

In mansonelliasis patients, the prominent cellular release of the eosinophil- and neutrophilactivating chemokines Eotaxin-2 (CCL24) (Fig. 1, Part A) and CXCL8 (IL-8) (Fig. 2, Part A) may enhance the capacity of granulocytes for activation and chemotaxis. Despite that CXCL8 (IL8) and Eotaxin-2 (CCL24) persisted at elevated levels, blood circulating microfilariae of *M. perstans* were not eliminated by granulocyte-mediated destruction. Immobilization and microfilariae destruction in infested tissues may trigger inflammatory responses as observed in sowda type onchocerciasis [27], in lymphatic filariasis patients following treatment with diethylcarbamazine (DEC) [28], and serious adverse events have occurred in ivermectintreated onchocerciasis patients heavily co-infected with *Loa loa* [29,30]. Cellular killing of microfilariae of *O. volvulus* was granulocyte-mediated and serum-dependent [31], and antigen extracts of *O. volvulus* which contain the symbiotic bacteria *Wolbachia* elicited strong macrophage and neutrophil activation and chemotaxis via induction of CXCL8 (IL-8) [32]. All study participants were repeatedly treated with ivermectin and it remains unknown to which extent repeated ivermectin treatments will modify or modulate in mansonelliasis patients their filaria-specific immune responses and whether such changes may enhance their resistance or susceptibility to co-infections with protozoa, bacterial and viral pathogens. In onchocerciasis patients, the Th2-type chemokines CCL22 (MDC) and CCL17 (TARC) increased temporarily and shortly after primary ivermectin treatment, and then diminished significantly with the reduced parasite load [33]. Enhanced levels of CCL17 (TARC) and CCL22 (MDC) may support elimination of Mf but both were not inducible by helminth antigens (AscAg, OvAg, SmAg) neither in Mp-PAT nor in CTRL. In the present study, only PHA and bacterial PPD and SLO enhanced their production in Mp-PAT, and such selective inducible cellular release of MDC and TARC chemokines indicates functional Th2type cell recruitment and tissue-specific migration of lymphocytes in Mp-PAT [34].

Th1-type and pro-inflammatory chemokine cellular production of MCP-4, MIG and IP-10

The monocyte chemoattractant protein 4 (CCL13 (MCP-4)) is found in many chronic inflammatory diseases, and it displays antimicrobial activity against Gram-negative bacteria [35]. In the present work, the cellular release of CCL13 (MCP-4) following stimulation with mitogen and bacteria- or helminth-derived antigens was significantly higher in Mp-PAT than in CTRLs. The “promiscuous” binding of CCL13 (MCP-4) to several chemokine receptors, i.e. CCR1, CCR2 and CCR3, may enhance cytokine secretion, activate further effecter cells and then facilitate the cell-mediated clearance of microfilaria of *M. perstans*.

The chemokines CXCL9 (MIG) and CXCL10 (IP-10) bind to their receptor CXCR3, they activate and recruit T cells, eosinophils, monocytes and NK cells to inflamed tissues and such may also contribute to tissue damage [36]. In both Mp-PAT and CTRL, helminth extracts (AscAg, OvAg, SmAg) as well as protozoan and bacterial antigens (PPD, SLO) induced the production of CXCL9 (MIG) being always higher in Mp-PAT. Such responsiveness indicated that helminth parasites will not only activate pro-inflammatory Th2-type chemokines. CXCL10 (IP10) and its receptor CXCR3 contribute to the pathogenesis of chronic inflammatory arthritis [36], and CXCL10 (IP10) and CXCL9 (MIG) were strongly augmented in children with severe malaria [37,38]. The lower secretion of CXCL10 (IP10) in Mp-PAT in response to AscAg and EhAg supported that IP10-mediated recruitment of inflammatory cells and the induction of inflammatory cytokines was less in Mp-PAT.

Regulatory and anti-inflammatory cytokine and chemokine production of IL-27 and PARC

Regulatory T cells (Treg) are potent suppressors of the adaptive immune response with the ability to guide monocyte differentiation toward alternatively activated macrophages (AAM) [39]. Chronic human filarial infection is associated with increased levels of immune regulatory cytokines produced by Treg and the presence of monocytes characterized by an AAM phenotype expressing genes encoding the alternative activation markers resistin, MRC1, CCL18 (PARC) and MGL [40]. A feature of AAM is an increased production of CCL18 (PARC) [40], and as observed in the present study, PBMC from Mp-PAT did not differ from CTRL in their capacity to produce CCL18 (PARC) in response to bacteria- or helminthspecific antigens

suggesting that cellular production of CCL18 (PARC) was equilibrated in both study groups.

The spontaneous IL-27 production by PBMC was higher in Mp-PAT than in CTRL, and IL-27 responses above baseline were inducible only in CTRL. The properties of IL-27 to modulate inflammation, both by promoting IL-10 as well as by antagonizing Th-17 responses, will limit infection-induced pathology [41]. In children, the levels of regulatory IL-27 rose with an increasing number of parasite infections [42], but with life-threatening severe malaria the plasma levels of IL-27 were reduced while pro-inflammatory chemokines were markedly high [37,38]. The elevated pro-inflammatory Th2-type Eotaxin-2 (CCL24) and regulatory IL-27 cytokine persisted in Mp-PAT, but to which extent such response profile may control, facilitate or prevent patent *M. perstans* infection, or limit pathogenesis, remains unanswered.

The mixed expression of chemokine and cytokine cellular responses with mansonelliasis

In patients with patent *M. perstans* infection, we found spontaneously elevated and distinctively inducible pro-inflammatory CXCL8 (IL-8), Th2-type Eotaxin-2 (CCL24), monocytederived chemokine MDC and regulatory IL-27 responses in Mp-PAT, while the cellular production levels of the Th1-type chemokine CXCL10 (IP-10: inducible by IFN-γ) were depressed. Such response profile suggests that blood circulating Mf of *M. perstans* may activate neutrophil and eosinophil granulocyte-mediated defense mechanisms and in parallel *M. perstans* will stimulate regulatory cytokine responses which dampen aberrant inflammation. A similar response profile was observed in mansonelliasis patients in Cameroon; their inflammatory type IL-17A and T helper type 1 and 2 cytokines IFN-γ, IL-10 and IL-13 cytokines were enhanced upon re-stimulation with *M. perstans* antigen extract, also the monokine inflammatory protein 1β (MIP-1 β) was measured at significantly higher concentrations while the serum levels of CXCL8 (IL-8) and CCL5 (RANTES) were less in patients than in *M. perstans* negative individuals [43]. In Mali patients with *W. bancrofti* and/or *M. perstans* infections, the plasma levels of IL-10 were elevated but the chemokine IP-10 concentration low, and concurrently, bystander malaria antigen-induced cellular production of IP-10 diminished while IL-10 was high [44]. In those infected with either *W. bancrofti* or *M. perstans*, the frequencies of malaria-specific Th1 and Th17 T cells were dramatically reduced, and such response profile may alter specific T cell responses to concomitant parasite infections [45]. In hookworm co-infected Mp-PAT, the cellular production of

CXCL10 (IP-10) was diminished in response to helminth AscAg, OvAg and SmAg and *Entamoeba* (Em) antigen extracts, and such lessened Th1-type CXCL10 (IP10) production in filariasis patients could attenuate inflammatory immune responses associated with e.g. severe malaria [38,46].

Conclusions

In patients with patent *M. perstans* infection the innate cellular chemokine and cytokine production was at higher levels than in mansonelliasis-free endemic controls, and cellular responses of both Type 1 and Type 2 were selectively inducible by helminth-, bacteria- and protozoa-specific antigens and mitogen stimulation. The observed mixed Th1-, Th2- and regulatory-type cellular reactivity supports that *M. perstans* will not broadly suppress innate and adaptive immunity in patients. Such non polarized cytokine and chemokine response profile with patent infection may facilitate *M. perstans* persistence and account for the lack of severe vascular and lymphatic immune-mediated pathology, but this immune adaptation may alter resistance and susceptibility to other protozoan and metazoan parasites which are co-endemic in the study area.

Conflict of Interest:

The authors have no conflicts of interest to declare.

Authors' contributions:

BW, KK, RGG and PTS conceived, designed and performed the experiments. MB, RGG and PTS recruited and examined patients. BW, KK, VA, PWP, PTS and CK analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements:

For support we thank the laboratory technical staff from the Centre Hospitalier Regional de Sokodé/Togo. This work was supported by the research program of the Bundesministerum für Bildung und Forschung (BMBF grant 01KA1008) and the Commission of the European Community FP7 Project (Grant Acronym E-PIAF #242131).

References

1. Downes BL, Jacobsen KH. A Systematic Review of the Epidemiology of Mansonelliasis. Afr J Infect Dis 2010; **4**: 7-14.
2. Simonsen PE, Onapa AW, Asio SM. *Mansonella perstans* filariasis in Africa. Acta Trop 2011; **120:**109-120.
3. Asio SM, Simonsen PE, Onapa AW. Analysis of the 24-h microfilarial periodicity of *Mansonella perstans*. Parasitol Res 2009; **104:**945-8.
4. Taylor MJ, Hoerauf A, Bockarie M. Lymphatic filariasis and onchocerciasis. Lancet 2010; **376**:1175-85.
5. Schulz-Key H, Albrecht W, Heuschkel C, Soboslay PT, Banla M, Görgen H. Efficacy of ivermectin in the treatment of concomitant *Mansonella perstans* infections in onchocerciasis patients. Trans R Soc Trop Med Hyg, 1993; **87:**227-9.
6. Korbmacher F, Komlan K, Gantin RG, Poutouli WP, Padjoudoum K, Karabou P, Soboslay PT, Köhler C. *Mansonella perstans*, *Onchocerca volvulus* and *Strongyloides stercoralis* infections in rural populations in central and southern Togo. Parasite Epidemiol Control 2018; **3:**77-87.
7. Kyelem D, Medlock J, Sanou S, Bonkoungou M, Boatin B, Molyneux DH. Short communication: impact of long-term (14 years) bi-annual ivermectin treatment on Wuchereria bancrofti microfilaraemia. Trop Med Int Health 2005; **10**:1002-4.
8. Drame PM, Montavon C, Pion SD, Kubofcik J, Fay MP, Nutman TB. Molecular Epidemiology of Blood-Borne Human Parasites in a Loa loa-, Mansonella perstans-, and Plasmodium falciparum-Endemic Region of Cameroon. Am J Trop Med Hyg 2016; **94:**1301-8.
9. Dolo H, Coulibaly YI, Kelly-Hope L, Konate S, Dembele B, Coulibaly SY, Sanogo D, Soumaoro L, Coulibaly ME, Doumbia SS, Diallo AA, Traore SF, Colebunders R, Nutman TB, Klion AD. Factors Associated with *Wuchereria bancrofti* Microfilaremia in an Endemic Area of Mali. Am J Trop Med Hyg 2018; **98:**1782-7.
10. Thylefors B, Alleman M. Towards the elimination of onchocerciasis. Ann Trop Med Parasitol 2006; **100:**733-46.
11. Katabarwa MN, Lakwo T, Habomugisha P, Unnasch TR, Garms R, Hudson-Davis L, Byamukama E, Khainza A, Ngorok J, Tukahebwa E, Richards FO. After 70 years of fighting an age-old scourge, onchocerciasis in Uganda, the end is in sight. Int Health 2018; **10:**i79-i88.
12. Asio SM, Simonsen PE, Onapa AW. *Mansonella perstans*: safety and efficacy of ivermectin alone, albendazole alone and the two drugs in combination. Ann Trop Med Parasitol 2009; **103:**31-7.
13. Soboslay PT, Dreweck CM, Hoffmann WH, Lüder CG, Heuschkel C, Görgen H, Banla M, Schulz-Key H. Ivermectin-facilitated immunity in onchocerciasis. Reversal of lymphocytopenia, cellular anergy and deficient cytokine production after single treatment. Clin Exp Immunol 1992; **89:**407-13.
14. Mai CS, Hamm DM, Banla M, Agossou A, Schulz-Key H, Heuschkel C, Soboslay PT. Onchocerca volvulus-specific antibody and cytokine responses in onchocerciasis patients after 16 years of repeated ivermectin therapy. Clin Exp Immunol 2007; **147:**504-12.
15. Lechner CJ, Gantin RG, Seeger T, Sarnecka A, Portillo J, Schulz-Key H, Karabou PK, Helling-Giese G, Heuschkel C, Banla M, Soboslay PT. Chemokines and cytokines in

patients with an occult Onchocerca volvulus infection. Microbes Infect 2012; **14:**438-46.

16. Arndts K, Specht S, Debrah AY, Tamarozzi F, Klarmann Schulz U, Mand S, Batsa L, Kwarteng A, Taylor M, Adjei O, Martin C, Layland LE, Hoerauf A. Immunoepidemiological profiling of onchocerciasis patients reveals associations with microfilaria loads and ivermectin intake on both individual and community levels. PLoS Negl Trop Dis 2014; **8:**e2679.
17. Arndts K, Klarmann-Schulz U, Batsa L, Debrah AY, Epp C, Fimmers R, Specht S, Layland LE, Hoerauf A. Reductions in microfilaridermia by repeated ivermectin treatment are associated with lower Plasmodium-specific Th17 immune responses in Onchocerca volvulus-infected individuals. Parasit Vectors 2015; **8:**184.
18. Wammes LJ, Hamid F, Wiria AE, May L, Kaisar MM, Prasetyani-Gieseler MA, Djuardi Y, Wibowo H, Kruize YC, Verweij JJ, de Jong SE, Tsonaka R, Houwing-Duistermaat JJ, Sartono E, Luty AJ, Supali T, Yazdanbakhsh M. Community deworming alleviates geohelminth-induced immune hyporesponsiveness. Proc Natl Acad Sci USA 2016; **113:**12526-31.
19. Soboslay PT, Hamm DM, Pfäfflin F, Fendt J, Banla M, Schulz-Key H. Cytokine and chemokine responses in patients co-infected with Entamoeba histolytica/dispar, Necator americanus and Mansonella perstans and changes after anti-parasite treatment. Microbes Infect 2006; **8**:238-47.
20. Schulz-Key H, Albiez EJ, Büttner DW. Isolation of living adult *Onchocerca volvulus* from nodules. Tropenmed Parasitol 1977; 28: 428–30.
21. Traore MO, Sarr MD, Badji A, Bissan Y, Diawara L, Doumbia K, Goita SF, Konate L, Mounkoro K, Seck AF, Toe L, Toure S, Remme JH. Proof-of-principle of onchocerciasis elimination with ivermectin treatment in endemic foci in Africa: final results of a study in Mali and Senegal. PLoS Negl Trop Dis 2012; **6:**e1825.
22. Katabarwa MN, Walsh F, Habomugisha P, Lakwo TL, Agunyo S, Oguttu DW, Unnasch TR, Unoba D, Byamukama E, Tukesiga E, Ndyomugyenyi R, Richards FO. Transmission of onchocerciasis in wadelai focus of northwestern Uganda has been interrupted and the disease eliminated. J Parasitol Res 2012; **2012:**748540.
23. Lovato R, Guevara A, Guderian R, Proaño R, Unnasch T, Criollo H, Hassan HK, Mackenzie CD. Interruption of infection transmission in the onchocerciasis focus of Ecuador leading to the cessation of ivermectin distribution. PLoS Negl Trop Dis 2014; **8:**e2821.
24. Tekle AH, Zouré HG, Noma M, Boussinesq M, Coffeng LE, Stolk WA, Remme JH.Progress towards onchocerciasis elimination in the participating countries of the African Programme for Onchocerciasis Control: epidemiological evaluation results. Infect Dis Poverty 2016; **5:**66.
25. Komlan K, Vossberg PS, Gantin RG, Solim T, Korbmacher F, Banla M, Padjoudoum K, Karabou P, Köhler C, Soboslay PT. Onchocerca volvulus infection and serological prevalence, ocular onchocerciasis and parasite transmission in northern and central Togo after decades of Simulium damnosum s.l. vector control and mass drug administration of ivermectin. PLoS Negl Trop Dis 2018; **12**:e0006312.
26. Wanji S, Tayong DB, Layland LE, Datchoua Poutcheu FR, Ndongmo WP, Kengne-Ouafo JA, Ritter M, Amvongo-Adjia N, Fombad FF, Njeshi CN, Nkwescheu AS, Enyong PA, Hoerauf A. Update on the distribution of Mansonella perstans in the southern part

of Cameroon: influence of ecological factors and mass drug administration with ivermectin. Parasit Vectors 2016; **9**:311.
27. Brattig NW. Pathogenesis and host responses in human onchocerciasis: impact of Onchocerca filariae and Wolbachia endobacteria. Microbes Infect 2004; **6:**113-28.
28. Ottesen EA. Immediate hypersensitivity responses in the immune pathogenesis of human onchocerciasis. Rev Infect Dis 1985; **7:**796-801.
29. Boussinesq M, Gardon J, Gardon-Wendel N, Chippaux JP. Clinical picture, epidemiology and outcome of Loa-associated serious adverse events related to mass ivermectin treatment of onchocerciasis in Cameroon. Filaria J 2003; **24:**S4.
30. Mackenzie CD, Geary TG, Gerlach JA. Possible pathogenic pathways in the adverse clinical events seen following ivermectin administration to mansonnelliasis patients. Filaria J 2003; **24:**S5.
31. Greene BM, Taylor HR, Aikawa M. Cellular killing of microfilariae of Onchocerca volvulus: eosinophil and neutrophil-mediated immune serum-dependent destruction. J Immunol 1981; **127:**1611-8.
32. Brattig NW, Büttner DW, Hoerauf A. Neutrophil accumulation around Onchocerca worms and chemotaxis of neutrophils are dependent on Wolbachia endobacteria. Microbes Infect 2001; **3:**439-46.
33. Fendt J, Hamm DM, Banla M, Schulz-Key H, Wolf H, Helling-Giese G, Heuschkel C, Soboslay PT. Chemokines in onchocerciasis patients after a single dose of ivermectin. Clin Exp Immunol 2005; **142:**318-26.
34. Kunkel EJ, Butcher EC. Chemokines and the tissue-specific migration of lymphocytes. Immunity 2002; **16:**1-4.
35. Mendez-Enriquez E, García-Zepeda EA. The multiple faces of CCL13 in immunity and inflammation. Inflammopharmacology 2013; **21:**397-406.
36. Lee EY, Lee ZH, Song YW. The interaction between CXCL10 and cytokines in chronic inflammatory arthritis. Autoimmun Rev 2013; **12:**554-7.
37. Ayimba E, Hegewald J, Ségbéna AY, Gantin RG, Lechner CJ, Agosssou A, Banla M, Soboslay PT. Proinflammatory and regulatory cytokines and chemokines in infants with uncomplicated and severe Plasmodium falciparum malaria. Clin Exp Immunol 2011; **166:**218-26.
38. Wangala B, Vovor A, Gantin RG, Agbeko YF, Lechner CJ, Huang X, Soboslay PT, Köhler C. Wangala B, Chemokine levels and parasite- and allergen-specific antibody responses in children and adults with severe or uncomplicated Plasmodium falciparum malaria. Eur J Microbiol Immunol (Bp) 2015; **5:**131-41.
39. Tiemessen MM, Jagger AL, Evans HG, van Herwijnen MJ, John S, Taams LS. CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages. Proc Natl Acad Sci USA 2007; **104:**19446-51.
40. Babu S, Nutman TB. Immunopathogenesis of lymphatic filarial disease. Semin Immunopathol 2012; **34:**847-61.
41. Hall AO, Beiting DP, Tato C, John B, Oldenhove G, Lombana CG, Pritchard GH, Silver JS, Bouladoux N, Stumhofer JS, et al. The cytokines interleukin 27 and interferon-gamma promote distinct Treg cell populations required to limit infection-induced pathology. Immunity 2012; **37:**511–523.
42. Hegewald J, Gantin RG, Lechner CJ, Huang X, Agosssou A, Agbeko YF, Soboslay PT, Köhler C. Cellular cytokine and chemokine responses to parasite antigens and

fungus and mite allergens in children co-infected with helminthes and protozoa parasites. J Inflamm 2015; **12:**5.

43. Ritter M, Ndongmo WPC, Njouendou AJ, Nghochuzie NN, Nchang LC, Tayong DB, Arndts K, Nausch N, Jacobsen M, Wanji S, Layland LE, Hoerauf A. Mansonella perstans microfilaremic individuals are characterized by enhanced type 2 helper T and regulatory T and B cell subsets and dampened systemic innate and adaptive immune responses. PLoS Negl Trop Dis 2018; **12:**e0006184.
44. Metenou S, Dembélé B, Konate S, Dolo H, Coulibaly SY, Coulibaly YI, Diallo AA, Soumaoro L, Coulibaly ME, Sanogo D, Doumbia SS, Wagner M, Traoré SF, Klion A, Mahanty S, Nutman TB. Patent filarial infection modulates malaria-specific type 1 cytokine responses in an IL-10-dependent manner in a filaria/malaria-coinfected population. J Immunol 2009; **183:**916-24.
45. Metenou S, Dembele B, Konate S, Dolo H, Coulibaly YI, Diallo AA, Soumaoro L, Coulibaly ME, Coulibaly SY, Sanogo D, Doumbia SS, Traoré SF, Mahanty S, Klion A, Nutman TB. Filarial infection suppresses malaria-specific multifunctional Th1 and Th17 responses in malaria and filarial coinfections. J Immunol 2011; **186:**4725-33.
46. Dolo H, Coulibaly YI, Dembele B, Konate S, Coulibaly SY, Doumbia SS, Diallo AA, Soumaoro L, Coulibaly ME, Diakite SA, Guindo A, Fay MP, Metenou S, Nutman TB, Klion AD. Filariasis attenuates anemia and proinflammatory responses associated with clinical malaria: a matched prospective study in children and young adults. PLoS Negl Trop Dis 2012; **6:**e1890.

The effects of taxanes, vorinostat and doxorubicin on growth and proliferation of *Echinococcus multilocularis* metacestodes assessed with magnetic resonance imaging and simultaneous positron emission tomography

Xiangsheng Huang[1&], Stefan Wiehr[2&], Anna-Maria Wild[2],
Patrick Voßberg[1], Wolfgang Hoffmann[1], Beate Grüner[3],
Carsten Köhler[1], Peter T. Soboslay[1*]

[1]Institute for Tropical Medicine, Eberhard Karls University, Tübingen, Germany
[2]Werner Siemens Imaging Center, Department of Preclinical Imaging and Radiopharmacy, Eberhard Karls University, Röntgenweg 13, 72076, Tübingen, Germany
[3]Section of Clinical Immunology and Infectiology, University Clinics Ulm, Robert-Koch Str. 8, 89081 Ulm, Germany

[&]Xiangsheng Huang and Stefan Wiehr contributed equally to this work.

Oncotarget. 2018 Jan 10;9(10):9073-9087

Abstract

Cytostatic drugs used in cancer therapy were evaluated for their capacity to inhibit *Echinococcus multilocularis* metacestode growth and proliferation. Metacestode tissues were exposed *in vitro* to docetaxel, doxorubicin, navelbine, paclitaxel, and vorinostat for 1 week, then incubated in drug-free culture, and thereafter metacestodes were injected into the peritoneum of *Meriones unguiculatus*. Magnetic resonance imaging (MRI) and simultaneous positron emission tomography (PET) were applied to monitor *in vivo* growth of drug-exposed *E. multilocularis* in *Meriones*. At 3 month p.i., docetaxel (at 10µM, 5µM and 2µM) inhibited *in vivo* growth and proliferation of *E. multilocularis*, and at 5 months p.i., only in the 2µM docetaxel exposure group 0.3cm^3 of parasite tissue was found. With paclitaxel and navelbine the *in vivo* growth of metacestodes was suppressed until 3 months p.i., thereafter, parasite tissues enlarged up to 3cm^3 in both groups. *E. multilocularis* tissues of more than 10g developed in *Meriones* injected with metacestodes which were previously exposed *in vitro* to doxorubicin, navelbine, paclitaxel or vorinostat. In *Meriones* infected with metacestodes previously exposed to docetaxel, the *in vivo* grown parasite tissues weighted 0.2g. *In vitro* cultured *E. multilocularis* metacestodes exposed to docetaxel did not produce vesicles until 7 weeks post drug exposure, while metacestodes exposed to doxorubicin, navelbine and vorinostat proliferated continuously. In summary, docetaxel, and less efficaciously paclitaxel, inhibited *in vivo* and *in vitro* parasite growth and proliferation, and these observations suggest further experimental studies with selected drug combinations which may translate into new treatment options against alveolar echinococcosis.

Introduction

Alveolar echinococcosis (AE), a life-threatening zoonosis for humans, is caused by the proliferative growth of the larval metacestode of *Echinococcus multilocularis* (Em) within tissues and organs, mostly the liver [1]. Surgical removal of the infested organs or tissues and long-lasting benzimidazole (BMZ) therapy will improve the survival rate of patients, however, the chemotherapeutic options remain limited and new treatments of AE are needed. Long-lasting BMZ treatment is parasitostatic and not parasitocidal, and as such, despite surgical resection of parasite tissues, undetected and residual larval metacestodes may restart growth with progression of disease as soon as chemotherapy is stopped.

E. multilocularis can be maintained in an experimental life cycle by intra-peritoneal inoculation of larval metacestodes into permissive hosts such as *Meriones unguiculatus* (gerbils). The metacestode larvae will progressively grow in gerbils and parasite tissues can be collected and used for research and diagnostic purposes. To evaluate the efficacy of chemotherapy, *E. multilocularis* infected gerbils can be treated with parasiticides or cytostatic drugs [2-4]. *In vitro* cultured metacestodes can selectively be exposed to anti-helminthic drugs or new compounds to evaluate their parasitocidal or parasitostatic efficacy [5-9], or else, after intra-peritoneal transfer of these drug-exposed metacestodes into permissive recipients, e.g. gerbils, the viability and proliferative capacity of the parasite tissues can be evaluated *in vivo* [10-12]. Cytostatic drugs used in cancer therapy were applied to determine their potential to inhibit *E. multilocularis* metacestode growth and proliferation [2-4,7,9,11,12]. The selection of cytostatic drugs was based on gene expression analysis of the *E. multilocularis* metacestode tissue, which disclosed that metacestodes expressed genes associated with proliferation of cancer cells and progressive tumor growth, which can be inhibited by specific anti-cancer compounds [11]. Inhibitors of tubulin genes were chosen for this study. The taxanes (docetaxel, paclitaxel) and vinorelbine (navelbine) are microtubule-stabilizing agents that function primarily by interfering with spindle microtubule dynamics causing cell cycle arrest and apoptosis [13]. Paclitaxel at clinically achievable concentrations inhibited *in vitro* the survival of larval cells, protoscoleces and metacestodes of *Echinococcus granulosus* [9], while metacestode vesicles of *E. multilocularis* when *in vitro* cultured and exposed to paclitaxel, docetaxel or vorinostat were not affected [12]. Navelbine has been tested *in vivo* against *E. multilocularis* by Hübner et al 2010, and *in vitro* by Stadelmann et al. 2014, and the drug did not did not show parasitocidal or clear parasitostatic effects [11,12]. Vorinostat (SAHA) is one of the most potent inhibitors of histone acetyltransferases and histone deacetylases (HDAC) and clinical trials have shown it to be effective against cutaneous T-cell lymphoma and other malignancies [14]. The anti-cancer agent doxorubicin is a membrane permeable drug which mediates DNA damage and inhibits DNA synthesis, promotes reactive oxygen species and cell senescence, it will cause cardiotoxicity and drug resistance while being of low bio-availability [15]. With doxorubicin, when bound to bio-degradable nanoparticles and applied into *E. multilocularis* infected mice, the hepatic parasite development and metacestode viability were reduced, but free doxorubicin had no anti-parasitic activity [4]. For the pre-clinical evaluations of

therapeutic effects of tumor suppressors in various types of cancers, *in vivo* positron emission tomography (PET) combined with *ex vivo* histology and nuclear magnetic resonance (NMR) metabolic fingerprinting was successfully applied for therapy monitoring [16,17]. Such *in vivo* imaging techniques have also been used for non-invasive diagnosis of invasive pulmonary aspergillosis [18]. The follow-up of patients with AE was accomplished with delayed glucose traced-assisted PET which facilitated the differentiation between active and inactive liver lesions [19]. In experimental animal models of AE magnetic resonance imaging [20] or ultrasound [21] were successfully applied to follow-up parasite growth in living animals during the treatment phase.

In this study, cytostatic drugs at present used in cancer therapy were evaluated for their capacity to inhibit *E. multilocularis* metacestode growth and proliferation. We have exposed *in vitro* parasite tissues to drug concentrations used for the therapy of cancer patients, this was to evaluate the parasitostatic or parasitocidal efficacy of these cytostatic drugs at concentrations not applicable and adapted for *in vivo* use with experimental animals. After the *in vitro* exposure, and one week of culture in drug-free medium to wash out residual drug from the parasite tissue blocks, the *E. multilocularis* metacestode tissues were injected into parasite-susceptible animals (*Meriones unguiculatus*, gerbils) and this approach evaluated whether the preceding drug-exposure would inhibit *in vivo* parasite growth or has had a parasitocidal effect. Magnetic resonance imaging (MRI) and simultaneous positron emission tomography (PET) with the 2-deoxy-2-[^{18}F]-fluoro-D-glucose ([^{18}F]FDG) tracer were applied [20] to monitor *in vivo* the growth of drug-exposed *E. multilocularis* metacestodes, and in parallel, drug-exposed parasite tissues were studied *in vitro* for growth and proliferative "budding" of metacestode vesicles.

Results

Selection of cytostatic drugs for *in vitro* exposure with *E. multilocularis* metacestodes

The analysis of *E. multilocularis* cDNA hybridization to human microarrays showed strongly expressed cancer-related genes in metacestodes. The signal strength of hybridization of *E. multilocularis* cDNA to human genes was prominent for member of the RAS oncogene family (RAB2), the folate receptor (FOLR1), the eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), tubulins (TUBA1A, TUBA1C, TUBB3), aquaporin, calreticulin, and synuclein alpha (Table 1). These microarray results suggested similarities between *E. multilocularis* metacestode proliferation and

cancer progression and tissue metastases, and thus, the selection of drugs for the evaluation of their capacity to inhibit *E. multilocularis* growth and proliferation, was on FDA-approved compounds that disrupt normal function of microtubules and interfere with the cell division or replication. The taxanes docetaxel, paclitaxel and navelbine and also the histone deacetylase inhibitor SAHA (vorinostat) as well as doxorubicin were investigated in this study. *E. multilocularis* metacestodes were exposed *in vitro* to cytostatic drugs, then those drug-exposed parasite tissues were injected into *Meriones unguiculatus* (gerbils), and parasite growth and proliferation studied by MRI and PET.

Table 1: The signal strength of hybridization of *Echinococcus multilocularis* cDNA to human microarray chip.

Genbank Accession No.	*Signal Strength Sample 1*	*Signal Strength Sample 2*	*Mean Signal Strength (S1+S2)*	*Gene Title*
AA535244	1.050	837,5	944	RAB2, member RAS oncogene family
AL515273	499,6	433,1	466	eukaryotic translation elongation factor 1 alpha 1
AK098740	440,4	468,6	455	hypothetical protein LOC202051
BE221212	631,5	140,3	386	collagen, type I, alpha 1
L36675	414,8	305,7	360	synuclein, alpha (non A4 component of amyloid precursor)
AJ006206	366,8	308,6	338	B1 for mucin /// similar to MUC-B1
AL581768	399,7	244,6	322	tubulin, alpha, ubiquitous
AF000381	376	229,3	303	folate receptor 1 (adult)
AW015506	284,9	298,9	292	aquaporin 2 (collecting duct)
NM_001402	294,3	237,2	266	eukaryotic translation elongation factor 1 alpha 1
BE964125	322,6	207,2	265	similar to eukaryotic translation elongation factor 1 alpha 1; eukaryotic translation elongation factor 1 alpha 1-like 14; CTCL tumor antigen; translation elongation factor 1 alpha 1-like 14; prostate tumor-inducing protein 1; EF1a-like protein;
AL137719	257,8	248,2	253	olfactory receptor, family 7, subfamily E, member 104 pseudogene
BE786672	299,2	203,3	251	eukaryotic translation elongation factor 1 alpha 1
AK098354	292,7	208,7	251	BS 3076
AI378706	214,6	204,5	210	Calreticulin
AW001777	235,1	176	206	hypothetical LOC400843
U15197	283	127,6	205	ABO blood group (transferase A, alpha 1-3-N-acetylgalactosaminyltransferase; transferase B, alpha 1-3-galactosyltransferase)
AW271225	220,4	163,1	192	oxysterol binding protein-like 5
BI912454	233,1	119,5	176	hypothetical locus LOC338799
AK096064	210	141,1	176	---
NM_024732	194,1	154,1	174	hypothetical protein FLJ14351
NM_152909	208,1	129,4	169	zinc finger protein 548
AW612342	196,9	127,3	162	Rho-associated, coiled-coil containing protein kinase 1
AK093104	179,3	123	151	hypothetical protein FLJ35785
AL133228	198,3	100,8	150	thymosin, beta 4, X-linked /// thymosin-like 3
AI820801	203,9	82,7	143	Transcribed locus
Z22814	155,6	124,7	140	atrophin 1
NM_153606	182,4	86,8	135	family with sequence similarity 71, member A
BF223582	180,9	87,9	134	---
AV710357	196	70,9	133	---
AA046650	172,7	89	131	TRIO and F-actin binding protein
BC005946	185,9	75,2	131	tubulin alpha 6 /// tubulin alpha 6
NM_001403	81,4	172	127	eukaryotic translation elongation factor 1 alpha 1
NM_014030	127,9	122	125	G protein-coupled receptor kinase interactor 1
BC004949	141,2	107,3	124	tubulin alpha 6
AI869532	113,6	131,1	122	Nuclear factor related to kappaB binding protein
W07773	109,2	129,4	119	chromosome 19 open reading frame 22
BU928170	139,2	98,4	119	Similar to F4N2.10

NM_001030	151,2	85,1	118	ribosomal protein S27 (metallopanstimulin 1)
BC013641	124,5	107,3	116	Homo sapiens, clone IMAGE:4151631, mRNA
Y15916	110,9	112,5	112	collagen, type I, alpha 1
AJ251708	177,6	45,1	111	putative microtubule-binding protein
U58856	108,5	114	111	mannose receptor, C type 2
AW015517	95,3	126,6	111	Follistatin-like 3 (secreted glycoprotein)
AL565749	125,1	94,71	110	tubulin, beta 3
AB009010	147,3	72,4	110	ubiquitin C
AJ296370	168,8	50	109	---
BC015443	99,21	119,1	109	Pseudogene similar to LOC112869 gene
BE300252	140,7	77,2	109	tubulin, alpha, ubiquitous
AL031186	126,6	89,59	108	EMI domain containing 1
BF246436	133,1	78,3	106	eukaryotic translation initiation factor 1
AF343666	112,8	97,7	105	Translocation associated fusion protein IRTA1/IGA1 (IRTA1/IGHA1) /// Translocation associated fusion protein IRTA1/IGA1 (IRTA1/IGHA1)
BC004952	129,4	75,3	102	polycomb group ring finger 1
AW974499	99,1	103,4	101	Rho GTPase activating protein 30
AI885873	122,4	78	100	transportin 2 (importin 3, karyopherin beta 2b)
BE552347	108,6	91,8	100	Kv channel interacting protein 2
BE813017	120,3	79,3	100	---
BC002778	96,3	102	99	myosin light chain 2, precursor lymphocyte-specific
U81961	104,1	88,4	96	sodium channel, nonvoltage-gated 1 alpha
W94546	87,5	103,5	96	hypothetical protein 284297
AA398062	100,9	87,03	94	aminopeptidase-like 1
AI251399	71,3	114,8	93	protein kinase D2
NM_000748	115,2	70,3	93	cholinergic receptor, nicotinic, beta polypeptide 2 (neuronal)
AK096064	155,9	29,2	93	---
AI304355	101,7	80,81	91	Chromosome 1 open reading frame 78
AW248552	143,2	32,9	88	NOL1/NOP2/Sun domain family, member 5
M80469	102,7	72,89	88	HLA-G histocompatibility antigen, class I, G /// major histocompatibility complex, class I, H (pseudogene)
AK024602	128,3	38,5	83	CDNA: FLJ20949 fis, clone ADSE01902
NM_002587	100	64,3	82	protocadherin 1 (cadherin-like 1)
BG701300	106,6	53,5	80	hypothetical gene supported by BC030123
NM_002375	109,1	40,8	75	microtubule-associated protein 4
NM_024671	104,5	42,3	73	hypothetical protein FLJ23436
AL390137	104	42	73	Eukaryotic translation initiation factor 3, subunit 10 theta, 150/170kDa
NM_032887	112,6	26,6	70	hypothetical protein MGC16037
BC014556	100,1	36,7	68	hypothetical protein FLJ35390
X07618	104,7	30,1	67	---
AA362254	119,8	13,8	67	CDNA FLJ30424 fis, clone BRACE2008881, weakly similar to ZINC FINGER PROTEIN 195
NM_022830	21	109,2	65	RNA binding motif protein 21
NM_006316	110,1	19,4	65	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian) opposite strand

***In vivo* growth of drug-exposed *E. multilocularis* in Meriones unguiculatus**

In vivo volumetric MRI measurements were performed at two time points after inoculation of drug-exposed *E. multilocularis* metacestode tissues into *Meriones*. Analysis of the MR images showed low or no parasite growth in infected *Meriones* if metacestodes were exposed to docetaxel *in vitro*, independent of the applied concentration (Figure 1, Part A and F). At 3 month post transfer of drug-exposed metacestodes in *Meriones*, the MRI measurement detected 0 mm^3 (n=2), 0 and 4934 mm^3 (n=2) and 0 and 6 mm^3 (n=2) of parasite tissues in the docetaxel 10µM, 5µM and 2µM exposure groups, respectively. At 5 months p.i., the MRI measurement did not detect any metacestodes in the docetaxel 10µM 5µM and 2µM exposure groups.

The *in vitro* exposure of metacestode tissues to paclitaxel did not prevent their growth after transfer into the peritoneum of *Meriones* (Figure 1, Part B and F). At 3 months p.i., metacestodes which were *in vitro* exposed to paclitaxel at concentrations of 10µM, 5µM and 2µM developed in *Meriones* into parasite tissues with volumes 1694 and 3316 mm^3 (n=2); 973 and 619 mm^3 (n=2) and 6932 and 577 mm^3 (n=2), respectively. At 5 months p.i. the MRI measurement determined 16370 mm^3 (n=1), 1692 and 10931 mm^3 (n=2) and 1933 and 18977 mm^3 (n=2) of parasite tissues in the paclitaxel 10µM, 5µgM and 2µM exposure groups, respectively. Following navelbine exposure, small metacestode tissue volumes were detected at 2 months post transfer in *Meriones*, but parasite volumes enlarged at 5 months post transfer (Figure 1, Part D and F).

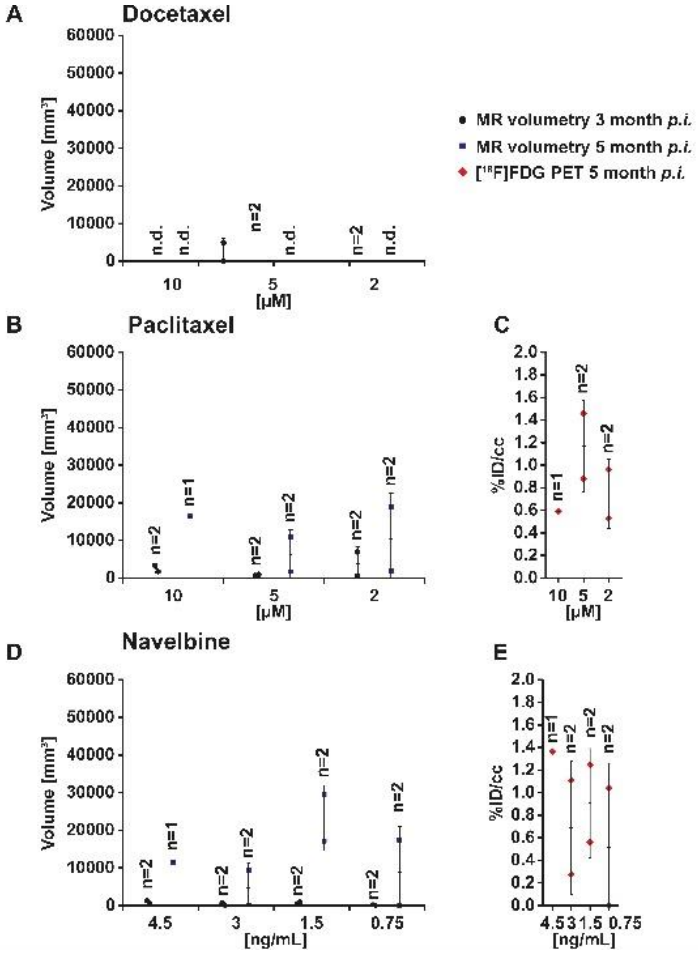
When exposed to navelbine at concentrations of 4.5ng/ml, 3ng/ml 1.5ng/ml and 0.75ng/ml, the tissue volumes detected by MRI at 2 months p.i. were 570 and 1290 mm^3 (n=2), 51 and 636 mm^3 (n=2), 890 and 468 mm^3 (n=2) and 0 and 177 mm^3 (n=2), respectively. At 5 months p.i., parasite tissue volumes of 11565 mm^3 (n=1), 78 and 9337 mm^3 (n=2), 17151 and 29422 mm^3 (n=2) and 0 and 17496 mm^3 (n=2) have grown in *Meriones* when the navelbine exposure concentrations were 4.5ng/ml, 3ng/ml 1.5ng/ml and 0.75ng/ml, respectively.

Animals inoculated with *E. multilocularis* metacestodes exposed to doxorubicin (Figure 2, Part A and H) showed comparable parasite growth as seen in the group treated with paclitaxel. At 2 months post inoculation, metacestodes exposed *in vitro* to doxorubicin at concentrations of 4.5µg/ml, 3µg/ml and 1.5µg/ml developed *in vivo* in *Meriones* parasite tissue volumes of 7385 and 15138 mm^3 (n=2), 2623 and 967 mm^3 (n=2) and 810 and 332 mm^3 (n=2), respectively. At 5 months p.i., tissue volumes of 13426 mm^3

(n=1), 39768 and 2297 mm^3 (n=2) and 11733 and 16953 mm^3 (n=2) were present in the respective doxorubicin exposure groups.

In vivo growth of *E. multilocularis* metacestode was observed following *in vitro* exposure with vorinostat (SAHA) (Figure 2, Part C and H). At 3 months post inoculation in *Meriones,* parasite tissues with volumes of 10879 and 412 mm^3 (n=2), 0 and 220 mm^3 (n=1), 10887 and 1470 mm^3 (n=2) and 8204 and 521 mm^3 (n=2) have developed from vorinostat (SAHA) 10μg/ml, 7.5μg/ml, 5μg/ml, 2.5μg/ml exposed metacestodes, respectively. At 5 months post inoculation, MRI measurements could be conducted in *Meriones* with vorinostat (SAHA) 7.5μg/ml, 2μg/ml and 1μg/ml exposed metacestodes, and 755 and 0 mm^3 (n=2), 20389 and 44256 mm^3 (n=2) and 24190 mm^3 (n=1) of tissues were found, respectively.

The *in vitro* DMSO-exposed metacestode tissues which were transferred into *Meriones* were prominently enlarged *in vivo* at the first time point of measurement with 19264±38127 mm^3 (n=5; at 3 months post transfer) and on the second measurement 7606 and 5049 mm^3 of parasite tissue was detected (n=2; at 5 months post transfer) (Figure 2, Part E and G control). Due to strong parasite growth in the DMSO control group, 3 animals had to be euthanized according to the animal welfare guidelines which resulted in a lower mean parasite burden at the second measurement time point.


A
Docetaxel
Volume [mm³]
MR volumetry 3 month p.i.
MR volumetry 5 month p.i.
[18F]FDG PET 5 month p.i.
n.d.
n=2
[µM]
B
Paclitaxel
C
%ID/cc
n=1
D
Navelbine
E
[ng/mL]

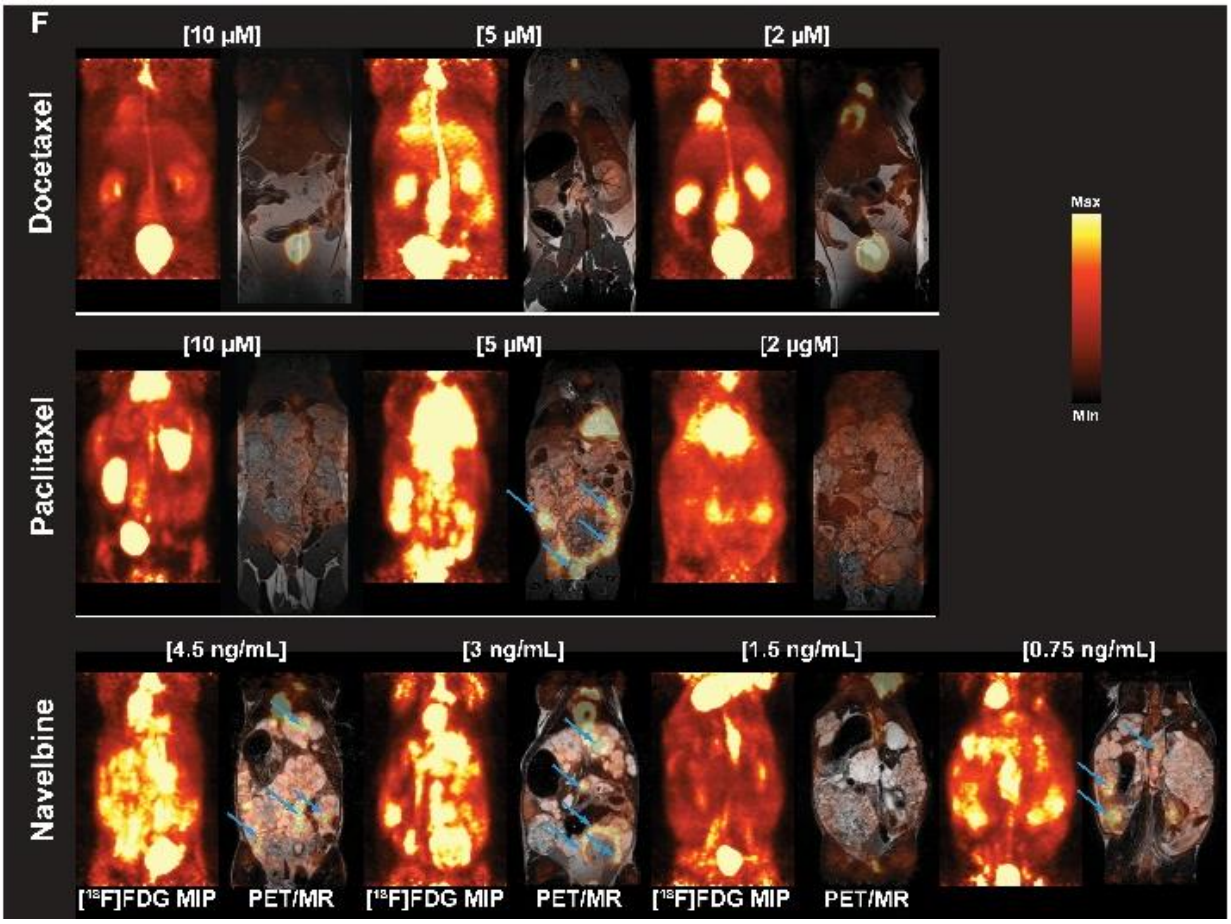

F
Docetaxel
Paclitaxel
Navelbine
[10 µM]
[5 µM]
[2 µM]
[2 µgM]
[4.5 ng/mL]
[3 ng/mL]
[1.5 ng/mL]
[0.75 ng/mL]
Max
Min
[18F]FDG MIP
PET/MR

Figure 1: PET- and MR-imaging, and quantification of parasite growth, in docetaxel, paclitaxel and vorinostat (SAHA) exposed *E.multilocularis* metacestodes. *In vivo* quantification of parasite growth was performed at two time points in all tested animals. The *in vivo* grown volumes of docetaxel (A), paclitaxel (C) and navelbine (D) exposed *E. multilocularis* metacestodes is shown. At the second measurement time point (Figure part F) and simultaneously to the MR acquisition all animals were PET imaged with [^{18}F]FDG. Quantification of the [^{18}F]FDG uptake in parasite tissue is presented as percentage of the injected dose per cubic centimeter (%ID/cc) and error bars represent one SD. Results are shown for docetaxel (C), paclitaxel and navelbine (E) exposure. Coronal [^{18}F]FDG maximum intensity projections (MIP) and fused PET/MR images from *E. multilocularis* metacestode infected gerbils are shown in the Figure Part F. Arrows indicate the positions of the [^{18}F]FDG uptake in the metacestode tissue. (n.d., non detected)

In vivo Positron Emission Tomography (PET) and Magnetic Resonanz Imaging (MRI) of Meriones unguiculatus infected with drug-exposed E. multilocularis

For the *in vivo* evaluation of the glucose metabolism, *E. multilocularis* infected and control *Meriones* were injected with the PET tracer [^{18}F]FDG for glucose consumption, and PET/MRI were simultaneously performed at 5 months post infection in one set of experiments. When *E. multilocularis* metacestodes were exposed *in vitro* to docetaxel and these metacestodes then transferred into the peritoneum of *Meriones*, the PET quantification showed no uptake of [^{18}F]FDG in these animals, independent of the *in vitro* applied concentrations of docetaxel. An increased [^{18}F]FDG tracer uptake of 0.6 %ID/cc (n=1), 0.9 and 1.5 %ID/cc (n=2) and 0.5 and 1.0 %ID/cc was seen in gerbils implanted with metacestode tissue exposed to paclitaxel at concentrations of 10µg/ml, 5µg/ml and 2 µg/ml, respectively (Figure 1, Part C). If *Meriones* inoculated with navelbine-exposed *E. multilocularis* metacestodes, the [^{18}F]FDG tracer uptake into parasite tissues was heterogeneous when compared to vorinostat and doxorubicin. Tracer uptake was 1.4 %ID/cc (n=1), 0.3 and 1.1 %ID/cc (n=2); 1.3 and 0.6 %ID/cc (n=2) and 0 and 1.0 %ID/cc (n=2) when metacestodes were exposed *in vitro* to navelbine at concentrations of 4.5µg/ml, 3µg/ml, 1.5µg/ml and 0.75µg/ml, respectively (Figure 1, Part D).

The preceding *in vitro* exposure of metacestodes to doxorubicin led to a dose dependent uptake of [^{18}F]FDG in parasite tissue in *Meriones* with 0.7 %ID/cc (n=1), 0.6 and 1.2 %ID/cc (n=2), and 1.4 and 1.2 %ID/cc (n=2) when doxorubicin was applied at concentrations of 4.5µg/ml, 3µg/ml and 1.5 µg/ml, respectively (Figure 2, Part B). Similarly, in *Meriones*, a dose dependent uptake of the [^{18}F]FDG tracer in *E. multilocularis* metacestode tissue was detected when the preceding *in vitro* exposure

was with vorinostat (SAHA). Animals inoculated with *E. multilocularis* metacestodes treated with 10µg/ml vorinostat showed no uptake of [^{18}F]FDG due to no parasite growth. Exposure with 7.5µg/ml of vorinostat resulted in low [^{18}F]FDG uptake with 0 and 0.7 %ID/cc (n=2); when metacestodes were exposed to 5µg/ml vorinostat then tracer uptake was 1.1 and 0.7 %ID/cc (n=2) and exposure with 2µg/ml showed a tracer uptake of 1.1 %ID/cc (Figure 2, Part D). In *Meriones* transferred with DMSO-exposed *E. multilocularis* metacestodes (positive control) the uptake of [^{18}F]FDG was at 1.3 and 1.2 %ID/cc (n=2) (Figure 2, Part F).

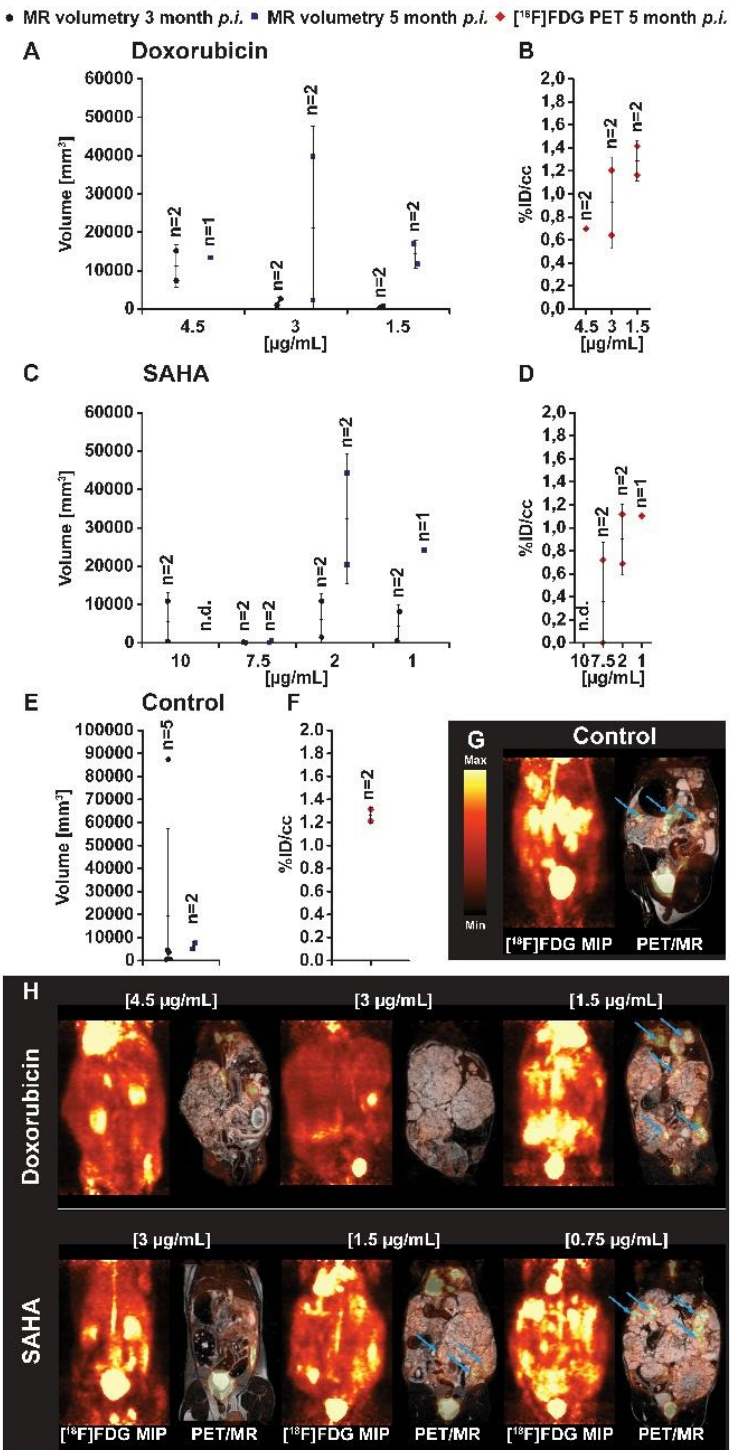


Figure 2: PET- and MR-imaging, and quantification of parasite volumes, in doxorubicin, vorinostat (SAHA) and DMSO (control) exposed *E. multilocularis* metacestodes. *In vivo* quantification of parasite growth was performed at two time points in all tested animals. The parasite tissue volumes of doxorubicin (A), vorinostat (SAHA; C) and DMSO control (E) exposed *E. multilocularis* metacestodes is shown. At the second measurement time point and simultaneously to the MR acquisition all animals were PET imaged with [^{18}F]FDG. Quantification of the [^{18}F]FDG uptake in parasite tissue is presented as percentage of the injected dose per cubic centimeter (%ID/cc) and error bars represent one SD. Results are shown for doxorubicin (B), vorinostat (SAHA; D) and DMSO control (F) treatment. Coronal [^{18}F]FDG maximum intensity projections (MIP) and fused PET/MR images from *E. multilocularis* metacestode infected gerbils are depicted in the Figure Part G and H. Arrows indicate the positions of the [^{18}F]FDG uptake in the metacestode tissue.

The *in vivo* weights of E. multilocularis metacestodes after *in vitro* drug-exposure

After the final PET scan at 5 months post infection, all *Meriones* were euthanized under deep anesthesia and parasite tissues were removed and weighted. *E. multilocularis* metacestode tissue masses of more than 10g developed in *Meriones* injected with metacestodes which were previously exposed *in vitro* to doxorubicin (mean parasite tissue weight: 17.8g), navelbine (12.0g), paclitaxel (11.6g) or vorinostat (SAHA) (20.1g), while in those animals which were injected with metacestodes previously exposed to docetaxel, the *in vivo* grown metacestodes weighted 0.2g (Figure 3). In Figure 3 the weights of the *in vivo* grown metacestode tissues from the animal groups with selected drug concentrations were merged.

The *in vitro* production "budding" of vesicles from E. multilocularis metacestode tissues after *in vitro* drug-exposure

E. multilocularis metacestode tissues were exposed to cytostatic drugs or drug-free culture media (control) for 1 week and maintained *in vitro* for another week drug-free, then the drug-exposed metacestode tissue culture media were changed weekly and the produced ("budded") *E. multilocularis* vesicles (diameter 2 to 4 mm) collected, counted and vesicle production scored (Figure 3). Metacestodes exposed to docetaxel did not produce vesicles until seven weeks post exposure, thereafter, few vesicles (n=1-5) were budding off the metacestode tissue blocks, and then vesicle production increased slightly (n=6-10) from 10 weeks post exposure onwards. Already at 2-3 weeks post drug exposure, few vesicles (n=1-5) were released from metacestode tissue blocks previously exposed to doxorubicin (4.5, 3 and 1.5μg/ml), navelbine (4.5,

3, 1.5 and 0.75μg/ml), paclitaxel (10μM, 5μM and 2μM), vorinostat (SAHA) (10, 7.5, 2 and 1μg/ml) (Figure 3), and the vesicle budding remained at this level until 14 weeks post drug exposure; thereafter cultures were ended. In Figure 3 (Part A) the respective vesicle productions at the selected drug concentrations are shown. The *E. multilocularis* metacestode tissue cultures exposed to the DMSO solvent control budded off vesicles shortly after exposure, the release of vesicles continued to increase for weeks and reached at 7 weeks post DMSO exposure a plateau level of production (n=20-30) which continued such until 14 weeks post drug exposure (Figure 3, Part B).

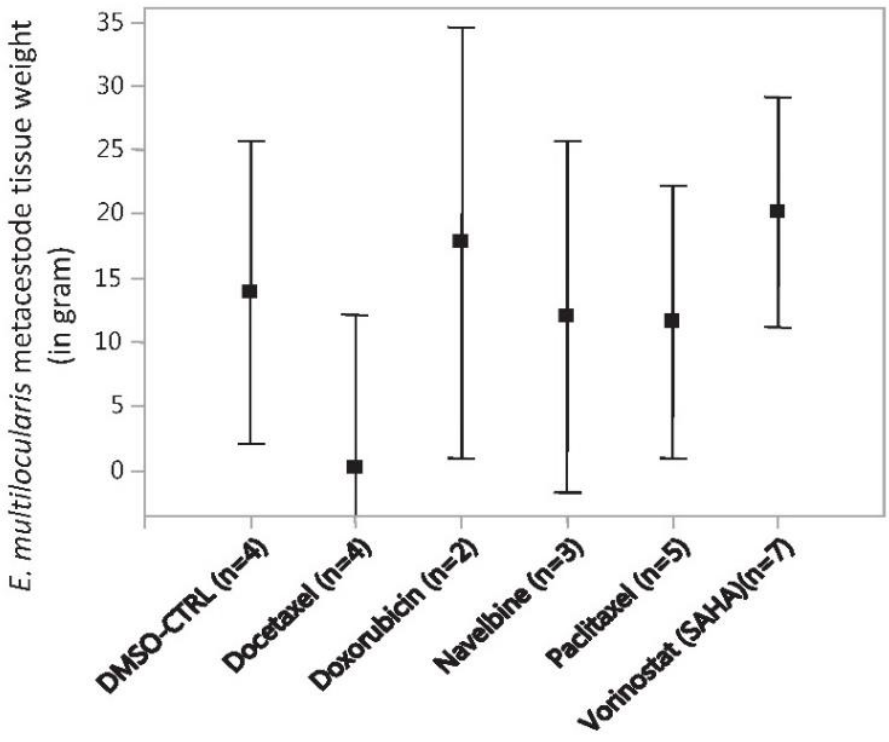
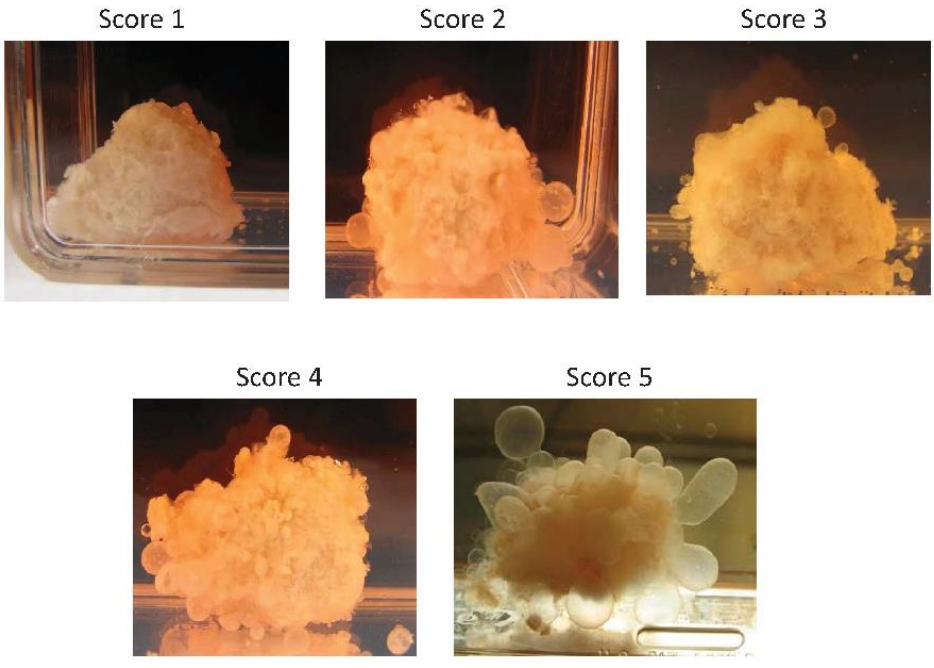


Figure 3: The weights of *E. multilocularis* metacestodes tissues isolated from infected *Meriones unguiculatus*. Metacestodes were exposed *in vitro* to the cytostatic drugs docetaxel (10μM, 5 μM, 2μM), doxorubicin (4.5, 3 and 1.5μg/ml), navelbine (4.5, 3, 1.5 and 0.75μg/ml), paclitaxel (10μM, 5μM and 2μM), vorinostat (SAHA) (10, 7.5, 2 and 1μg/ml) and DMSO (0.1%, 0.05%, solvent control, CTRL) at the indicated concentrations for 7 days, subsequently metacestodes rested in drug-free media for another 7 days, and then the drug-exposed metacestodes were injected into the peritoneum of *M. unguiculatus*. At 4 and 5 months post infection, the grown metacestode tissues were collected from *M. unguiculatus* and weighted. The drug concentration groups at which *E. multilocularis* metacestodes tissues were exposed to the cytostatic drugs are merged. The Figure shows the treatment groups, the mean metacestode tissue weights and the 95% confidence intervals. No significant differences in weights were observable between the treatment groups.

Discussion

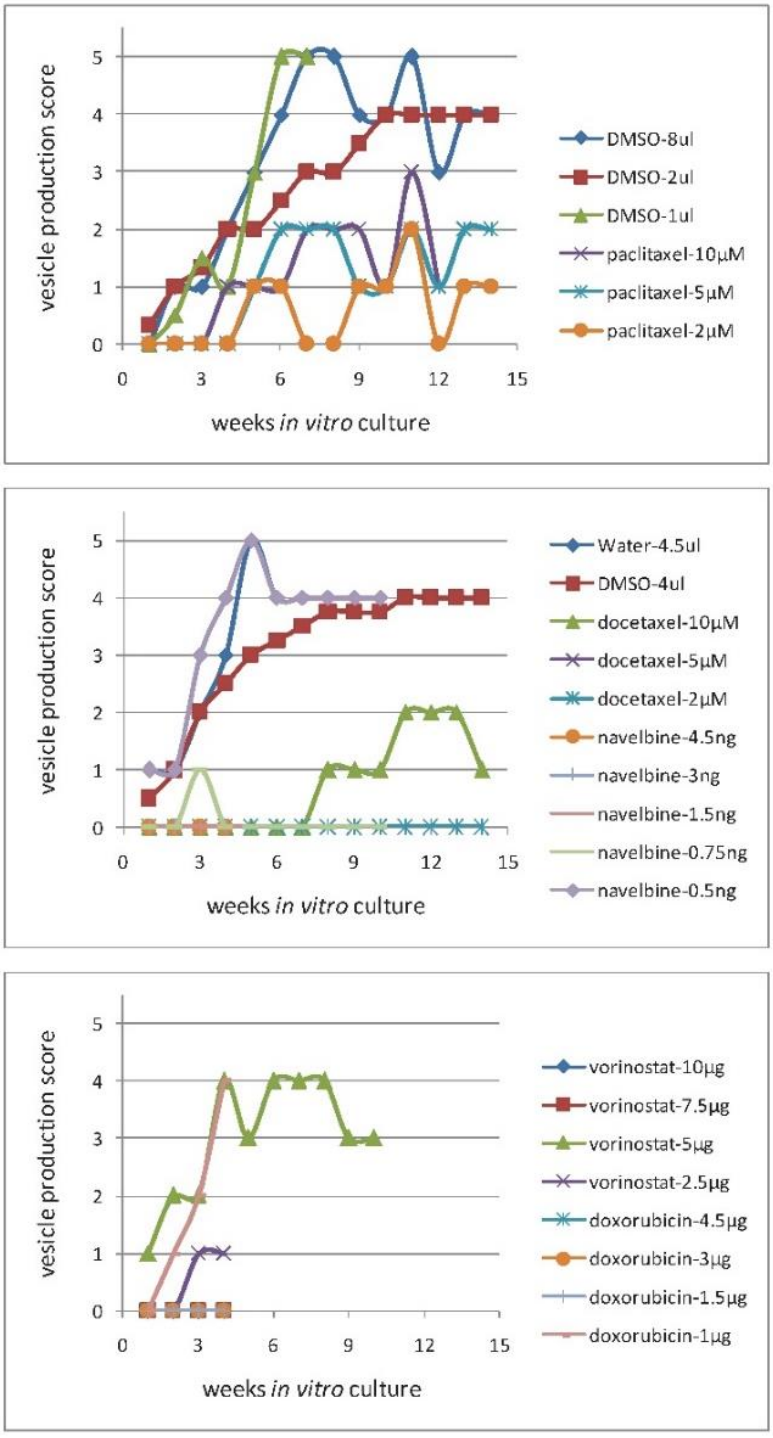
For AE patients treated with albendazole or mebendazole who experience severe side effects there are no alternative chemotherapeutics which reach beyond these classical benzimidazoles [1,22]. We have applied gene microarray profiling of *E.multilocularis* metacestodes which showed strongly expressed human cancer-related genes suggesting similarities between metacestode proliferation and malignancies. In this pre-clinical study we exposed *in vitro E. multilocularis* metacestodes *in vitro* to cytostatic drugs, then implanted those drug-exposed parasite tissues into *Meriones unguiculatus* (gerbils), and studied *in vivo* parasite growth and proliferation by MRI and PET. *In vivo* growth and proliferation of *E. multilocularis* metacestode tissues was inhibited by docetaxel, while with paclitaxel and navelbine the *in vivo* growth of metacestodes was suppressed only until 3 months post infection, thereafter, parasite tissues enlarged in both drug-exposure groups. The histone deacetylase inhibitor vorinostat (SAHA), and doxorubicin which mediates DNA damage and inhibits DNA synthesis, were both not effective to inhibit *E. multilocularis* metacestode growth and proliferation, which is consistent with previous findings [4, 12].

Figure 4: The *in vitro* "budding" of vesicles from E. multilocularis metacestode tissues after drug-exposure. The "budding" of *E. multilocularis* vesicles from *in vitro* cultured metacestodes tissue previously exposed *in vitro* to cytostatic drugs was evaluated during 14 weeks post drug exposure. The number of vesicles produced in culture was scored, i.e. Score 0 = no vesicle, Score 1 = very few vesicles (1-5), Score 2 = few vesicles (6-10), Score 3 = vesicles (11-20), Score 4 = vesicles (21-30), Score 5 = vesicles (>31). The production scores 1-5 of *E. multilocularis* metacestodes tissues are shown.



New research approaches have been suggested which should explore new therapeutic molecules, exploit parasite gene signaling pathways, target *E. multilocularis* stem cells and dissect the metabolic metamorphosis of *E. multilocularis* metacestodes [23, 24]. There are several observations on antigenic similarities between *E. granulosus* and various tumor types [25, 26], and together with the finding on highly expressed tumor-related genes in metacestodes this suggested the evaluation of anti-cancer cytostatic drugs as treatment options for AE. The standard readout method for the assessment of drug efficacy is parasite weight determination subsequent to cyst resection from experimentally *E. multilocularis* infected mice, rats or gerbils. This method has pitfalls because necropsy can change the parasite mass due to cyst rupture and release of vesicle fluid, and the assessments of parasite viability based on parasite weights may not be exact because host connective tissue encapsulating the parasite makes a complete resection of the parasite mass difficult [21, 27]. Previous works on *in vitro* drug-exposure followed by *in vivo* growth monitoring, have exposed *E. multilocularis* metacestodes to mebendazole, then transferred these metacestodes into parasite-permissive gerbils *Meriones unguiculatus* where tissues did not grow [28], as determined by weight monitoring after dissection. Thus, the exposure of *E. multilocularis* metacestodes *in vitro* to mebendazole at concentrations above 0.1μM was parasitocidal [28], and similarly effective was treatment using mefloquine (20 μM) against *in vitro* cultures of metacestodes but oral application of mefloquine to *E. multilocularis*-infected mice was ineffective, whereas oral albendazole application was highly effective [29]. Monitoring *in vivo* of the intra-peritoneal parasite growth without sacrificing the animal is possible by MRI and ultrasound allowing the assessments of parasite tissue volumes and its *in vivo* growth [11, 21], and by using PET tracers the metabolic activity of parasite tissues can be monitored, e.g. after application of therapeutic drugs [20]. The application of non-invasive imaging techniques, notably delayed [^{18}F]FDG PET, greatly facilitated the differentiation between active and inactive liver lesions in AE patients, and the results suggested that the combination of delayed [^{18}F]FDG PET and specific serology may help to prevent recurrences observed after premature interruption of treatment [19]. Further, imaging methods, using disease specific tracers for immuno-PET, have significant potential as effective tools to visualize infected tissues and cells, i.e. invasive pulmonary aspergillosis [18], and ligand-based targeting of specific cells or malignant tissues may help and guide surgeons to adequately resect infected while sparing critical tissues [30-32].

Figure 5: **The *in vitro* production of vesicles from E. multilocularis metacestode tissues after *in vitro* drug-exposure.** *In vitro* cultured *E. multilocularis* metacestode tissue blocks (1cm^3) were exposed to 10μM, 5 μM, 2μM of docetaxel, to 4.5 μg/ml, 3 μg/ml and 1.5μg/ml of doxorubicin, to 4.5μg/ml, 3μg/ml, 1.5μg/ml and 0.75μg/ml of navelbine, to 10μM, 5μM and 2μM paclitaxel, to 10μg/ml, 7.5μg/ml, 2μg/ml and 1μg/ml of vorinostat (SAHA), and to DMSO (solvent control) at the indicated concentrations. The effects of these cytostatic compounds on the *in vitro* production by *E. multilocularis* vesicles was scored and studied for 14 weeks. The number of vesicles produced in culture was scored (Figure 5), i.e. Score 0 = no vesicle, Score 1 = very few vesicles (1-5), Score 2 = few vesicles (6-10), Score 3 = vesicles (11-20), Score 4 = vesicles (21-30), Score 5 = vesicles (>31).



The taxanes paclitaxel, and docetaxel, a semi-synthetic analogue of paclitaxel, are proven anti-cancer drugs and FDA-approved formulations of first line against advanced prostate cancer. Paclitaxel and docetaxel have a similar mechanism of action, they promote tubulin assembly and inhibit microtubule disassembly, stabilizing microtubule polymerization and thus blocking cells in the G2/M phase of the cell cycle thus triggering the signaling pathway that leads to apoptosis. Docetaxel is effective against tumor cells by inducing cell death, it inhibits the transcription of androgen receptors thus improving survival in metastatic hormone-resistant prostate cancer and also with tumors at earlier stages [33]. Navelbine is approved for the treatment of non-small cell lung cancer and metastatic breast cancer [34, 35]. Paclitaxel and docetaxel distribute into most tissues of mice and rats, including tumor tissue, but despite similarity in chemical structures their metabolic profile is distinct. Whereas paclitaxel metabolism is largely species dependent, docetaxel metabolism is similar across species, and for both taxanes, hepatobiliary excretion is the major pathway of elimination, and a major fraction of the dose is excreted in feces as parent drug or hydroxylated metabolites [36]. Albendazole remains the most common and effective treatment for AE, it targets tubulin but has its limitation, such as poor solubility and intestinal absorption and often there is no complete recovery after treatment. High dosage and lifelong uptake is required for albendazole in AE patients, which may lead to severe adverse effects [22]. Thus, less uptake time and high efficiency with paclitaxel and docetaxel may decreases the adverse effects and lead to potential treatments.

Recently, the anti-cancer drug bortezomib, a proteasome inhibitor developed for the chemotherapy of myeloma, displayed high anti-metacestodal activity, and Balb/c mice experimentally infected with *E. multilocularis* metacestodes presented with reduced parasite weights, but bortezomib treatment induced adverse effects such as diarrhea and neurological symptoms [12]. Previously, we found that navelbine suppressed *in vivo E. multilocularis* metacestode growth and proliferation [11], and the present results show that docetaxel visibly, and paclitaxel to a lesser extent, inhibited parasite growth. Similar results were reported by Pensel PE *et.al.*, who showed the paclitaxel can inhibit the survival of larval cell, protoscoleces and metacetodes of *Echinococcus granulosus* [9]. There are clinically relevant differences between docetaxel and paclitaxel, docetaxel is more cytotoxic than paclitaxel against a variety of murine and human tumor cell lines [37]. Both have been serving as important drugs for the treatment of various cancers, but drug resistance imposes limitations in their application since both

have high affinity for multidrug-resistance proteins, in particular the drug efflux pump P-glycoprotein [38].

In conclusion, our observations advocate for drug combinations to be applied in experimental pre-clinical studies; which may provide essential information on their efficacy against *E. multilocularis* metacestodes, and ultimately this may translate into new treatment options against alveolar echinococcosis.

Materials and methods

Animal model of alveolar echinococcosis

All experiments were performed according to the German Animal Protection Law with permission from the Regierungspräsidium Tübingen as per guidelines from the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). Ten-week-old female gerbils (*Meriones unguiculatus*) were purchased from Charles River Laboratories (Sulzfeld, Germany) or bred in our animal facility. The animals were kept under standardized and sterile environmental conditions (20°C ± 1°C room temperature, 50% ± 10 % relative humidity, 12h light-dark cycle) and received food and water *ad lib*.

E. multilocularis metacestode tissue was routinely maintained in gerbils using a modified method of serial implantation of parasite tissue as described previously [39, 11]. The metacestode tissue was passed through a metal sieve with 1 mm^2 width. *M. unguiculatus* (gerbils) were anesthetized with 2% isoflurane mixed with 100% oxygen and 0.5ml of the metacestode tissue cell suspension was injected into the peritoneum of each gerbil. After sufficient growth of the metacestode tissue, the gerbils were euthanized with CO_2, and the parasite tissues were removed and used for *in vitro* culture assays and for *in vivo* transfer and maintenance of the *E. multilocularis* metacestodes.

In vitro culture of *E. multilocularis* metacestodes

For *in vitro* cultivation of *E. multilocularis*, metacestode blocks were freshly and aseptically removed from the peritoneal cavity of experimentally infected gerbils (*Meriones unguiculatus*) [11, 39] and incubated with RPMI 1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin (Biochrom GmbH, Berlin, Germany) at 37°C and 5% CO_2. Medium was changed once a week for all cultures.

Purification of *E. multilocularis* metacestodes total RNA, cDNA generation, labeling and oligonucleotide hybridization and microarray

E. multilocularis metacestodes were cultured *in vitro* as described [11]. Metacestode tissue blocks which were not drug-exposed were snap frozen in liquid nitrogen, the deep frozen tissues were minced and homogenized with a tissue grinder, total RNA was purified by RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA was quantified with a Nanodrop UV spectrofluorometer and quality of RNA determined by Agilent Bioanalyzer 2100 (Agilent, CA, USA). Double-stranded cDNA was synthesized from 100 ng of total RNA and subsequently linearly amplified and biotinylated using the GeneChip® WT cDNA Synthesis and Amplification Kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. Microarrays were analyzed with 15µg of labeled and fragmented cDNA hybridized to GeneChip® HumanGene 1.0 ST arrays (Affymetrix). After hybridization, the arrays were washed and stained in a Fluidics Station 450 (Affymetrix) with the recommended washing procedure. Biotinylated cDNA bound to target molecules was detected with streptavidin-coupled phycoerythrin, biotinylated anti-streptavidin IgG antibodies and again streptavidin-coupled phycoerythrin according to the protocol. Arrays were scanned using the GCS3000 GeneChip scanner (Affymetrix) and AGCC 3.0 software. Scanned images were subjected to visual inspection to check for hybridization artifacts and proper grid alignment and analyzed with Expression Console 1.0 (Affymetrix) to generate report files for quality control. Normalization of raw data was performed by the Partek Software 6.6, applying an RMA (Robust Multichip Average) algorithm. For analysis, microarray hybridization data were converted to signal values using ArrayAssist 3.4 (Stratagene), and the signal strength of hybridization of the *E. multilocularis* cDNA samples to the human micro-array chip greater than 100 above background were selected. Two *E. multilocularis* metacestode samples, which were cultured as described above without having been exposed to anti-cancer drugs, were applied and the hybridization signals of both samples and their mean signal strength were aligned and are shown in Table 1.

Selection of cytostatic drugs

Gene expression profiling in *E. multilocularis* metacestodes showed strongly expressed human cancer-related genes which suggested similarities between *E. multilocularis* metacestode proliferation and cancer progression and tissue metastases. The signal strength of hybridization to human genes was prominent for member of the RAS oncogene family, the folate receptor, the eukaryotic translation elongation factor 1 alpha 1, tubulin, aquaporin, calreticulin, and synuclein alpha (Table

1). Based on these hybridization signals and specific gene expression FDA-approved formulations against advanced cancer were selected for the *in vitro* exposure of *E. multilocularis* metacestodes. Taxanes were selected for their capacity to stabilize microtubule polymerization thus blocking the cell cycle which leads to apoptosis. vorinostat (SAHA) was selected for inducing cell cycle arrest, doxorubicin for its capacity to mediate DNA damage and to inhibit DNA synthesis and proliferation, and both SAHA and doxorubicin may act in synergy to inhibit growth of tumor cells.

Exposure of *E. multilocularis* to cytostatic drugs

In vitro cultured *E. multilocularis* metacestode tissue blocks (1cm^3) were exposed to docetaxel, paclitaxel, navelbine, doxorubicin, or vorinostat in concentrations according to their recommended dosage for cancer treatment in humans. Metacestode tissues were exposed *in vitro* to 10μM, 5 μM, 2μM of docetaxel (Sigma-Aldrich; #01885-F), to 4.5 μg/ml, 3 μg/ml and 1.5μg/ml of doxorubicin (Sigma-Aldrich; #44583), to 4.5μg/ml, 3μg/ml, 1.5μg/ml and 0.75μg/ml of navelbine (Pierre Fabre, Freiburg, Germany, 10mg/ml; UKT#2698), to 10μM, 5μM and 2μM paclitaxel (Sigma-Aldrich; #T7191), to 10μg/ml, 7.5μg/ml, 2μg/ml and 1μg/ml of vorinostat (SAHA) (Sigma-Aldrich; SML0061) and to DMSO (Sigma-Aldrich; #D8418) (0.1%, 0.05%; solvent control) at the indicated concentrations, and the effects of these cytostatic compounds on growth and proliferation of *E. multilocularis* metacestodes were evaluated *in vitro* and *in vivo*.

E. multilocularis infection in vivo

Metacestodes of *E. multilocularis* were cultured *in vitro* by established techniques [11] and the infection of *M. unguiculatus* was carried out according to the previous study [11]. In brief, metacestodes were exposed to cytostatic drugs or drug-free culture media (as above) for 1 week and then *in vitro* culture continued for another week in drug-free culture media. Thereafter, metacestode tissue blocks were split in half, one for further *in vitro* culture and monitoring of vesicle production and the other half was used to prepare the metacestode suspension for intra peritoneal injection (i.p.) in *M. unguiculatus*. The growth of drug-treated and untreated *E. multilocularis* metacestodes was monitored *in vivo*. All animals were examined for metacestode growth by *in vivo* magnetic resonance imaging (MRI) and tracer-guided positron emission tomography (PET). After the last PET scan at 5 months p.i., *M. unguiculatus* were autopsied, the *Em*-metacestode tissues removed and weighted. Most metacestode tissues were recovered from the peritoneal cavity either as singularly isolated masses or dissected from liver, kidney or gut tissues.

Drug-exposure of *in vitro* cultured *E. multilocularis* metacestodes

Drug-exposed metacestode tissue blocks were cultured *in vitro* as described previously [11]. One half of the drug exposed parasite tissue was incubated with RPMI 1640 medium supplemented with 10 % FCS and 1 % penicillin/streptomycin (Biochrom GmbH, Berlin, Germany) at 37 °C and 5 % CO_2. The culture medium was changed once a week and *E. multilocularis* vesicles were collected from the cell culture and the number of produced vesicles (diameter 2 to 4 mm) counted. The number of vesicles produced in culture was scored (Figure 5), i.e. Score 0 = no vesicle, Score 1 = very few vesicles (1-5), Score 2 = few vesicles (6-10), Score 3 = vesicles (11-20), Score 4 = vesicles (21-30), Score 5 = vesicles (>31).

***In vivo* volumetric quantization of parasite growth**

For the volumetric evaluation of *E. multilocularis* metacestode growth in infected *Meriones*, *in vivo* MRI was performed using a 7T, 300Mhz small animal MR tomograph (Bruker Biospin MRI GmbH, Ettlingen, Germany) for the acquisition of anatomical information. The animals were anesthetized during the measurements with 1.5 % isoflurane mixed with 100 % oxygen under respiration monitoring. The images were acquired using a T2 fat saturated 3D sequence with a TE/TR of 90.51/1800.000ms and data were analyzed using Inveon Research Workplace software (IRW, Siemens Preclinical Solutions, Knoxville, TN, USA). Parasite tissues were delineated from other organs based on the anatomical information obtained from the MR images and marked as regions of interest (ROIs) and quantified volumes are expressed as cubic millimeters.

PET Tracer Production

Fluorine-18 was produced as ^{18}F-fluoride at the PET trace cyclotron (General Electric Medical Systems, GEMS, Uppsala, Sweden) using the $^{18}O(p,n)^{18}F$ nuclear reaction, and [^{18}F]FDG was synthesized as described [40].

PET/MR Imaging

Simultaneous PET/MR imaging was performed with *E. multilocularis* infected gerbils 5 months p.i*. In vivo* bio-distribution of the PET tracer [^{18}F]FDG was assessed using a small animal PET insert (Bruker Biospin GmbH, Ettlingen, Germany) yielding a spatial resolution of approximately 1.3 mm in the reconstructed images [41]. All animals were shortly anaesthetized with isoflurane and *i.v.* injected with 10-12 MBq of the tracer via a lateral tail vein. Static (10 min) PET scans were acquired after the injection of the tracer. During PET/MR imaging, the animals were anesthetized with 1.5 % isoflurane

mixed with 100 % oxygen. Anesthesia was monitored by measuring the respiratory frequency, and the body temperature was kept at 37 °C using a heating pad. PET data were acquired in list-mode, histograms collected in one 10 min time frame and reconstructed using an iterative ordered subset expectation maximization (OSEM) algorithm. No attenuation correction was applied. MR imaging was performed as described above on a 7T, 300 Mhz dedicated small animal MR tomograph obtaining anatomical information for parasite delineation. In addition to the T2 fat saturated 3D sequence, a T1 3D fast low angle shot (FLASH) sequence with a TE/TR of 6.000/30.000ms was performed. PET images were normalized to each other, subsequently fused to the respective MR images and analyzed using IRW. ROIs were drawn around the respective tissue based on the anatomical information obtained from the MR images. Absolute quantification of the PET data is expressed as percentage of the injected dose per cubic centimeter (%ID/cc). After the final PET scan, all animals were euthanized under deep anesthesia and parasite tissue was removed and weighted.

Statistical Analysis

For the analysis of microarray data, significance was calculated using a t-test without corrections for multiple testing selecting all transcripts with a minimum change in expression level of 1.5-fold together with a p-value of less than 0.05. The signal strength of hybridization of the *E. multilocularis* cDNA samples to the human microarray chip greater than 100 above background were selected.

LIST OF ABBREVIATIONS

Alveolar echinococcosis (AE); benzimidazole (BMZ); *Echinococcus multilocularis* (Em); Food and Drug Administration (FDA); intra peritoneal (i.p.); Magnetic resonance imaging (MRI); maximum intensity projections (MIP); non detected (n.d.); nuclear magnetic resonance (NMR); positron emission tomography (PET); post infection (p.i.); 2-deoxy-2-[^{18}F]-fluoro-D-glucose ([^{18}F]FDG); percentage of the injected dose per cubic centimeter (%ID/cc);

AUTHOR CONTRIBUTIONS

XH, SW and PTS designed the experiments. XH, SW, AMR, PV, WH and PTS performed experiments and analyzed data. XH, SW, AMR and PTS wrote the manuscript. BG and CK advised on experimental design and provided critical feedback. All authors reviewed the manuscript.

FUNDING

This work was supported by the Baden-Württemberg Stiftung (Grant P-LS-AL2/06), the Bundesministerum für Bildung und Forschung (BMBF grant 01KA1008), the Deutsche Forschungsgemeinschaft (DFG grant WI3777/1-2) and the Werner Siemens Fundation.

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

References

01. Kern P, Bardonnet K, Renner E, Auer H, Pawlowski Z, AmmannRW, Vuitton DA, Kern P; European Echinococcosis Registry. European echinococcosis registry: human alveolar echinococcosis, Europe, 1982-2000. Emerging Infectious Diseases. 2003; 9:343-349.
02. Lubinsky G, Lee CF, Baron RW. Attempts at chemotherapy of Echinococcus multilocularis infections in rodents. II. A study of some parasiticides and cytostatic agents. Canadian journal of zoology. 1971; 49:1301-1304.
03. Novak M. Efficacy of mitomycin C against alveolar Echinococcus. International journal of parasitology. 1990; 20:119-120.
04. Liance M, Nemati F, Bories C, Couvreur P. Experience with doxorubicin-bound polyisohexylcyanoacrylate nanoparticles on murine alveolar echinococcosis of the liver. Int J Parasitol. 1993 May;23(3):427-9.
05. Naguleswaran A, Spicher M, Vonlaufen N, Ortega-Mora LM, Torgerson P, Gottstein B, Hemphill A. *In vitro* metacestodicidal activities of genistein and other isoflavones against Echinococcus multilocularis and Echinococcus granulosus. Antimicrobial Agents and Chemotherapy 2006; 50:3770-3778.
06. Stadelmann B, Scholl S, Müller J, Hemphill A. Application of an *in vitro* drug screening assay based on the release of phosphoglucose isomerase to determine the structure-activity relationship of thiazolides against Echinococcus multilocularis metacestodes. Journal of Antimicrobial Chemotherapy. 2010; 65:512-519.
07. Hemer S, Brehm K. *In vitro* efficacy of the anticancer drug imatinib on Echinococcus multilocularis larvae. International journal of antimicrobial agents. 2012; 40:458-462. doi: 10.1016/j.ijantimicag.2012.07.007.
08. Küster T, Kriegel N, Stadelmann B, Wang X, Dong Y, Vennerstrom JL, Keiser J, Hemphill A. Amino ozonides exhibit *in vitro* activity against Echinococcus multilocularis metacestodes. International journal of antimicrobial agents. 2014; 43:40-46.

09. Pensel PE, Albani C, Gamboa GU, Benoit JP, Elissondo MC. *In vitro* effect of 5-fluorouracil and paclitaxel on Echinococcus granulosus larvae and cells. Acta tropica. 2014; 140:1-9. doi: 10.1016/j.actatropica.2014.07.013.
10. Spicher M, Naguleswaran A, Ortega-Mora LM, Müller J, Gottstein B, Hemphill A. *In vitro* and *in vivo* effects of 2-methoxyestradiol, either alone or combined with albendazole, against Echinococcus metacestodes. Experimental parasitology 2008; 119:475-482. doi: 10.1128/AAC.00553-08.
11. Hübner C, Wiehr S, Kocherscheidt L et al. Effects of *in vitro* exposure of Echinococcus multilocularis metacestodes to cytostatic drugs on *in vivo* growth and proliferation of the parasite. Parasitology research 2010; 107:459–463.
12. Stadelmann B, Aeschbacher D, Huber C, Spiliotis M, Müller J, Hemphill A. Profound activity of the anti-cancer drug bortezomib against Echinococcus multilocularis metacestodes identifies the proteasome as a novel drug target for cestodes. PLoS neglected tropical diseases. 2014; 8(12):e3352.
13. McGrogan BT, Gilmartin B, Carney DN, McCann A. Taxanes, microtubules and chemoresistant breast cancer. Biochimica et biophysica acta. 2008; 1785:96-132.
14. Sato A. Vorinostat approved in Japan for treatment of cutaneous T-cell lymphomas: status and prospects. Onco targets and therapy. 2012; 5:67-76.
15. Cappetta D, Rossi F, Piegari E, Quaini F, Berrino L, Urbanek K, De Angelis A. Doxorubicin targets multiple players: A new view of an old problem. Pharmacological research. 2017;. pii: S1043-6618(17)30045-2. doi: 10.1016/j.phrs.2017.03.016.
16. Judenhofer MS, Wehrl HF, Newport DF, Catana C, Siegel SB, Becker M, Thielscher A, Kneilling M, Lichy MP, Eichner M, Klingel K, Reischl G, Widmaier S, Röcken M, Nutt RE, Machulla HJ, Uludag K, Cherry SR, Claussen CD, Pichler BJ. Simultaneous PET-MRI: a new approach for functional and morphological imaging. Nat Med. 2008; 14:459-465. doi: 10.1038/nm1700.
17. Honndorf VS, Wiehr S, Rolle AM, Schmitt J, Kreft L, Quintanilla-Martinez L, Kohlhofer U, Reischl G, Maurer A, Boldt K, Schwarz M, Schmidt H, Pichler BJ. Preclinical evaluation of the anti-tumor effects of the natural isoflavone genistein in two xenograft mouse models monitored by [18F]FDG, [18F]FLT, and [64Cu]NODAGA-cetuximab small animal PET. Oncotarget. 2016; 7:28247282-61. doi: 10.18632/oncotarget.8625.
18. Rolle AM, Hasenberg M, Thornton CR, Solouk-Saran D, Männ L, Weski J, Maurer A, Fischer E, Spycher PR, Schibli R, Boschetti F, Stegemann-Koniszewski S,

Bruder D, Severin GW, Autenrieth SE, Krappmann S, Davies G, Pichler BJ, Gunzer M, Wiehr S. ImmunoPET/MR imaging allows specific detection of Aspergillus fumigatus lung infection *in vivo*. Proceedings of the national academy of science USA. 2016; 113:E1026-1033. doi: 10.1073/pnas.1518836113.

19. Caoduro C, Porot C, Vuitton DA, Bresson-Hadni S, Grenouillet F, Richou C, Boulahdour H, Blagosklonov O. The role of delayed 18F-FDG PET imaging in the follow-up of patients with alveolar echinococcosis. Journal of nuclear medicine. 2013; 54):358-363. doi: 10.2967/jnumed.112.109942.
20. Rolle AM, Soboslay PT, Reischl G, Hoffmann WH, Pichler BJ, Wiehr S. Evaluation of the Metabolic Activity of Echinococcus multilocularis in Rodents Using Positron Emission Tomography Tracers. Molecular imaging and biology. 2015; 17:512-520. doi: 10.1007/s11307-014-0815-3.
21. Gorgas D, Marreros N, Rufener R, Hemphill A, Lundström-Stadelmann B. To see or not to see: non-invasive imaging for improved readout of drug treatment trials in the murine model of secondary alveolar echinococcosis. Parasitology. 2017; 8:1-8. doi: 10.1017/S0031182017000051.
22. Budke CM, Casulli A, Kern P, Vuitton DA. Cystic and alveolar echinococcosis: Successes and continuing challenges. PLoS neglected tropical diseases. 2017; 11:e0005477. doi: 10.1371/journal.pntd.0005477.
23. Brehm K, Koziol U. Echinococcus-Host Interactions at Cellular and Molecular Levels. Adv Parasitology. 2017; 95:147-212. doi: 10.1016/bs.apar.2016.09.001.
24. Hemphill A, Spicher M, Stadelmann B, Mueller J, Naguleswaran A, Gottstein B, Walker M. Innovative chemotherapeutical treatment options for alveolar and cystic echinococcosis. Parasitology. 2007 Nov;134(Pt 12):1657-70. Epub 2007 Jul 16.
25. van Knapen F. Echinococcus granulosus infection and malignancy. British Medical Journal. 1980;vol. 281, no. 6234, pp. 195–196.
26. Alvarez-Errico D, Medeiros A, Miguez M, Casaravilla C, Malgor R, Carmona C, Nieto A, Osinaga E. O-glycosylation in Echinococcus granulosus: identification and characterization of the carcinoma-associated Tn antigen. Experimental Parasitology. 2001;98:100–109..
27. Hemphill A, Stadelmann B, Rufener R, Spiliotis M, Boubaker G, Müller J, Müller N, Gorgas D, Gottstein B. Treatment of echinococcosis: albendazole and mebendazole -what else? Parasite. 2014; 21:70. doi: 10.1051/parasite/2014073.

28. Jura H, Bader A, Frosch M. *In vitro* activities of benzimidazoles against Echinococcus multilocularis metacestodes. Antimicrobial agents and chemotherapy. 1998; 42:1052-1056.
29. Küster T, Stadelmann B, Hermann C, Scholl S, Keiser J, Hemphill A. *In vitro* and *in vivo* efficacies of mefloquine-based treatment against alveolar echinococcosis. Antimicrobial agents chemotherapy. 2011; 55:713-721. doi: 10.1128/AAC.01392-10.
30. Harmsen S, Teraphongphom N, Tweedle MF, Basilion JP, Rosenthal EL. Optical Surgical Navigation for Precision in Tumor Resections. Molecular imaging and biology. 2017; 19:357-362. doi: 10.1007/s11307-017-1054-1.
31. Claudon M, Bessieres M, Regent D, Rodde A, Bazin C, Gerard A, Bresler L. Alveolar echinococcosis of the liver: MR findings. Journal of computer assisted tomography. 1990; 14:608-14.
32. Duewell S, Marincek B, von Schulthess GK, Ammann R. [MRT and CT in alveolar echinococcosis of the liver]. Rofo. 1990; 152:441-445.
33. Ojima I, Lichtenthal B, Lee S, Wang C, Wang X. Taxane anticancer agents: a patent perspective. Expert opinion on therapeutic patents. 2016; 26:1-20. doi: 10.1517/13543776.2016.1111872.
34. Grossi F, Kubota K, Cappuzzo F, de Marinis F, Gridelli C, Aita M, Douillard JY. Future scenarios for the treatment of advanced non-small cell lung cancer: focus on taxane-containing regimens. Oncologist. 2010; 15:1102-1112. doi: 10.1634/theoncologist.2010-0322.
35. Xu YC, Wang HX, Tang L, Ma Y, Zhang FC. A systematic review of vinorelbine for the treatment of breast cancer. Breast journal. 2013; 19:180-188. doi: 10.1111/tbj.12071.
36. Sparreboom A, van Tellingen O, Nooijen WJ, Beijnen JH. Preclinical pharmacokinetics of paclitaxel and docetaxel. Anticancer Drugs. 1998; 9:1-17.
37. Crown J. Docetaxel: overview of an active drug for breast cancer. Oncologist. 2001; 6 Suppl 3:1-4.
38. Goldstein LJ. MDR1 gene expression in solid tumors. European journal of cancer. 1996; 32A:1039–1050.
39. Hemphill A, Gottstein B. Immunology and morphology studies on the proliferation of *in vitro* cultivated Echinococcus multilocularis metacestodes. Parasitology research. 1995; 81:605-614.

40. Hamacher K, Coenen HH, Stocklin G. Efficient stereo-specific synthesis of no-carrier-added 2-[18F]-fluoro-2-deoxy-D-glucose using aminopolyether supported nucleophilic substitution. Journal of nuclear medicine 1986; 27:235–238.
41. Wehrl HF, Hossain M, Lankes K, Liu CC, Bezrukov I, Martirosian P, et al. Simultaneous PET-MRI reveals brain function in activated and resting state on metabolic, hemodynamic and multiple temporal scales. Nature Medicine. 2013; 19:1184-1189.

Curriculum vitae

Name	Patrick Voßberg
Date of Birth	01.04.1989
Nationality	German

Academic education

04/2016 - 03/2019	Doctoral Thesis at the University Clinics of Tübingen, Institute for Tropical Medicine
03/2016 - 05/2016	Research stay in Togo/ West Africa
05/2015 - 03/2016	Master Thesis at the University Clinics of Tübingen, Institute for Tropical Medicine “Immunepidemiologische Untersuchung des O*nchocerca volvulus* Infektionsstatus bei Personengruppen in einem Onchozerkose-Endemiegebiet in Nord- und Zentraltogo“
09/2015 - 11/2015	Research stay in Togo/ West Africa
10/2013 - 03/2016	Master of Science in Biology at the University of Tübingen Major subject: Molecular Cell Biology and Immunology Minor subject: Parasitology
10/2012- 09/2013	Bachelor Thesis at the University Clinics of Tübingen, Institute for Tropical Medicine “Die Antikörper-Reaktivität und zelluläre Cytokin- und Chemokinantworten bei Filariose-Patienten und Kontrollpersonen auf Peptid-Antigene von *Onchocerca volvulus“*
01/2013 - 04/2013	Research stay in Togo/ West Africa
10/2009 - 09/2013	Bachelor of Science Biology at the University of Tübingen

Military Service

10/2008 - 06/2009	JaboG 32, Lagerlechfeld

Acknowledgements

In the past years I worked in several research projects, not only in Tübingen, but also in Togo. The result of these works is the present dissertation. Without a lot of help from supporters and friends this would not have been possible.

I especially want to thank Prof. Dr. Hans-Georg Rammensee for his willingness to act as my PhD supervisor.

In addition, I would like to thank Prof. Dr. Peter Soboslay for allowing me, after completing my bachelor and master thesis in his laboratory, to perform and complete my PhD in his laboratory, and for his support and advice throughout these past years.

Dr. Dr. Carsten Köhler I would like to thank for his multiple support and advice.
Prof. Dr. Stefan Stevanović and his team have supported my works through the identification and synthesis of peptides.

From Prof. Dr. Peter Kern and Dr. Beate Grüner at the University Clinics of Ulm I have received a lot of support and cooperation throughout the works and studies with echinococcosis.

I am thankful to my colleagues in Tübingen and Togo, notably Dr. Richard Gantin, Dr. Wolfgang Hoffmann, Dr. Christian Lechner and Dr. Xiangsheng Huang for their support and the great time during and after work.

For their technical support, I would like to thank all colleagues and employees of the Centre Hospitalier Regional de Sokodé and the Institute for Tropical Medicine in Tübingen, and notably, for their practical advices and many unforgettable moments.
Last but not least, I want to thank my parents and my friends for their inimitable support in all matters during the last years.