Evaluation of *Wolbachia* depletion after antibiotic treatment using real-time PCR in Onchocerciasis and Lymphatic Filariasis, and analysis of genetic associations in Lymphatic Filariasis

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1. Introduction

1.1 Filariasis

Infections with filarial nematodes affect more than 150 million people mainly in the tropics. Official estimates state that a total of 120 million people are infected with the causative agents of lymphatic filariasis (LF), *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* and >1.34 billion people are at risk of infection living in one of the 81 endemic countries [1]. For *Onchocerca volvulus*, which is endemic in 19 african countires, >102 million people are at risk of infection [2] with approximately 37 million people being already infected [3].

1.2 Lymphatic filariasis (LF)

Lymphatic filariasis is endemic in 81 countries of the world. These comprise sub-Saharan Africa (plus a small focus in the Nile Delta), South Asia (with a large population infected in the Indian subcontinent), Southeast Asia, many tropical Pacific islands and some areas in South America (Figure 1) [4].

More than 1 billion people are living in endemic areas and thus are at risk to acquire infection [5]. Infective third stage larvae (L3) are transmitted by insect vectors, *Anopheles* in rural African and *Culex* in urban/sub-saharan areas. In Southeast Asia the vectors are mainly *Mansonia* and *Aedes* spp..

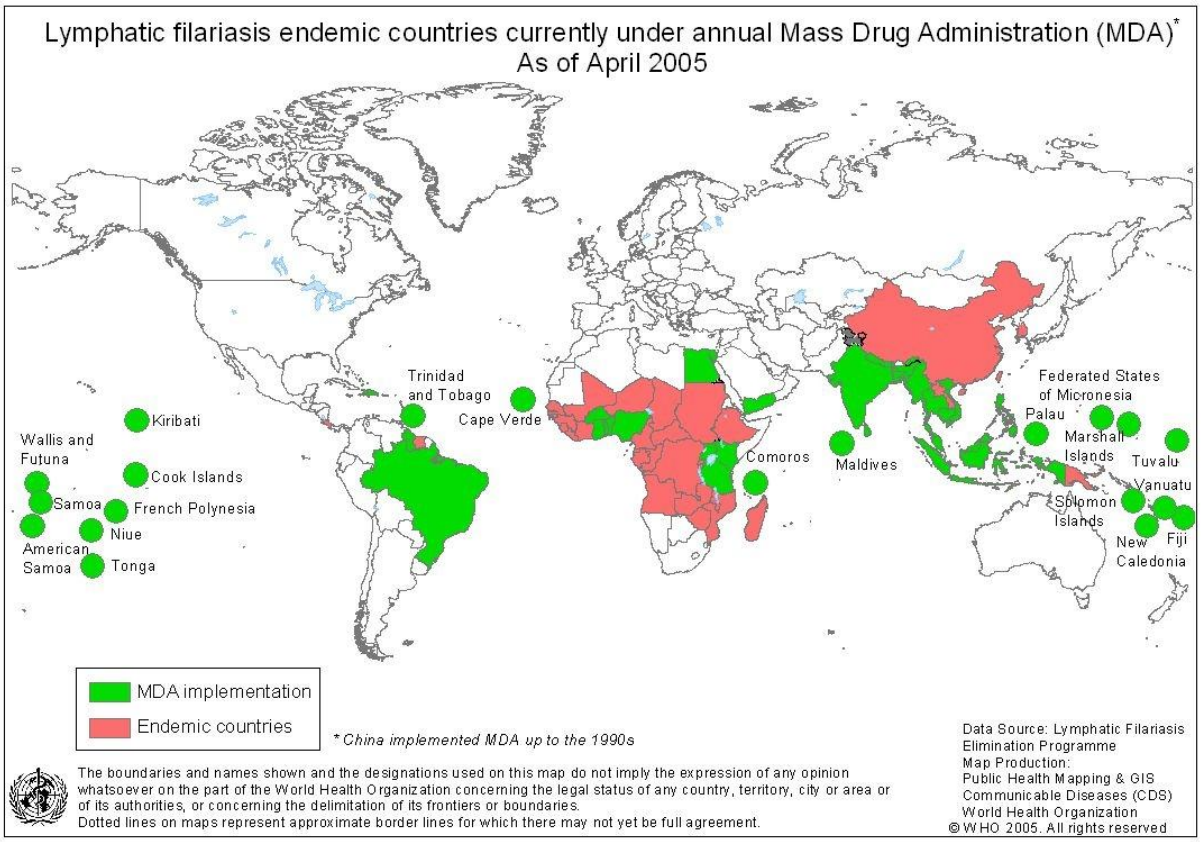


Figure 1 Global distribution of lymphatic filariasis www.who.int

The first stage larvae (Microfilariae, Mf) are picked up by the vector and develop with two moults into infective L3 (Figure 2). During a blood meal, these are deposited on the skin surface and actively invade the host through the biting area. There, the L3 develop to the L4 stage and finally to adult worms. Adult filarial parasites are sexually dimorphic and reside in the lymphatic vessels, where they mate and produce thousands of Mf for up to eight years. In adult men, *W. bancrofti* [6-8] but not *B. malayi* [9] are usually seen in the scrotal areas (Figure 3) where they can be visualized via ultrasonography (USG) in dilated lymphatics.

After 6-12 months, Mf can be detected in approximately 50% of the infected individuals. They show a periodicity in adaption to the preferred biting hours of their vector. *Culex*, *Anopheles* and *Mansonia* spp. are vectors active at night whereas *Aedes* spp. are daytime vectors.

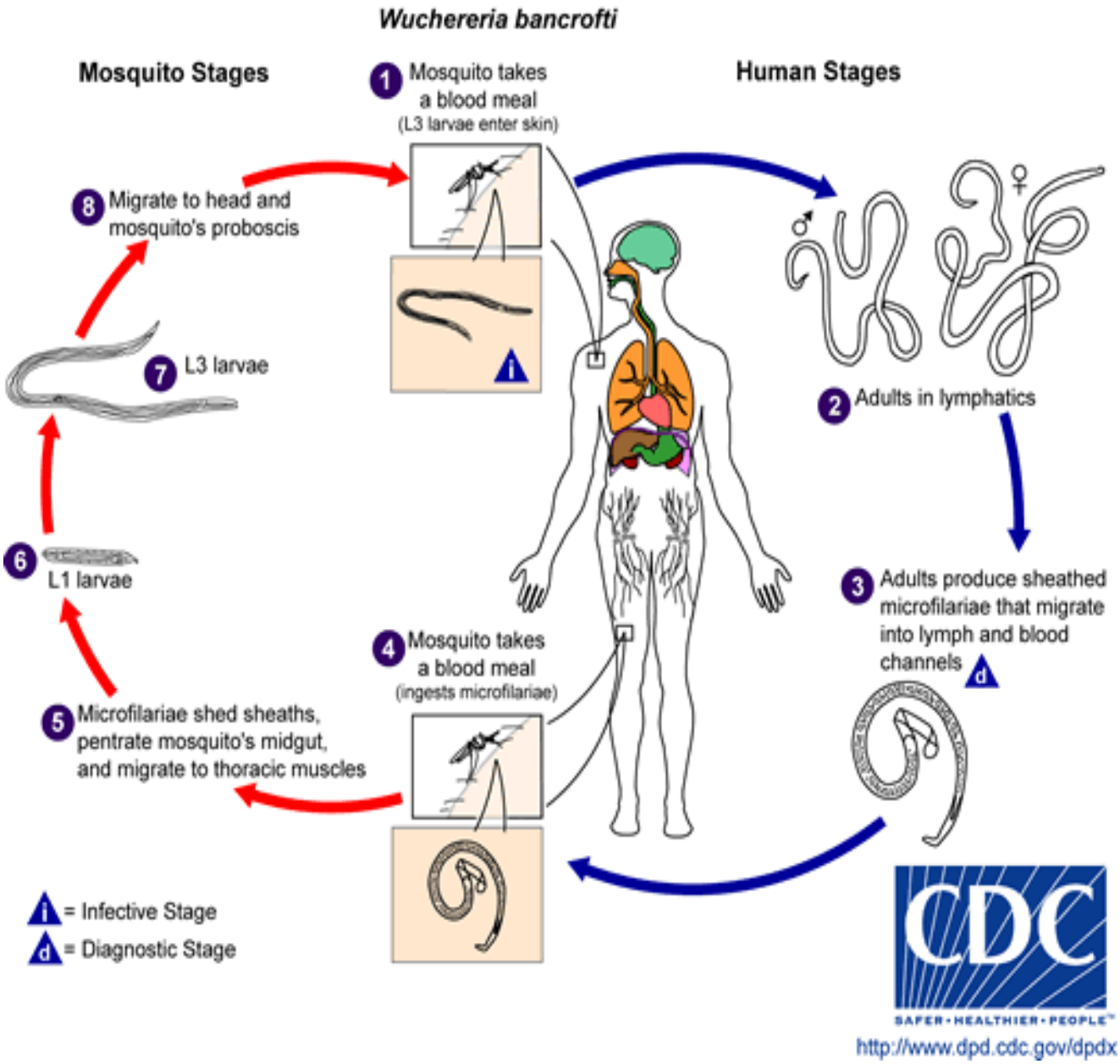


Figure 2 Life cycle of *W. bancrofti*, causative agent of LF

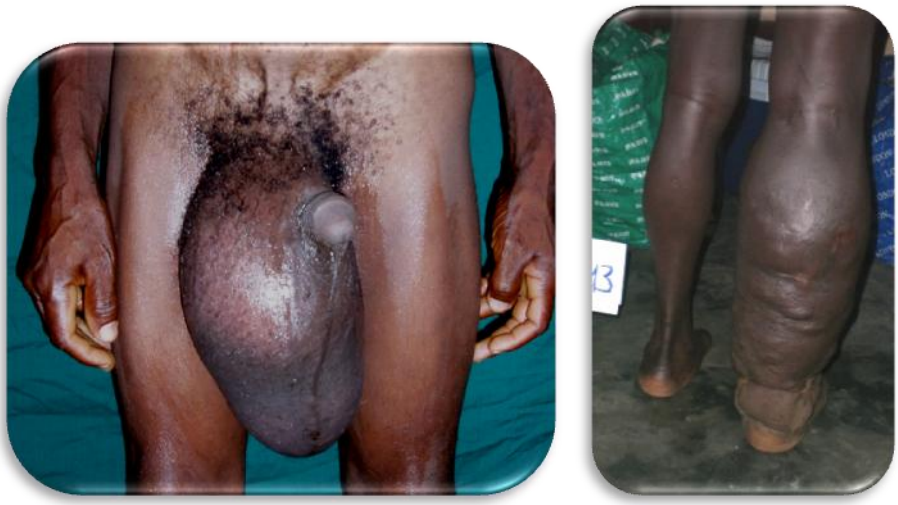


Figure 3 Hydrocele and lymphedema Pictures © Sabine Mand

Except for the rare disease of tropical pulmonary eosinophilia, which is a hyper-reactivity to Mf while they are passing through the lung, Mf do not induce major disease. The major pathologies of LF are attributed to death and destruction of adult worms within the lymphatic vessels. Clinical studies have also implicated a role for secondary opportunistic infections along with other cofactors in advancing the chronic pathologies of LF [10]. The pathology in LF has a spectrum of clinical states with two major poles. One pole is represented by microfilaremic patients with high parasite numbers and down regulated cell-mediated immune responses, and the other by patients with lymphedema (LE) or hydrocele (Figure 1) who typically have few or no parasites but specific immune reactions [11].

Approximately 40 million people suffer from the stigmatizing and disabling clinical manifestations of the disease, including 15 million who have lymphoedema (elephantiasis) and 25 million men who have urogenital swelling, principally scrotal hydrocele [1]. LE and hydrocele are not mutually exclusive and both involve dilation of the lymphatic vessels and flow of lymph into surrounding tissues, indicating a shared pathogenesis.

Both pathologies develop progressively and not all affected individuals will progress to the most severe form of pathology.

1.3 Onchocerciasis

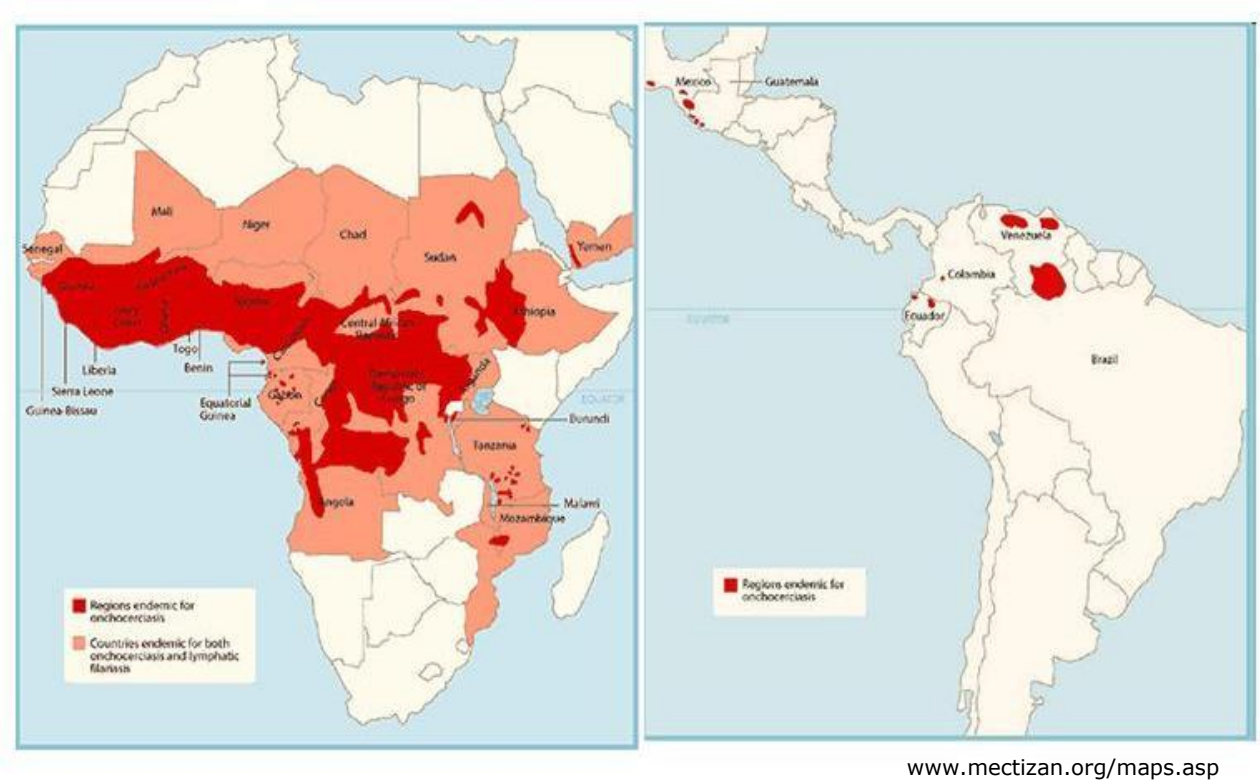
The parasite *Onchocerca volvulus* is transmitted by small blackflies of the genus *Simulium*, which breed in fast-flowing, highly oxygenated rivers and streams [12]. An infected blackfly deposits one or more infective L3 which have developed within 10 days from Mf acquired from other humans during a preceding blood meal. The L3 develop into adults within a year and accumulate in subcutaneous or deep nodules, so-called onchocercomata (Figure 4).

Figure 4 Life cycle *O.volvulus*, causative agent of onchocerciasis

Pictures © D. W. Büttner, S. Mand and S. Specht

In onchocerciasis, Mf are born from viviparous females and induce disease symptoms, including chronic dermatitis and skin atrophy, lymphadenitis and fibrosis. The Mf migrate through the nodular tissue and then to the dermal part of the skin where they can be taken up by blackflies during a blood meal. Invasion of the cornea by Mf leads to corneal opacity and thus, can lead to blindness. Onchocerciasis is the second most common cause of blindness induced by an infectious agent in the developing world. About 50% of adults are affected by blindness in some West african Savannah communities with an estimated economic loss of US$ 30 Million [13]. The WHO estimated in 1995 that 800,000 people are visually impaired and 270,000 are blind due to infection with *O. volvulus* [14]. Very important, although second to blindness, are the various forms of dermatitis that Mf induce in the skin. In a highly infected individual with more than five palpable nodules, one can calculate a daily turnover of 50,000 Mf [15]. The females produce millions of Mf during their lifetime, which lasts up to 14 years [16]. Thus, infected persons are constantly exposed to worm antigenic molecules. *O. volvulus* develops only in human and presumably has no animal reservoir.

The disease is most closely associated with West and Central Africa with 19 endemic countries, but it is also prevalent in Yemen and six countries in Latin America (Figure 5) [2, 13].



www.mectizan.org/maps.asp

Figure 5 Global distibution of onchocerciasis in Africa and America. Endemic areas for onchocerciasis are in red, co-endemic areas for onchocerciasis and LF are in orange.

1.4 Treatment strategies

The World Health Assembly, in its 1997 resolution WHA 50.35, has called for the elimination of onchocerciasis and lymphatic filariasis as public health problems [17]. There are three standard drugs in use for filarial infections: diethylcarbamazine (DEC), albendazole (ALB) and ivermectin (IVM). DEC is the oldest of the three and is still used for LF, since it has a strong microfilaricidal and partial macrofilaricidal effect [18]. In individuals with infection, adverse reactions can arise after DEC is given. Reactions are associated with the rapid killing of Mf wich induces systemic inflamation caused by release of *Wolbachia* endobacteria [19]. DEC is known to cause irreversible ocular damage in onchocerciasis patients with moderate-to-high Mf counts. This has precluded its use for onchocerciasis as well as for LF or loiasis in areas where onchocerciasis is coendemic.

A broad-spectrum anthelmintic which is effective against nematodes, flatworms and cestodes is ALB [20]. The drug inhibits polymerisation of β-tubulin and microtubule formation.

Ivermectin, a macrocyclic lactone [21], was introduced as treatment for onchocerciasis in the 1980s. It acts through inhibition of glutamate-gated chloride channels of Mf, effectively immobilizing them so that they are carried with the lymphatic flow from their usual locations into the regional lymph nodes. Since Mf are killed away from the eye, there is no ocular damage following IVM.

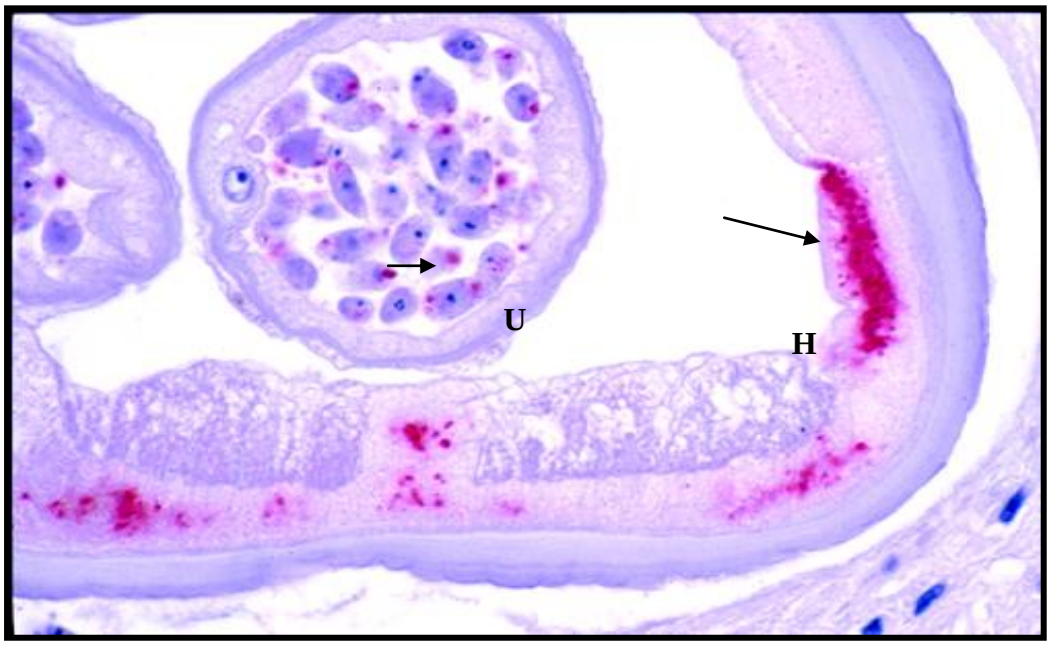
Mass drug administration (MDA) is the major tool of intervention in the different control programs: the African Programme of Onchocerciasis Control (APOC) [2, 22-24], the Onchocerciasis Elimination Programme for the Americas (OEPA) [24, 25] and the Global Programme to Eliminate Lymphatic Filariasis (GPELF) [1, 5, 26]. All programs use different treatment regimes ranging from a single dose of IVM per year (APOC) or IVM and ALB (GPELF) in Africa to twice-yearly application of IVM (OEPA).

The general idea in mass chemotherapy is to abolish Mf in the skin and blood in a large enough proportion of the population so that the cycle of transmission is inhibited or blocked. However, even after the end of transmission, mass treatment has to continue at least as long as adult worms survive in humans (up to 14 years) [27]. A major problem with the current control strategy is that while IVM and DEC kill Mf, they do not effectively kill adult worms nor permanently stop Mf production.

For almost 30 years, it has been known that filarial nematodes contain endosymbiotic bacteria. The endobacteria are found in the hypodermis of male and female worms, and in oocytes, embryos and all larval stages (Figure 6). Apart from many animal filarial species, endobacteria are present in the human filariae *W. bancrofti*, *Brugia* spp. and *O. volvulus* [28, 29], *Mansonella perstans* [30] and *M. ozzardi* [31], but not in *L. loa* [32, 33].

These endosymbionts were classified at the molecular level to be of the genus *Wolbachia*, which are also common endosymbionts of arthropods [34, 35].

In filarial species that harbour *Wolbachia* as endosymbionts, it seems to be a mutualistic adaption that has lasted for many million years [36], with vertical transmission of the bacteria via oocytes from one worm generation to the next.



Picture © by D. W. Büttner

Figure 6 Female *O. volvulus* worm with *Wolbachia* endosymbionts (purple colour)

U: Uterus

H: Hypodermis

The efficacy of tetracycline antibiotics to deplete the *Wolbachia* endosymbionts, which are essential for larval moulting and adult female worm fertility in those filarial species that harbour them, was first shown in several animal models of filariasis [37-39]. On the molecular level, there is circumstantial evidence that one of the metabolic pathways which the *Wolbachia* provide to the worm may be the synthesis of nucleotides because filarial worms lack most of the genes necessary for nucleotide

synthesis [36], while *Wolbachia* have kept all of them [40]. Lack of nucleotide synthesis would particularly affect cell division in embryos, and indeed this is the first parasitological feature that can be observed after *Wolbachia* depletion [41]. Trials on doxycycline involving humans have been ongoing for more than 10 years [41-49]. The first human trial involved onchocerciasis patients that were treated daily with a dose of 100 mg doxycycline for 6 weeks, followed by IVM 4 months later [41, 44, 47]. This first study [47] showed that *Wolbachia* depletion is antiparasitic (in this case, sterilizing female worms) in human filariasis (Figure 7). Depletion of *Wolbachia* by doxycycline was also demonstrated in human LF patients infected with *W. bancrofti* [42, 43, 49, 50].

The mode of action of doxycycline, which belongs to the tetracyclines, is inhibition of protein biosynthesis by blocking the binding of the 16S part of the 30S ribosomal subunit. This prevents the amino-acyl tRNA from binding to the A site of the ribosome, and thereby inhibits translation of mRNA to protein and finally growth of the cells.

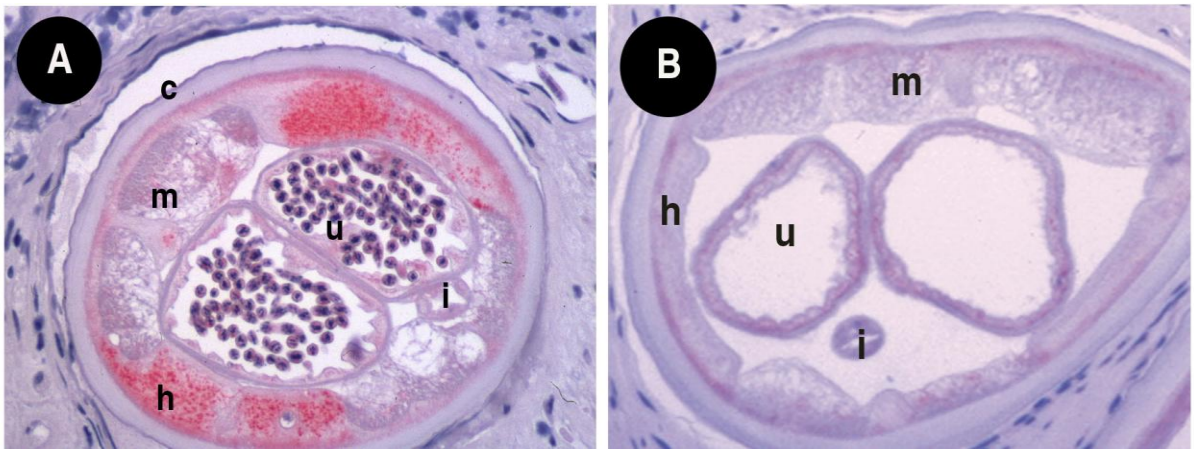


Figure 7 Doxycycline treatment and *Wolbachia* depletion

A= untreated

B=Doxycycline treated 6 weeks 100 mg/day

Results of this thesis show trials with doxycycline in different dosages and treatment times, as well as trials with other antibiotics (rifampicin and azithromycin) targeting the *Wolbachia* endosymbionts in onchocerciasis

and LF. Treatment of bancroftian filariasis with a doxycycline 4-, 6- or 8-week treatment with a 200 mg dose resulted in long-term sterility and eventual death of adult worms [42, 43, 49]. The anti-wolbachial therapy showed substantial improvements in lymphatic pathology with decreased severty in LE and hydrocele in infected individuals [43, 51]. In onchocerciasis, a 6-week treatment with 100 mg/day doxycycline resulted in long-term (>24 months) sterilization of female worms and absence of skin Mf [41, 44, 47]. The slow drug action on adult worm populations of doxycycline and activity against Mf give a good safety profile of this antibiotc and avoids inflammatory adverse reactions due to *Wolbachia* endobacteria. Considering the long duration of 6-week treatment and the fact that young children must not be treated with doxycycline, other antibiotics also have to be analyzed for different activities against the worms and their endobacteria. Azithromycin showed a good activity against intracellular bacteria such as *Chlamydia* spp. and *Mycoplasma* spp. [52] which are related to *Wolbachia*. It prevents bacteria from growing by interfering with their protein synthesis when it binds to the 50S subunit of the bacterial ribosome, and thus inhibits translation of mRNA. Nucleic acid synthesis is not affected. It is a safe drug that can be given to children. This also applies to Rifampicin, which has already shown good activity against *Wolbachia* in experimental trials [53, 54]. Rifampicin inhibits the DNA-dependent RNA polymerase in bacterial cells by binding its beta-subunit, thus preventing the transcription to RNA and subsequently translation to proteins.

1.5 Diagnosis of onchocerciasis and lymphatic filariasis

Diagnosis of filarial infection is required to determine individuals who need treatment, or, with mass treatment programs, where treatment should be delivered. It is also important to ascertain the impact an intervention is having on the infection and whether or not there is reappearance of the infection or renewed transmission in a given area. Moreover, diagnostics for filarial infections are important for validating model predictions, and in

the future for helping to determine when and where mass-treatment activities can be stopped.

First steps always involve the clinical symptoms; in onchocerciasis, these are skin pathology (acute or chronic palpular dermatitis, skin atrophy, lichenification and leopard skin), ocular pathology and the presence of subcutaneous onchocercomas, mainly around the hips, but also at the median side of knees, on the thorax, at the rima ani and on the head. In LF, it is mainly signs of lymphatic pathology, such as lymphangitis (filarial fewer), lymphedema and pains in the scrotal area due to disturbance in the local lymph flow [26].

Current methods of determining prevalence and intensity of onchocerciasis involve palpating nodules of individuals in suspected endemic areas and counting of Mf from skin biopsies. Both methods involve patient material to identify the worms. This is time consuming and requires medical training. Added to that is that a skin biopsy for onchocerciasis can be very painful.

In LF, blood is used for microscopic examination of Mf. This method is very specific but insensitive when Mf counts are low or absent, and so it is inadequate for detecting early, low-intensity (e.g. after MDA) or pre-patent infections.

In bancroftian filariasis, circulating filarial antigen (CFA), which is secreted by adult worms before microfilaremia, can be determined from plasma through a rapid card test or enzyme-linked immunosorbent assay (ELISA) [55, 56]. It has revealed that approximately twice as many individuals are CFA positive compared with being microfilaremic, so that a proportion equal to the MF positives harbours cryptic infections. Visualizing adult worms by USG is also possible in *W. bancrofti* infections [8]. In brugian LF, however, neither the CFA nor the USG methods [9] are able to detect cryptic infections, although statistically they must exist. The only way to further estimate exposure to brugian parasites is with brugian specific IgG4 serology, which can be used as a screening test for filarial exposure, since strong and filarial-specific antibody responses are induced upon

infection. Several recombinant *B. malayi* antigens have been used in ELISAs or rapid-format assays (Brugia Rapid$^{TM)}$[57] (CELISA)[58] to detect filarial specific immunoglobulin IgG4 antibodies. Anti-filarial IgG4 levels have been demonstrated to be elevated in active filarial infection [59-62] and decline post-treatment [63-66]. The advantage of an antibody assay is the low cost of the test, high sensitivity (95% in Mf positive samples) and availability for *B. malayi*. However, the anti-filarial IgG4 assay has limited use in cross-sectional surveys since it cannot discriminate between active infection and past exposure.

Improved diagnostic methods detecting pre-patent and latent LF infections are required. A method of monitoring onchocerciasis and LF before, during and after anti-filarial administration that does not rely on skin biopsies or night-blood collections would be an improvement in time and labour costs, as well as compliance of the study volunteers.

In the last few years, PCR based assays have become the preferred methods for the diagnosis of many parasitic infections in diagnostic laboratories for several reasons. First, these assays do not require intensive training, experience, or skill in microscopy. Second, the equipment and practical skills required to perform PCR are now widespread even in the developing world. In recent years, real-time PCR has increasingly replaced conventional PCR (C-PCR) for technical reasons (improved sensitivity) and practical reasons (faster results). In addition, real-time PCR assays have the potential to be used as high-throughput diagnostic tools for the screening of DNA extracted from blood and tissue, for epidemiological studies, or the monitoring of control programs [67-69].

1.6 Real-time PCR

Quantitative real-time PCR (qPCR) is a powerful system for detection and quantification of pathogens. Using this system the amount of a given pathogen can be precisely estimated, allowing comparison of the pathogen load in different samples. Real-time PCR is a highly sensitive technique enabling amplification and quantification of a specific nucleic acid sequence with detection of the PCR product in real time. In an optimized reaction, the target quantity will approximately double during each amplification cycle. In quantitative PCR, the amount of amplified product is linked to fluorescence intensity using a fluorescent reporter molecule. The fluorescence at each cycle of the amplification progress is measured. The fluorescence intensity increases proportionally with each amplification cycle in response to the increased amplicon concentration. Quantification of DNA, cDNA or RNA targets can easily be achieved by determining the cycle when the PCR product can first be detected.

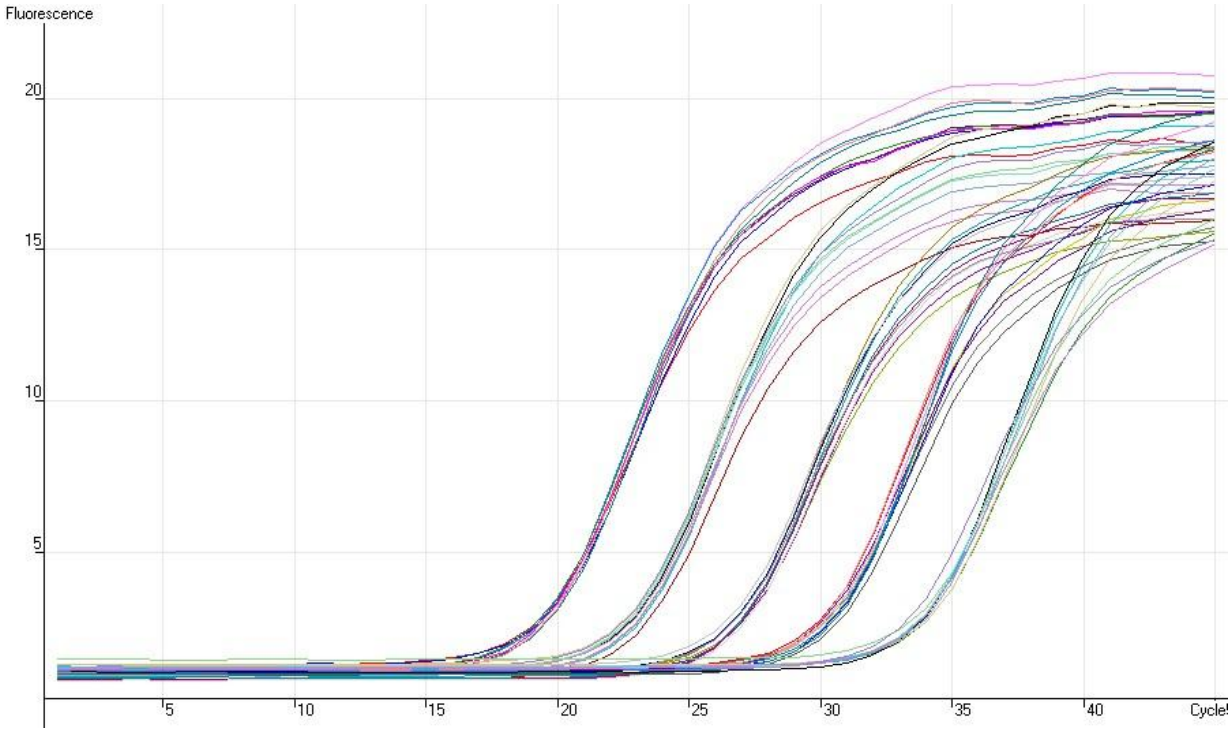


Figure 8 Amplification of standard plasmid dilution series

This is in contrast to end-point detection in conventional PCR, which does not enable accurate quantification of nucleic acids. In real-time PCR, the generation of the amplification products is monitored cycle-by-cycle (Figure 8). This allows measurement of the exponential phase of the reaction and to fix a threshold cycle (C_t). The threshold cycle is the amplification cycle in which the amplification signal passes the threshold line (Figure 9). Fluorescence is measured during each cycle, which greatly increases the dynamic range of the reaction, since the amount of fluorescence is proportional to the amount of PCR product.

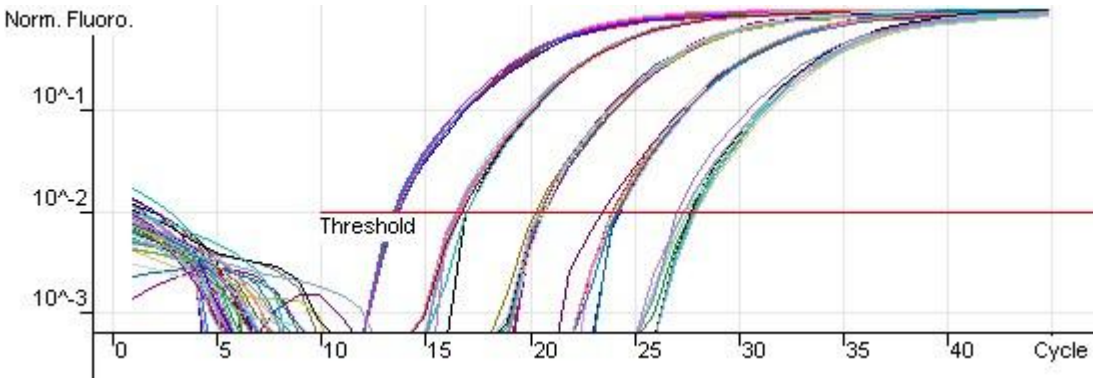


Figure 9 Threshold set in the exponential phase

PCR products can be detected using either fluorescent dyes that bind to double-stranded DNA (SYBR® Green) or fluorescently labelled sequence-specific probes (TaqMan®, Molecular Beacons, Scorpions, FRET, MGB) (Figure 10).

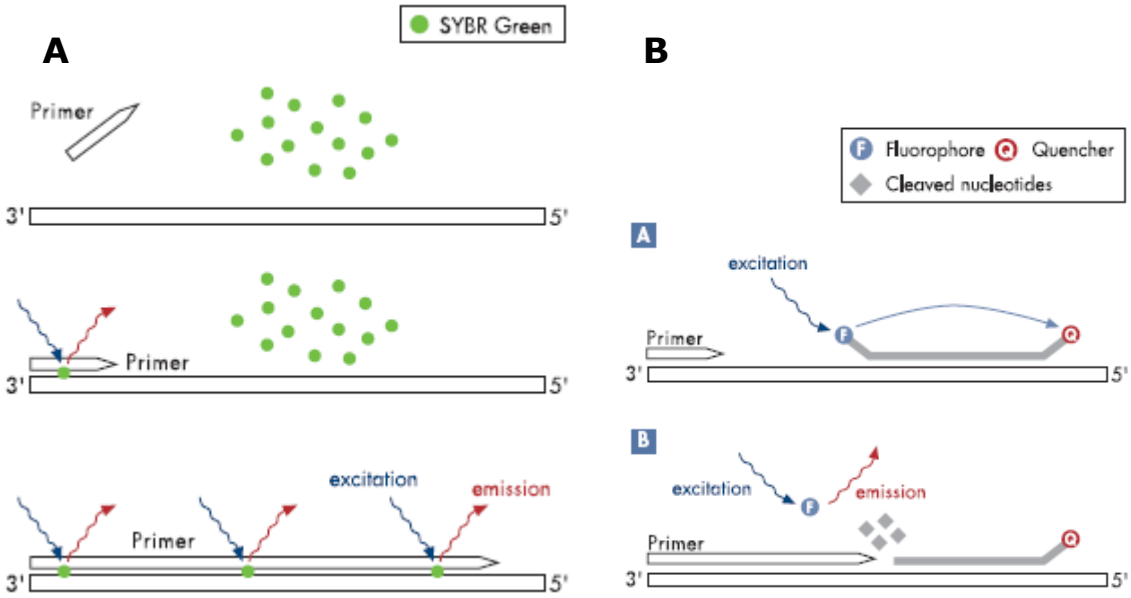


Figure 10 SYBR® Green (A) and TaqMan® (B) principle

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When using SYBR® Green, a melt curve analysis must follow the amplification reaction to exclude false positive samples (Figure 11).

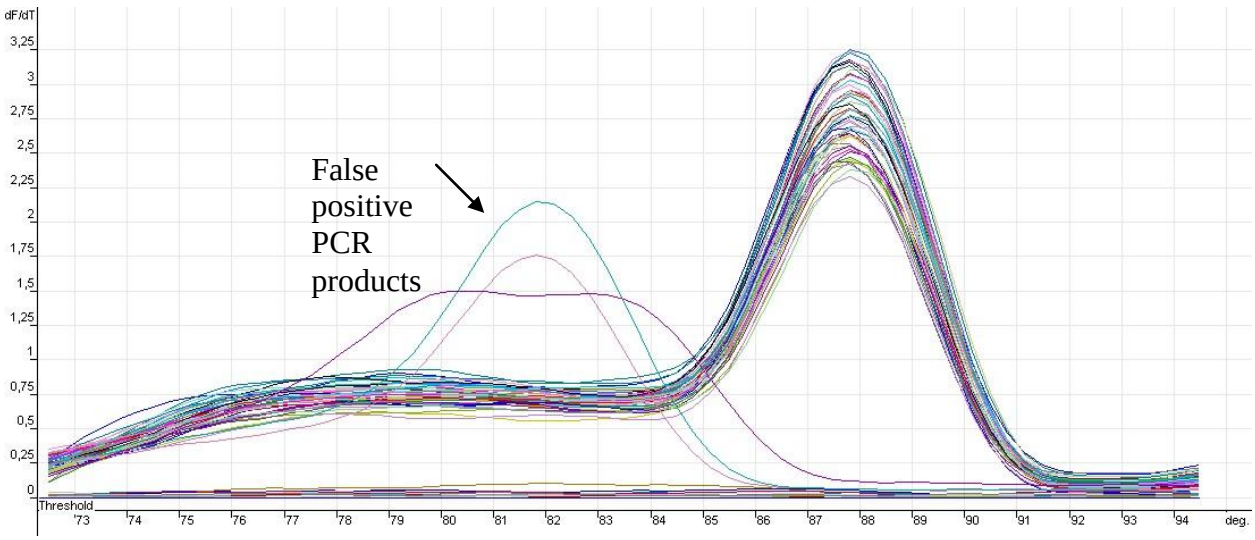

Figure 11 Melt curve analysis in a SYBR® Green real-time PCR assay

Quantification is usually done using a plasmid dilution series as a standard curve with a known copy number of the target sequence in each dilution.

A standard curve (plot of C_t values/crossing points of different standard dilutions against log of amount of standard) is generated using a dilution series of at least 5 different concentrations of the standard (Figure 9). The amount of the unknown target sequence should fall within the range tested. The C_t value of the unknown sample DNA is compared with the standard curve to determine the amount of the target sequence in the unknown sample. For more accurate quantification of difficult samples, where efficiencies are not always the same as the plasmid reference used (<100%), a modified formula of the comparative quantification can be used, which is described in more detail in the patients, materials and methods section (2.1.8).

1.7 Genetic associations in filariasis

The nematodes of bancroftian and brugian filariasis cause lymphatic filariasis that shows a spectrum of clinical states with two major poles. One pole is represented by microfilaremic patients with high parasite numbers and down-regulated cell-mediated responses (asymptomatic), and the other pole by symptomatic patients with LE, tropical eosinophilia or hydrocele, who typically have few or no parasites, but vigorous specific immune reactions [10]. The pathologies develop progressively. It has been known that in areas endemic for LF, not all patients that show exposure to worms become microfilaremic [70] and that infection status (i.e. presence or absence of adult worms) and not the Mf status determines cellular responses, although the Mf stage probably contributes to a lowered immune response. The actual causes of heterogeneity in infection and disease are not well understood, but have been attributed to differences in inflammatory processes that are immune-mediated, to secondary bacterial infections and to host immunogenetics [71-75].

In animal models, T_h2 responses, in particular those dependent on interleukin (IL)-4, have been shown to limit the occurrence and the extent of microfilaremia. Using filarial animal models, it has been shown that genetic factors are also involved in the susceptibility to infection in different mouse strains [76, 77]. Studies in humans have shown that susceptibility to infection, parasite load and lymphatic pathology cluster in families independent of household and environment [78-82]. In Indonesia, Mf levels have been demonstrated to cluster in families even though rates of exposure are equal [81, 82]. An association of the variant Arg110Gln in IL-13, thought to lead to higher IL-13 bioactivity, with severe skin pathology in onchocerciasis infection has been previously shown and is one of the first descriptions of a specific genetic basis for the variation seen in filarial pathology [83]. In LF, previous studies have shown the association between polymorphisms in various host genes [e.g., human leucocyte antigen (HLA), Chitotriosidase (CHIT1), mannose-

binding lectin (MBL), vascular endothelial growth factor A (VEGF-A) and Toll-like receptor 2 (TLR2)] and susceptibility as well as clinical types of LF [84-90]. Additionally, a variant in the transforming growth factor-beta 1 (TGF-β-1) gene in LF patients was found to be associated with the lack of Mf and differential Mf loads in blood [91].
Results of this thesis show an association of pathology in LF and SNPs in the interleukin-18 (IL-18) and the interleukin-4 receptor gene (IL-4R) in an Indonesian population infected with *B. timori*.

IL-18 is a pro-inflammatory cytokine that stimulates both T_h1 and T_h2 responses, depending on the cytokine environment (Figure 12) [92].
Together with IL-12, its primary function is the induction of Interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNFα) in T-cells and natural killer (NK) cells and therefore up-regulation of T_h1 cytokines. It is expressed by many different cells including macrophages, dendritic cells, keratinocytes and osteoblasts [93]. In the absence of IL-12, IL-18 can also induce the production of T_h2 cytokines by T-cells, mast cells and basophiles [94, 95]. In humans, up-regulation of IL-18 production was shown in Crohn's disease [96] and rheumatoid arthritis [97]. The expulsion of gastrointestinal nematodes such as *Strongyloides venezuelensis* depends on T_h2 responses, and the protective role of IL-18 in the *S. venezuelensis* mouse model indicates that IL-18 exerts its function against helminth infection through activation of T-cells, basophiles and mast cells to produce IL-4, IL-9 and IL-13 [98]. Another study analyzing the antigen-specific cellular responsiveness in patients exposed to *O. volvulus* and *Entamoeba histolytica* infection showed the importance of IL-18 in nematode infections [99]. IL-18 reduced parasite-antigen driven IL-10 secretion by peripheral blood mononuclear cells (PBMCs) in individuals exposed to *O.volvulus* and *E.histolytica,* whereas neutralization of IL-18 clearly enhanced IL-10 release. Chronic pathology in LF is associated with a pro-inflammatory response with elevated IFN-γ

levels [72-74] and decreased IL-10 levels compared to microfilaremic patients [73, 100].

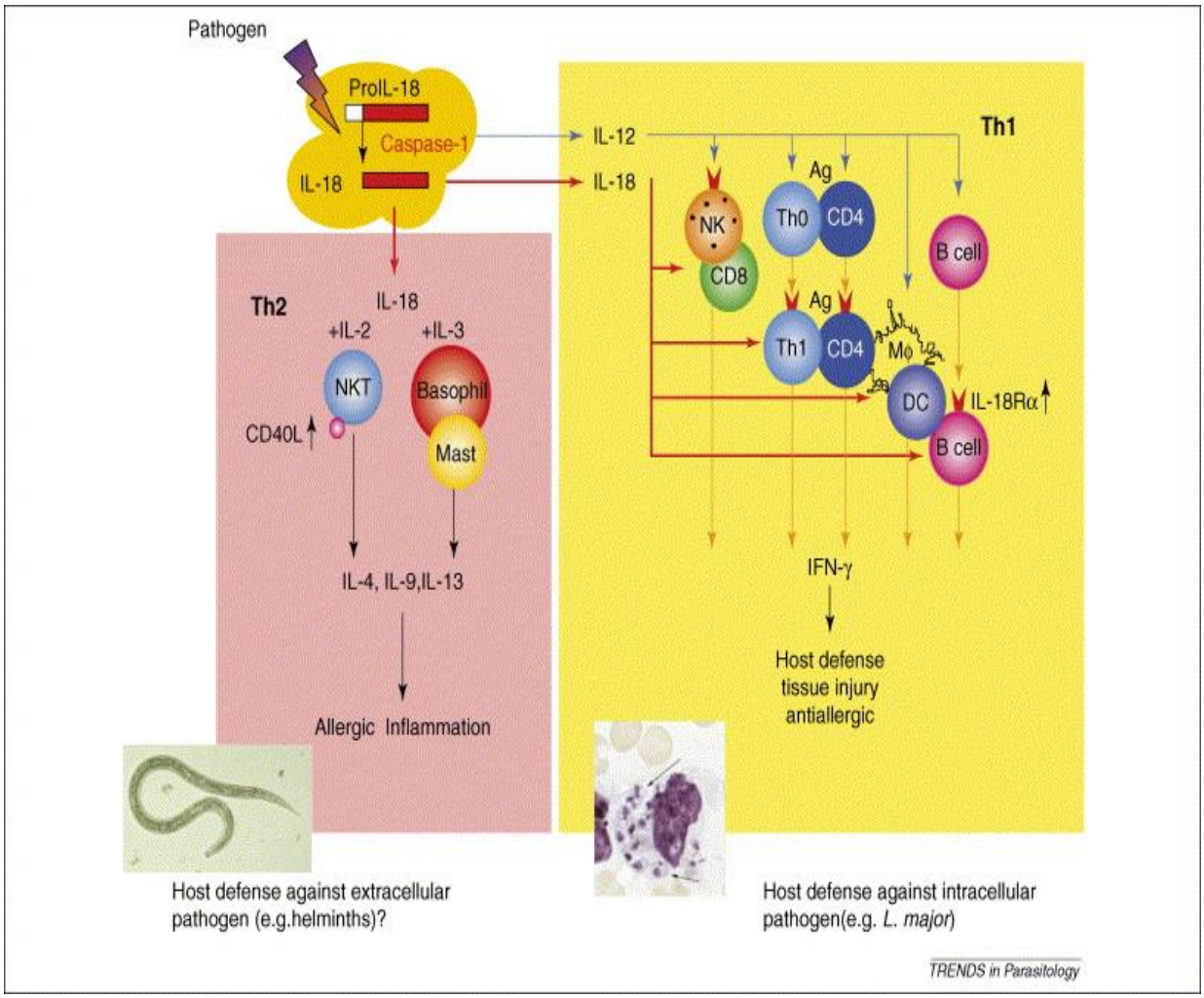

Figure 12 IL-18

IL-18 stimulates both T_h1 and T_h2 responses, depending on the cytokine environment. The combination of IL-18 and IL-12 strongly stimulates various types of cells to produce IFN-γ, which, in turn, activates macrophages to produce nitric oxide, leading to the eradication of intracellular pathogens such as *Leishmania major*. However, without IL-12, IL-18 promotes production of T_h2 cytokines by T-cells, basophils and mast cells. Thus, IL-18 can regulate both T_h1 (pink box) and T_h2 (yellow box) responses. Abbreviations: Ag, antigen; DC, dendritic cell; MΦ, macrophage; NKT, natural killer T-cell.

Giedraitis *et al*. first reported that IL-18 promoter gene polymorphisms influenced the expression of IL-18 mRNA and potentially also IFN-γ expression after stimulation with PMA/ionomycin [101]. As part of this thesis we analyzed two SNPs in the IL-18 promoter in patients infected

with *B. timori* from Alor Island, Indonesia. *B. timori* shares a high degree of sequence homology with *B. malayi* and LE is the most severe pathology seen with this infection. We found an association of LE pathology with the -607 (rs1946518) and -137 (rs187238) promoter SNPs of the IL-18 gene (GeneID: 3606) responsible for elevated IL-18 levels. These SNPs have previously been associated with inflammatory diseases such as Systemic Lupus Erythematosus (SLE) [102] and sarcoidosis [103].

Additionally two SNPs in the IL-4R gene were found to be associated with LF. The IL-4R (GeneID: 3566) is a type 1 transmembrane protein that binds the T_H2 cytokines IL-4 and IL-13. Both cytokines play key roles in the development of T_H2 cells, a hallmark of many worm infections. Both cytokines use the same receptor alpha chain and seem to share many physiological functions. One of their functions is the activation of B-cells and induction of proliferation and differentiation of the IgG1 and IgE subtypes. Because of their specific signalling pathways they are attractive targets for allergy and asthma treatment [104]. The importance of IL-4 for the control of Mf in murine infection with the filarial nematode *Litomosoides sigmodontis* has previously been described [105, 106]. IL-4 binds with high affinity to the IL-4R, leading to dimerization with a gamma chain to form a type I receptor, or dimerization with the IL-13 receptor alpha-1 chain to form a type II receptor (Figure 13). The intracellular signal via STAT6 activates IL-4 and IL-13 regulated genes [107, 108]. It has been proposed that subtle differences in IL-4 and IL-13 signalling due to polymorphisms near docking sites in the IL-4R gene may have profound implications for allergy and asthma [104].

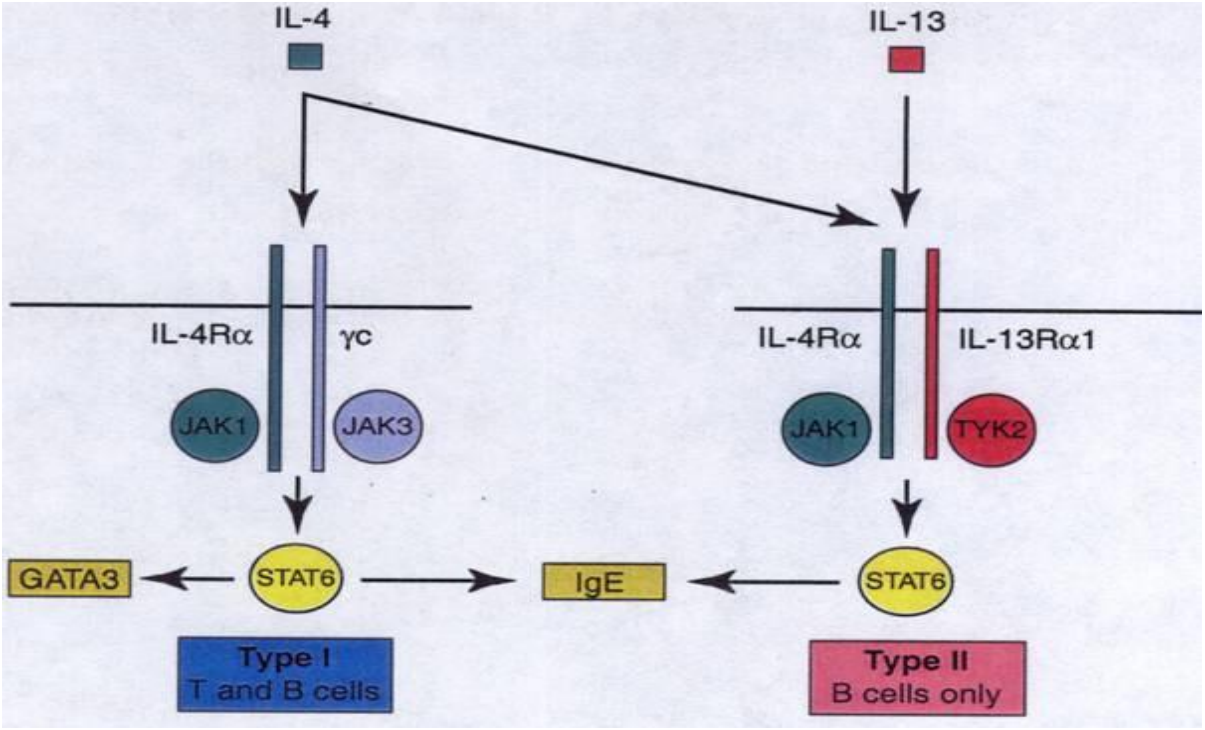

Figure 13 IL-4R http://bionmr-c1.unl.edu/IL13/IL13.htm

The IL-4 gene encodes the alpha chain of the IL-4 receptor, a type I transmembrane protein that can bind IL-4 and IL-13 to regulate IgE antibody production in B-cells. Among T-cells, the encoded protein also can bind IL-4 to promote differentiation of T_h2 cells.

The Ile50Val SNP (rs 1805010) in the IL-4R gene on chromosome 16 leads to a change from isoleucine to valine at amino acid position 50 and is associated with atopy, asthma and elevated total serum IgE levels [109-111]. Previous studies in mouse and human B-cell lines have shown a significant association of the Ile 50 form with a 3X greater cell growth and luciferase activation (expression under control of the IgE promoter) in response to IL-4 compared with Val 50 transfected cells [111]. Additionally, in human B-cell lines, increased STAT6 activation in cells with the Ile 50 form was observed [111]. These data suggested that the Ile 50 variant significantly up-regulates receptor response to IL-4.

The second IL-4R SNP analyzed in our study is in the 3`UTR region of the IL-4R gene at mRNA position +3111 (rs 1049631). It has been postulated that this SNP is a marker for asthma and atopy [112].

Knowledge of genomic loci linked to pathology would help in understanding pathogenesis which could lead to development of

strategies, beside supportive care, to ameliorate pathology in the 40 million people affected by LE and hydrocele. Knowing genetic markers for LE or hydrocele could provide a way to identify persons at risk before pathology is seen and might become the basis for development of a rapid screening test.

1.8 Aims and objectives

1. The first part of this thesis was the establishment of a DNA extraction method and quantification of the *Wolbachia ftsZ* gene in patients treated with doxycycline, rifampicin or azithromycin. The PCR results were used to support histological results of patients infected with *O. volvulus* or *W. bancrofti* and confirm a depletion of *Wolbachia* endobacteria after antibiotic treatment.

2. The effect of doxycycline treatment on *O. volvulus* larvae was analyzed. The question was, if Mf of doxycycline treated individuals were able to develop in the *Simulium* vector without their *Wolbachia* endosymbionts.

3. A PCR for *B. malayi* infected patients was developed detecting the *Brugia HhaI* gene to establish a detection method for Mf negative patients, who may have a pre-patent or latent infection.

4. To get more knowledge of the two poles of pathology in LF, a genetic association study of several markers was done to see if there are genotypes which could influence the outcome of pathology in LF.

2. Patients, materials and methods

2.1 Treatment of 4 and 6 weeks doxycycline in onchocerciasis

2.1.1 Study site

In the central region of Ghana, the area south of the river Offin and west of the Pra river is endemic for onchocerciasis but not for other human filarial infections. Both rivers are breeding sites of the vector blackflies *Simulium sanctipauli* that have flight ranges of up to 12 km [113]. The study participants were recruited in three neighbouring villages in the Assin district. In 1999, the focus was found to be hyperendemic during a rapid assessment of 30 men aged more than 19 years in each village: 75, 86 and 93% nodule carriers and 80, 89 and 90% Mf carriers were found (R.Horstmann, Bernard Nocht Institute, personal communication). Mass treatment with IVM started in 1999 in the district. This focus lies south of the area of the Onchocerciasis Control Program (OCP) and transmission is ongoing. Due to remoteness of the villages, the coverage of IVM was rather low until the end of our study in 2005 [113]. During another assessment of transmission in 2006 the *Simulium* infectivity parameters closely resembled those from other hyperendemic areas without intervention. The entomologists concluded that a person might easily receive one or several infective bites per week (R.Garms, Bernard Nocht Institute, unpublished report). The nodule loads found during recruitment for this study in September 2003 also indicated a high endemicity.

2.1.2 Ethical aspects and study design

The study was designed as a randomized, placebo-controlled, double-blind study and was approved by the Committee on Human Research and Ethics of the school of Medical Sciences of the Kumasi University of Science and Technology (KNUST), Kumasi, Ghana, as well as by the Research Ethics Committee of the Liverpool School of Tropical Medicine. The study conformed to the principles of the Helsinki Declaration of 1964 (last amended 2008).

2.1.3 Informed consent, recruitment and examination of the patients

All participants were asked to sign an Informed Consent Form after explaining the study in English and in the local language Twi. Individuals eligible for participation were: nodule carriers of both sexes, aged 18-62 years, with a body weight of more than 40 kg, in good health, and without any clinical condition requiring chronic medication. Physical examinations included inspection and palpation of onchocercomas. The Mf density was assessed by two skin biopsies as described previously [114]. Before doxycycline respectively placebo treatment and again 3 weeks after treatment onset, hepatic and renal functions as well as pregnancy were assessed by dip-stick chemistry using venous blood. Exclusion criteria were: palpation of less than two onchocercomas, abnormal hepatic and renal enzymes and creatinine, pregnancy, breast-feeding, intolerance to doxycycline, and alcohol or drug abuse.

2.1.4 Interventions

Patients were treated in September 2003 until October 2003 (Figure 14). There were three treatment arms: (1) 6 weeks 200 mg/day doxycycline; (2) 4 weeks 200 mg/day doxycycline, followed by 2 weeks matching

placebo; (3) 6 weeks matching placebo. Participants received two 100 mg capsules of Vibramycin® or matching placebo supplied by Pfizer. There was a daily monitoring in the villages of adverse side effects. After 6 months, a limited number of nodules were excised from some patients of the 6-week doxycycline and the placebo groups harbouring many onchocercomas, in order to confirm the depletion of *Wolbachia*. Six months after the start of the study and after the first round of nodulectomy, the patients received 0.15 mg/kg IVM to deplete skin Mf.

Based on the results of previous studies [114], patients were nodulectomised 20 and/or 27 months after the start of the study in May 2005 and November 2005. For the nodulectomies patients were admitted to the district hospital in Dunkwa. The nodules were excised aseptically under local anaesthesia as previously described [41, 115].

The study patients had been asked not to participate in IVM mass treatment between September 2003 and November 2005. However, they were treated with IVM in November 2005-2006 as part of our study protocol.

2.1.5 Objectives

The study had two objectives:

(1) To study in a placebo-controlled manner the efficacy of 4- and 6-week treatment with doxycycline followed by IVM, in order to define the minimum regimen needed to achieve *Wolbachia* depletion and complete sterilization of adult female worms, leading to a reduction in skin Mf over a long period. 2- and 3-week treatment was not sufficient in previous studies (unpublished data).

(2) To assess a potential macrofilaricidal activity of doxycycline on *O. volvulus*.

Part of my diploma thesis (2005) and this PhD thesis was the establishment of real-time PCR assays for quantification of *Wolbachia* in nodule and skin samples of the participants from this study.

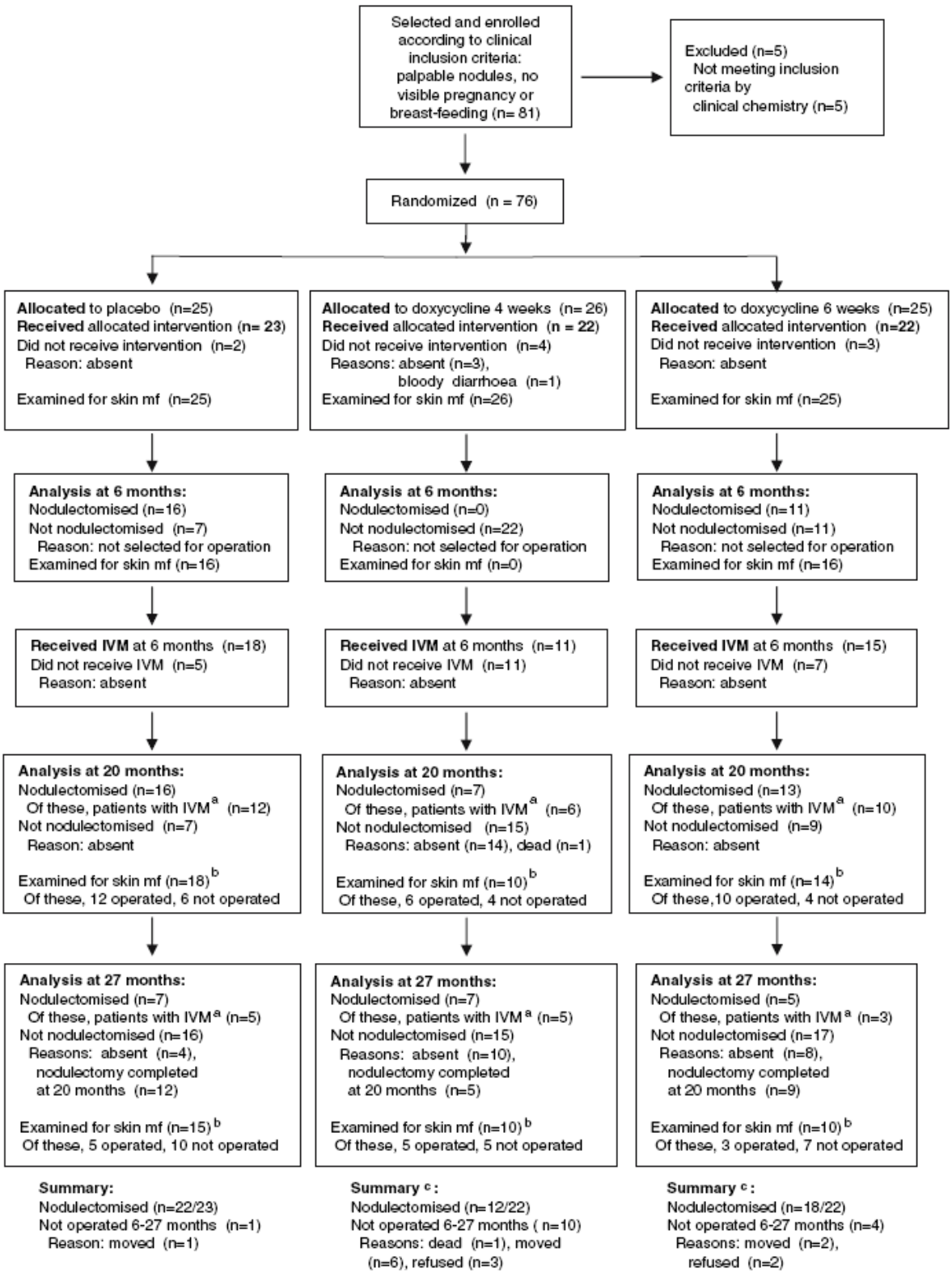

Figure 14 Patient Flow Chart (Published by Hoerauf *et al.*, 2008)

2.1.6 Nodule and skin preservation

Nodules were prepared for histology and PCR and separated in small nodules or more than half of medium or large nodules. Definitions for "medium" and "large" were diameters of 5-10 mm and more than 10 mm, respectively. For PCR analysis the minor portions of the nodules were kept at 4°C overnight and then frozen until further processing. Skin biopsies of 1-3 mg were taken during the nodulectomies from skin tissue near the nodule. Biopsies were stored in 80% ethanol until further DNA extraction.

2.1.7 DNA extraction

DNA was extracted using Trizol reagent following the manufacturer's protocol (Invitrogen, Karlsruhe, Germany) by homogenizing 4x30 sec at 6800 rpm in a Precellys 24 (PeqLab, Erlangen, Germany) using 2.8 mm steel beads. DNA was dissolved in 0.8 mM NaOH, pH adjusted to 7.4 with 1M HEPES (Invitrogen) and further purified using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the protocol for crude cell lysates.

2.1.8 PCR

For the analysis of the extent of *Wolbachia* depletion by quantitative PCR, only nodule and skin samples positive for worm *β-actin* were included in the analysis. The *O.volvulus β-actin* gene (M84916) was quantified from the purified DNA by real-time PCR with the following conditions: 1X HotStar® Taq Polymerase buffer (Qiagen), 200 µm dNTP, 300 nM each of forward and reverse primer (Appendix I), 0,2 µl SYBR® Green (1:1000 diluted in DMSO, Roche, Manheim, Germany), 2.5 units HotStar® Taq, and 2 µl DNA in a 20 µl reaction. The gene was amplified in a Rotor Gene 3000 (Corbett Research, Sydney, Australia) using the following conditions: 1x15 min at 95°C, 35 cycles of 94°C for 15 sec, 52°C for 15 sec, 72°C for 15

sec. Fluorescence was acquired on the FAM channel. To exclude unspecific SYBR® Green products, a melting curve from 62°C-99°C was performed after each run. To quantify the amount of *O. volvulus Wolbachia ftsZ* (AJ276501) the same PCR set up was used, except using 200 nM each of forward and reverse primer (Appendix I). The profile for the *ftsZ* gene used same conditions as the *β-actin* PCR, but annealing temperature was 55°C for 30 sec.

Copy numbers of each gene were calculated using a modification of the comparative quantification formula as described previously [116]. Using the Rotorgene 6000 version 6.0 software the amplification (A) of each sample was calculated and the mean taken from the replicate samples (sam). A plasmid containing either the *ftsZ* or the *β-actin* sequence was quantified and copies/µl determined for use as a reference (ref) in each PCR run. The amplification factor of the sample compared to the specific reference was then calculated using the following formula:

A_{ref} ^ take off_{ref} / A_{sam} ^ take off_{sam}

The take off is defined as the crossing point cycle where all measured samples are 20% above background. The amplification factor was multiplied by the known concentration of the reference to give copies/µl and the *ftsZ*/actin ratio was calculated.

Particularly in the skin samples of African decent, it was previously found that inhibition of the PCR reaction can occurre due to melanin contamination [117, 118]. Therefore an inhibition test with a reference plasmid (murine IFN-γ) was done. The samples were set up for real-time PCR with a mastermix containing 1X HotStar® Taq Polymerase buffer (Qiagen), 200 µm dNTP, 400 nM each of forward and reverse primer (Appendix I), 0,2 µl SYBR® Green (1:1000 diluted in DMSO, Roche, Manheim, Germany), 2.5 units HotStar® Taq, and 2 µl reference plasmid DNA and 2 µl samples DNA in a 20 µl reaction. The PCR profile was: 1x15 min at 95°C, 45 cycles of 94°C for 15 sec, 58°C for 20 sec, 72°C for 20 sec. Fluorescence was acquired on the FAM channel.

Samples were considered uninhibited when the reactions with sample DNA were detected at the same level as the reference plasmid control sample.

2.1.9 Statistics

Differences in *ftsZ*/*β-actin* ratios between doxycycline and placebo treated patients were analyzed using the Mann-Whitney-U Test with the GraphPad Prism Software, version 5.01 (GraphPad Software, La Jolla, CA, USA). *P* values ≤0.05 were considered significant.

2.2 Treatment of 5 weeks doxycycline in onchocerciasis

2.2.1 Study site

This open trial was conducted between August 2003 and November 2006 in the Assin district, Central Region of Ghana (detailed description in 2.1.1).

2.2.2 Ethical aspects, informed consent and inclusion criteria

As described in 2.1.2 and 2.1.3.

2.2.3 Patient treatment and nodulectomies

Twenty-four volunteers were included in the study (Figure 15). They received 100 mg capsules of doxycycline daily for 5 weeks (Vibramycin®, supplied by Pfizer, Karlsruhe, Germany). Doxycycline was administered after patients had taken a meal and the treatment was monitored daily by a physician. Two individuals did not complete the treatment. All participants were requested not to take part in IVM mass treatment during the study. They received IVM as part of our study after the nodulectomies at 27 months (11/2005) and after re-examination at 39 months (11/2006).

At 21 and 27 months after the beginning of doxycycline treatment, nodulectomies were performed aseptically under local anaesthesia in the Dunkwa Regional Hospital, as described [115].

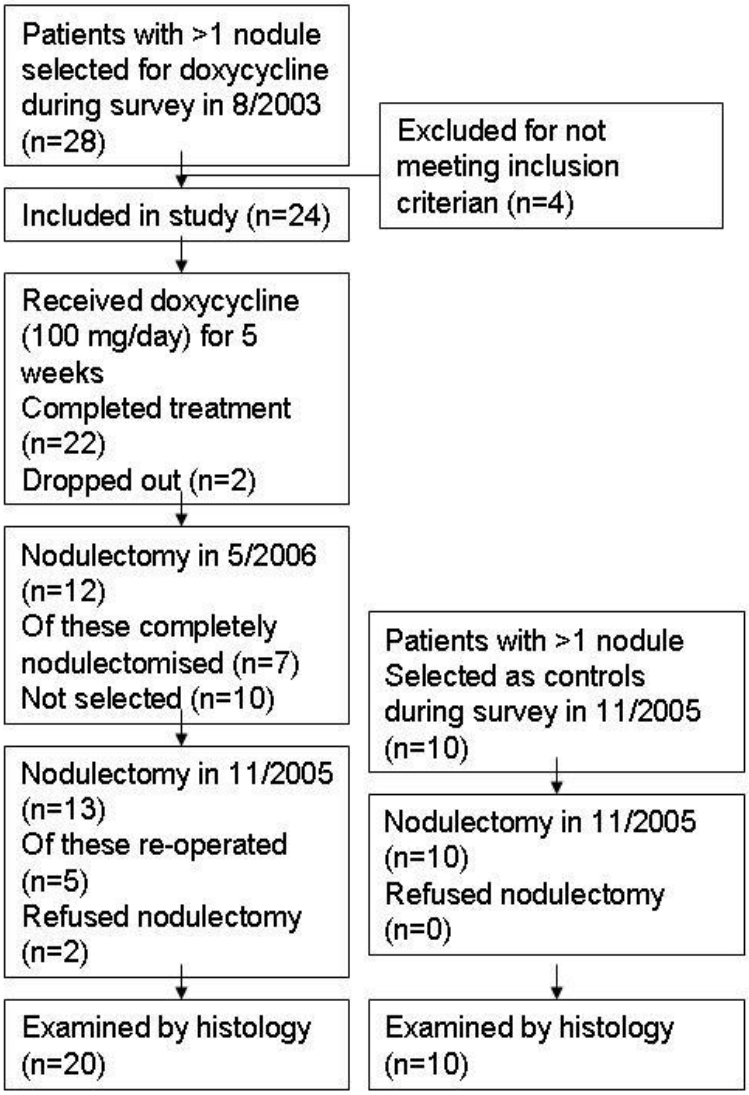

Figure 15 Patient Flow Chart

In addition to the doxycycline-treated patients, ten patients from the same village, who had not been enrolled in the study and had received neither doxycycline nor IVM, were enrolled for nodulectomy at 21 and 27 months as controls.

2.2.4 Objectives and Outcome measurements

Previously we had studied the efficacy of 100 or 200 mg/day doxycycline administered for 6 weeks [41, 47]. The aim of this study was to investigate the effects of a shortened regimen of only 5 weeks doxycycline

treatment on the depletion of *Wolbachia* endobacteria from *O. volvulus*, on interruption of embryogenesis and Mf production, and to detect macrofilaricidal activity.

The primary outcome measurements were female worm fertility, adult worm survival, and the presence of *Wolbachia* endobacteria. All parameters were assessed by immunohistology of extirpated nodules. Presence of *Wolbachia* was additionally assessed by quantification of the bacterial *ftsZ* gene normalized to the worm *β-actin* gene (part of this thesis).

The secondary measurement was the analysis of skin Mf from two skin biopsies that were taken from the doxycycline-treated and untreated control patients at study onset as well as at 21 and/or 27 months later.

2.2.5 Nodule and skin preservation

Immediately after extirpation, the nodules and skin samples were fixed in 80% ethanol or in buffered 4% formaldehyde solution for immunohistology.

Further preparation of nodule and skin tissue was as described in 2.1.6.

2.2.6 DNA extraction

DNA was prepared as described in 2.1.7.

2.2.7 PCR

PCR was performed for *Wolbachia ftsZ* and worm *β-actin* as described in 2.1.8. Same primers and PCR profiles were used.

2.2.8 Statistics

Differences in *ftsZ*/*β-actin* ratios between doxycycline and placebo treated patients were analyzed using the Mann-Whitney U Test as discribed in 2.1.9. Treatment of 6 weeks azithromycin in onchocerciasis

2.3 Treatment of 6 weeks azithromycin in onchocerciasis

2.3.1 Study site, ethical aspects and study design

This open trial was conducted in the Upper Denkyira district in the Central region of Ghana from 2002-2003. In 1999, a rapid assessment of 30 men older than 19 years in each village had identified many hyperendemic foci in the district (R.Horstmann and D.W. Büttner, Bernard Nocht Institute). Three villages were selected 2-9 km south of the river Offin, where *Simulium sanctipauli* vectors breed [113]. IVM mass treatment had started in Denkyira in 1999, but not all villages had been included until 2003 and not all eligible villagers had participated.

The study was approved by the Ethical Research Committee of the School of Medical Science of the Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. It conformed to the principles of the Helsinki Declaration of 1964 (last amended 2008). Informed consent was obtained from each participant.

Individuals eligible for treatment were adult men aged 18-60 years. In order to respond to the request by the villagers that women could also be part of the study, women were included as untreated controls for later nodulectomy. Further inclusion criteria were as described in 2.1.3. Here, exclusion criteria additionally encompassed a history of intolerance to azithromycin or erythromycin.

2.3.2 Treatment and nodulectomy

In the Praprababida community, 26 patients were recruited in January 2002 (Figure 16). Twenty-three patients were treated 6 weeks with 250 mg/day azithromycin (Zithromax® 250-mg tablets, Pfizer, Karlsruhe, Germany). In the Asma Camp community, 18 patients were recruited and

14 of them completed treatment with 1,200 mg azithromycin once per week for 6 weeks (two Ultreon® tablets, Pfizer).

At 6 or/and 12 months after the onset of treatment, nodulectomies were performed aseptically under local anaesthesia as described in 2.1.4.

In addition to the azithromycin-treated patients, nine untreated onchocerciasis patients from the Ayebiahwe community were nodulectomised at 12 months. All participants were administered IVM after the nodulelectomies at 12 months.

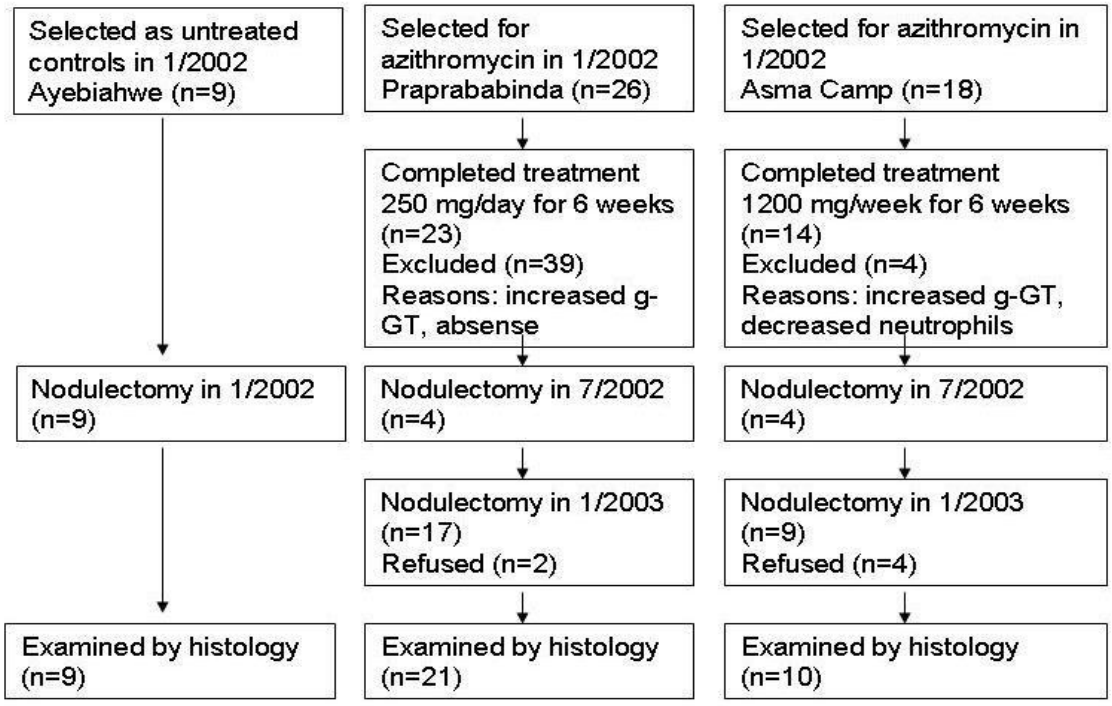

Figure 16 Patient Flow Chart (Published by Hoerauf *et al.*, 2008)

2.3.3 Objectives and Outcome measurements

The aim of the study was to analyse the effects of azithromycin on the presence of *Wolbachia* endobacteria and on embryogenesis and Mf production of *O.volvulus*. The primary outcome measurements were presence of *Wolbachia* and female worm fertility assessed by immunohistology of extirpated nodules as published by Hoerauf *et al*. in

2008 [119]. To expand and support histological results, presence of *Wolbachia* in extracted DNA of nodule tissue and quantification by real-time PCR was done as part of this thesis.

2.3.4 DNA extraction and PCR

DNA of nodules was extracted as described in 2.1.7. PCR conditions for the *Wolbachia ftsZ* gene and the *β-actin* gene of *O. volvulus* were the same as in 2.1.8, except the DNA was diluted 1:100 prior the PCR run due to a high amount of DNA after extraction.

2.3.5 Statistics

Differences in *ftsZ*/*β-actin* ratios between azithromycin treated or untreated patients were analyzed using the Mann-Whitney U Test as discribed in 2.1.9.

2.4 Treatment with rifampicin and/or doxycycline in bancroftian filariasis

2.4.1 Study site, ethical aspects and study design

This randomized open trial was conducted in the coastal villages of Asanta, Sanwoma, Agyambra and Miamia in the Nzema East and the Ahanta West District in the Western Region of Ghana from 2005-2008. The study site was selected based on an established occurrence of *W. bancrofti* infection within the surrounding region and clinical observations consistent with symptomatic disease in the villagers [43, 120]. Written informed consent was obtained from all participants and the study was approved by the Ethical and Research Committee of the School of Medical Sciences of the Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. The study conformed to the principles of the Helsinki Declaration of 1964 (last amended 2008).

Individuals eligible for participation were adult men aged 18-60 years, with a minimum body weight of 40 kg, in good health without any clinical condition requiring chronic medication. Exclusion criteria encompassed a microfilarial load <20 Mf/ml, abnormal hepatic and renal enzymes (above AST [0-40 IU/L], ALT [0-45 IU/L], creatinine [53-126 µmol/L]) assessed by dipstick chemistry, and alcohol or drug abuse. Leukocyte and differential counts of thin blood smears were also done.

2.4.2 Doxycycline and rifampicin treatment

In all, 48 men were recruited for the study and 39 completed the treatment course (Figure 17). Nine men dropped out and could not complete the treatment because they either moved from the villages or could not comply with the daily-observed treatment because of fishing activities. Of the 39 men, 12 were treated with 200 mg doxycycline/day

for 4 weeks, 16 patients with 200 mg doxycycline/day + 10mg/kg/day rifampicin for 2 weeks + 2 weeks placebo matching doxycycline and 11 patients were treated with placebo matching doxycycline for 4 weeks. Due to unavailability of rifampicin placebo at the time, the rifampicin study was an open study while for the doxycycline capsules matching placebo were available and this part of the trial was placebo-controlled. Four months after the start of treatment, all participants received a standard oral dose of 400mg ALB (GlaxoSmithKline) and 150 μg/kg IVM (Mectizan®, Merck, Sharp & Dohme).

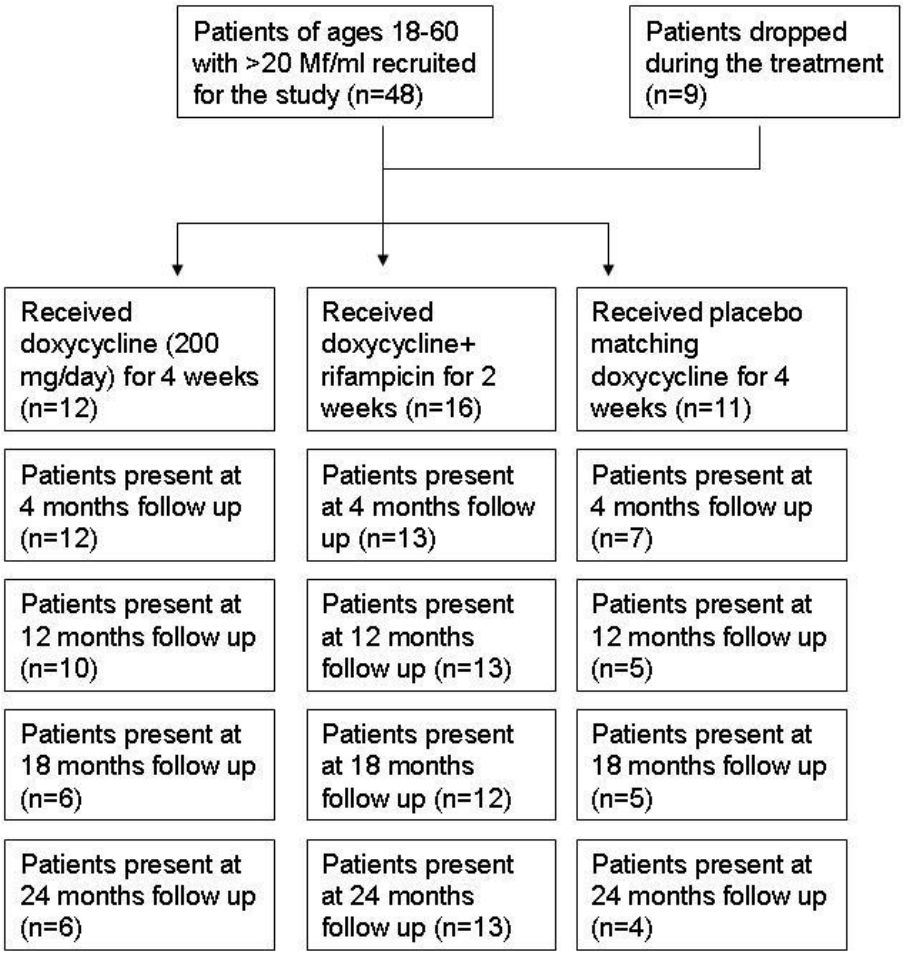

Figure 17 Patient Flow Chart (Published by Debrah *et al*., 2011)

2.4.3 Determination of *Wolbachia* levels in Mf by real-time PCR

One outcome measurement of this study was to quantify *Wolbachia ftsZ* single copy genes per Mf at pre-treatment and 4 months after treatment onset. The numbers of *Wolbachia*/Mf and microfilaremia were determined from a 10 ml blood sample taken between 21h and 23h. One ml of the blood was filtered through a 5 µm Whatman Nucleopore filter to hold back Mf. The filter was stained using the Giemsa method and Mf were counted using a microscope [43, 120]. A second filter was used to filter a blood volume calculated to have 500-1000 Mf. This filter was stored at –80°C until later analysis of *Wolbachia* gene copies by quantitative PCR. Due to a reduced sample number of Mf positive patients at 12, 18 and 24 months after treatment, PCR was only done at pre-treatment and 4 months after treatment onset.

2.4.4 DNA extraction

DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the tissue 2a protocol. A calculated number of 500-1000 Mf trapped on a Whatman filter was digested with proteinase K. Instead the recommended short incubation with proteinase K, incubation was extended to an overnight incubation at 56°C.

2.4.5 Determination of *Wolbachia* levels in Mf by quantitative PCR

The *Wolbachia* in Mf content was quantified by real-time PCR of the *W. bancrofti Wolbachia ftsZ* gene (AF081198) derived from 500-1000 Mf. Purified DNA was quantified by real-time PCR on a Rotor Gene 6000 (Corbett Research, Sydney, Australia). The mastermix contained: 1x HotStar® Taq Polymerase buffer (Qiagen), 200 µM dNTP, 4.5mM $MgCl_2$, 300 nM of forward and 900 nM of reverse primer (Appendix I), 0.1 µl of

hybridisation probe (Appendix I), 2.5 U HotStar® Taq Polymerase, and 2 µl DNA in a 10 µl reaction. The 2-step profile started with a 95°C activation step for 15 min, followed by 45 cycles with 95°C for 15 s and 60°C for 30 s.

The products were quantified by comparing with a standard curve of the plasmid containing the *ftsZ* fragment.

To verify the worm content of the individual sample, a second PCR assay for *W.bancrofti β-actin* (AF 184961) was done.

The mastermix contained 1x HotStar® Taq Polymerase buffer (Qiagen), 200 µM dNTP, 4 mM $MgCl_2$, 900 nM of forward and reverse primer (Appendix I), 0.1 µl of SYBR® Green (1:1000 diluted in DMSO, Roche, Mannheim, Germany), 2.5 U HotStar® Taq Polymerase, and 2 µl DNA in a 10 µl reaction.

The PCR profile contained a 15 min initiation with 95°C, followed by 40 cycles of 95°C 10s, 58°C 30s and final extension at 72°C for 20s. Melt curve analysis showed a specific peak at 80°C for all positive samples.

For the analysis of the extent of *Wolbachia* depletion by quantitative PCR, only samples which were positive for worm *β-actin* were included in the analysis.

2.4.6 Statistics

Depletion of *Wolbachia* loads from Mf by doxycycline alone and doxycycline+rifampicin, and comparison of microfilaremia between various treated groups were summarized as medians. Differences in medians at baseline and subsequent follow-ups were analyzed using Wilcoxon Signed Rank test of raw data. All analyses were done using tests included in the PASW® software version 18.

2.4.7 Additionally outcome measurements

PCR was only one part of the study. Additionally, determination of circulating filarial antigenaemia and ultrasound examinations (USG) for filarial dance sign (FDS) were done by Dr. Sabine Mand and Dr. Alex Yaw Debrah [121].

2.5 Retarded *O. volvulus* L1 to L3 larval development in the *Simulium damnosum* vector after anti-wolbachial treatment of the human host

2.5.1 Ethical approval

This study received ethical clearance from the Institutional Review Board of the Tropical Medicine Research Station, Kumba and was conducted in accordance with the Helsinki Declaration of 1964 (last amended 2008).

2.5.2 Selection of study patients and treatment

In November and December 2006, participants for the study were recruited in four neighbouring villages in an onchocerciasis-endemic region of South West Cameroon. Patients for the study were selected from male volunteers, aged between 21 and 50 years after informed consent was signed. Exclusion criteria were abnormal hepatic (SGPT and SGOT) and renal (creatinine) functions, persons with chronic infections and under prolonged medication, alcohol abuse and intolerance to doxycycline. As there was mass treatment with IVM in the area in April 2006, all patients were asked whether they took their dose at this time or any time before, but all volunteers answered in the negative. Inclusion criteria were absence of other clinically manifest diseases as assessed by a medical doctor and $\geq$ 10 Mf in skin biopsies. Two bloodless skin biopsies, one from each iliac crest of each participant, were aseptically obtained using a Holth corneoscleral punch. Each biopsy was immersed in 2 drops of 0.9% NaCl solution in a separate well of a labelled 96-well round-bottom microtiter plate.

The plates were transported to the laboratory and skin biopsies were incubated overnight at room temperature to allow the emergence of Mf

into the saline solution. The Mf were counted using 10-fold magnification of a microscope and the numbers of Mf were expressed as Mf per skin biopsy.

Twelve volunteers were blindly allocated into two treatment groups. One group of 7 volunteers received doxycycline (200 mg/day) for 6 weeks while the second group of 5 volunteers received matching placebo.

2.5.3 Infection of wild *Simulium* flies with *O. volvulus*

Before and after treatment, the volunteers were brought to a pre-selected capture point near a *Simulium* breeding site on the bank of the River Mungo to allow *Simulium damnosum* flies, the vector of *O. volvulus*, to take a blood meal. The site was chosen based on the results of dissection of wild *Simulium* flies to determine their natural parous, infection and infective rates. These parameters were monitored throughout the study period. The fed flies were captured on the volunteers in small plastic containers. Containers for each volunteer were kept separately in plastic baskets. At least four hundred flies were caught from each patient in four days at each time point. Transport to the insectarium was carried out in cold boxes. The flies were then kept for 7 days at 25°C and 80% relative humidity to allow the Mf to develop into infective third stage larvae (L3).

Larvae were isolated from the flies by dissection using a dissecting microscope and their developmental stages were scored. The number of flies dissected at each time point varied due to different fly mortality rates. The isolated developmental stages of the L1 (first larval stage), L2 (second larval stage) and L3 (third, infective larval stage) were stored separately in 80% ethanol. At least two tubes each containing different numbers of L1, L2 and L3 isolated from flies fed on each volunteer at each time point were stored for later DNA extraction and quantification of *Wolbachia*.

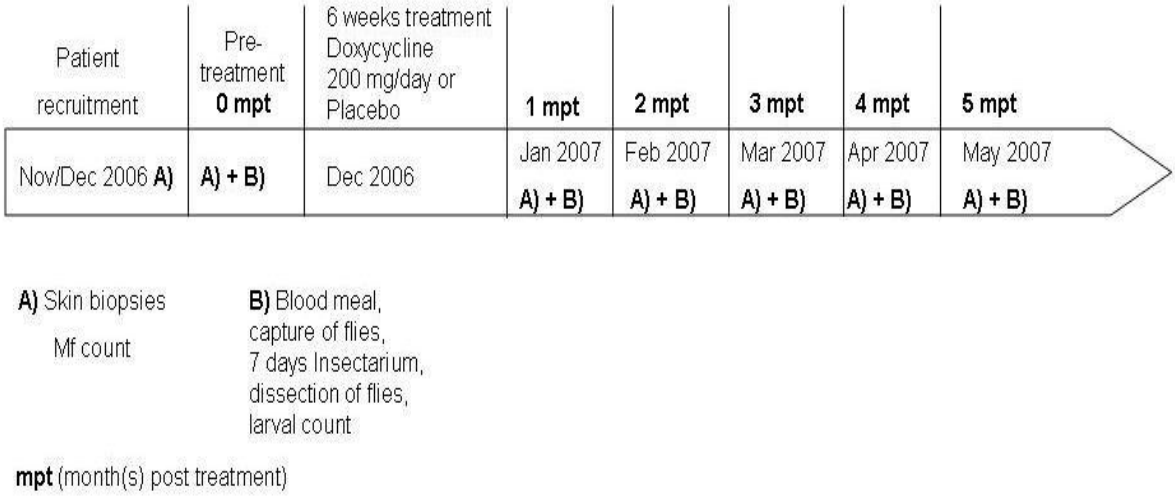

Figure 18 Time line

2.5.4 Follow-up of volunteers and monitoring of Mf load after treatment

During the following 5 months, flies were captured on a monthly basis on the volunteers as described above (Figure 18). Skin biopsies were also obtained from the patients at each time point to assess their Mf load after the antibiotic treatment. After counting, the Mf were stored in 80% ethanol for later quantitative PCR analysis to determine their *Wolbachia* content.

2.5.5 Kinetics of larval development

The total number of L1, L2 and L3 collected after dissection of the flies was calculated from doxycycline and placebo treated individuals. The proportion of each larval stage generated for that month in each group was calculated and plotted against the month of dissection to follow the kinetics of the development of larvae in both treatment groups. At pre-

treatment and one month follow-up, the L1 and L2 larvae counts were combined.

2.5.6 DNA extraction from larvae

One tube each of the different stages of larvae (L1, L2 and L3) per time point preserved in ethanol was randomly selected for DNA extraction and quantification of *Wolbachia*. The larvae preserved in ethanol were first centrifuged at 1800 rpm in a LH 4000 rotor (Multifuge 4KR, Heraeus, Haunau, Germany) for five minutes. The ethanol was slowly removed with a pipette without disturbing the larvae, leaving 100 µl of volume. These larvae were then suspended in the remaining ethanol and transferred to a new tube. DNA was extracted using the QIAamp® DNA mini Kit (Qiagen, Hilden, Germany) following the tissue protocol of the kit with the following modifications to achieve the maximum amount of quality DNA. The incubation period with proteinase K (Qiagen) was extended from 10 min to overnight at 56°. Wash buffers 1 and 2 were increased to 700 µl followed by elution with 2X50 µl of AE buffer. For each elution step, the columns were incubated with AE buffer for 5 minutes at room temperature prior to centrifugation.

2.5.7 Quantitative real-time PCR

To determine the amount of *Wolbachia* in the larvae isolated from the vector captured after having fed on doxycycline or placebo treated individuals, the purified DNA samples of randomly selected larvae for each volunteer and time point were analyzed by quantitative real-time PCR.
Primers were used from previous analysis (2.1-2.3) and a hybridization probe was designed with Primer3 software [122] for the *O. volvulus Wolbachia ftsZ* gene (GenBank accession no. AJ276501). For all samples the following master mix was used: 1x QuantiTect® Virus NR Master Mix

(Qiagen), 300 nM each forward and reverse primers (Appendix I), 50nM TaqMan hybridisation probe (Appendix I) (Biomers, Ulm, Germany), 2.5 U HotStar® Taq Polymerase and 10 µl of sample DNA in a 20 µl reaction. The PCR program utilized a two-step reaction of the following conditions in a Rotor Gene 6000 (Corbett Research, Sydney, Australia): 1x 5 min at 95°C, 45 cycles of 94°C for 10 s and 58°C for 45s. Fluorescence was acquired on the FAM channel at 58°C.

A second PCR was performed which quantified the amount of *O.volvulus β-actin* (GenBank accession no. M84916) to normalize the *ftsZ* values [123] as discribed in 2.1.8. In both PCR assays, each sample was analyzed in triplicate. Every run contained a plasmid specific for each gene with a known number of copies for use as a standard curve reference. A no-template control of water instead of sample DNA was used as negative control. Copy numbers for each gene were calculated using a modification of the comparative quantification formula as described in 2.1.8.

To exclude inhibition of the PCR reaction by inhibitors in DNA a third PCR with a plasmid containing a fragment of the murine INF-γ was performed in the presence of the extracted DNA as discribed in 2.1.8.

2.5.8 Data analysis and statistics used

The proportions of larvae were analyzed by calculating the L3/total larvae and L1+L2/total larvae ratios. Statistical significance between larvae of vectors fed on doxycycline or placebo treated individuals was determined with the Mann-Whitney U test ($P \leq 0.05$ was considered significant) with the PASW® 18 software (IBM, Chicago, USA). Results were presented using the median with 95% CI.

Larval recoveries were expressed as median with 10-90th percentiles.

Wolbachia quantities of the PCR results were expressed as the ratio of *ftsZ*/*β-actin*.

2.6 Detection of *HhaI* gene in infection with *B. malayi* by real-time PCR

2.6.1 Plasma from *B.malayi* infected patients from Indonesia

Samples were received from a larger study conducted in Budong-budong [124, 125], a district of Mamuju Regency in South-Sulawesi, Indonesia, which is endemic for periodic nocturnal *B. malayi* [126]. A transmigrant population had traveled to their new homesteads in groups coming from the same village or region in Bali or certain Lesser Sunda islands as part of the government-sponsored relocation program between 1990 and 1996. A total of 6 transmigrant units, settled between several months and 6 years prior to the survey, were included in the study together with 2 villages of indigenous Sulawesian villages, which were situated closely to the transmigration areas. Transmigrants from areas where filariasis is endemic (South Sulawesi and other Lesser Sunda islands) were excluded from the analysis. In co-operation with the medical doctors and health workers of the local District Health Centre and the head of each transmigration unit or village, all residents were informed about the study and invited to participate. Informed consent was obtained from all study participants or parents of underage children before parasitological studies and blood withdrawal in accordance with the guidelines of the Indonesian Department of Health and Human Services. The study was approved by the ethics committee of the Hasanudin University, Indonesia. A total number of 247 transmigrants and 133 life-long residents (LLR) were enrolled in the study [125].

2.6.2 Blood tests (done by Terhell and colleagues)

Venous blood samples of 10 ml were collected between 20:00h-24:00h and preserved with EDTA at a final concentration of 0.05 M. The tubes

were centrifuged and plasma was stored at -20°C for several months until shipment to Europe, where it was stored at -70°C until use. Parasitological examination and detection of filarial spesific IgG4 antibodies using a soluable worm antigen (SWA) based ELISA and BmR1 dipstick assay (Brugia Rapid™) were done as previously described [124, 125].

2.6.3 DNA extraction and PCR

DNA was extracted from 100 µl plasma with a QIAcube® (Qiagen, Hilden, Germany) using the QIAamp® DNA Blood Mini Kit. The recommended protocol for blood and body fluid was used and DNA was eluted in 100 µl AE-Buffer.

For the PCR assay, a QuantiTect® custom assay was designed with specific primers and a minor groove binding probe (MGB, QuantiProbe®) for detection of 120 bp of the *HhaI* repeat in *Brugia* species. The *HhaI* repeat (M12691 and AF499109 to AF499129) was used as the target sequence for several reasons. This sequence is arranged as a tandem repeat and is present in several thousand copies per haploid genome [127]. Furthermore, most conventional PCR assays for *Brugia* have used this target, and no consistent sequence variation has been reported in various strains of *B. malayi* and *B. timori*. The *HhaI* repeat is AT rich and closely related sequence are present in zoonotic filarial species such as *B. pahangi*; this makes it difficult to design primers and probes specific for the *Brugia* species that are parasitic in humans (*B.malayi* and *B. timori*). In addition, even within one parasite, the numbers of *HhaI* copies is not identical, and there are conserved and more variable areas. Conserved regions rarely span more than 30 bp, and selection of long primers and probes can decrease detection sensitivity.

The use of modified nucleotides (“superbases”) together with a minor groove binding probe (MGB) increases the melting temperatures of oligonucleotides to complementary sequences.

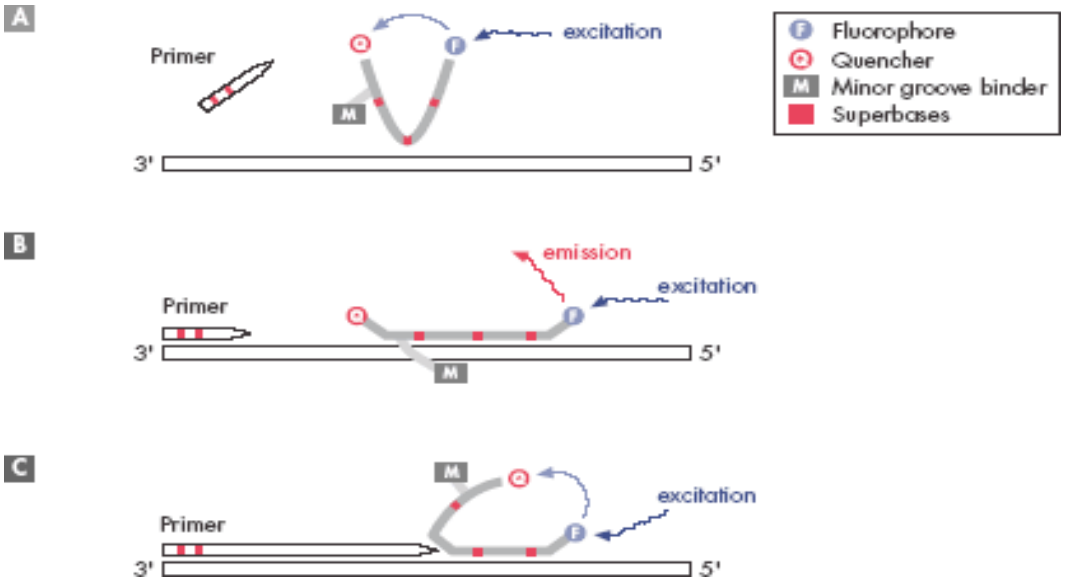

Figure 17 QuantiProbe® Principle QuantiProbes, included in QuantiTect Custom Assays, operate as hybridization probes, with Superbases for increased stability. **A** When not bound to its target sequence, the QuantiProbe forms a random structure in solution. The proximity of the fluorescent reporter with the quencher prevents the reporter from fluorescing. **B** During the PCR annealing step, the QuantiProbe hybridizes to its target sequence. This separates the fluorescent dye and quencher, resulting in a fluorescent signal. The amount of signal is proportional to the amount of target sequence, and is measured in real-time to allow quantification of the amount of target sequence. **C** During the extension step of PCR, the probe is displaced from the target sequence, bringing the fluorophore and quencher into closer proximity, resulting in a decrease in fluorescence without hydrolysis of the QuantiProbe. This means that the concentration of the QuantiProbe remains constant during the PCR, enabling reliable quantification.

The primer and probe sequences were obtained from previously published results of Rao *et al.* [128], and were purchased from Qiagen as 20x concentrates. The reaction mixture for the real-time PCR consisted of 10 µl of 2x QuantiTect® Probe PCR Master Mix (containing HotStar® Taq DNA Polymerase, QuantiTect® Probe PCR buffer, dNTP mix and 8 mM $MgCl_2$), 1 µl Primer Mix 20x and 0.5 ml QuantiProbe® solution 20x (Appendix I) in a 20 µl reaction. Cycling conditions on the RotorGene 6000 (Corbett Research, Sydney, Australia) included an initial activation for 15 min with 95°C and 45 cycles with 15 sec at 94°C, 30 sec at 56°C and extension for

30 sec at 76°C. Fluorescence was acquired on the FAM channel during the annealing step at 56°C. Target sequence copy numbers were calculated from the C_t values by using an external standard curve generated with a *HhaI* plasmid dilution series, which allowed 100% detection in samples with >200 copies/µl and 85% in the range between 20 and 200 copies/µl. For generating the external standard curve, the higher dilutions were measured in triplicates, lowest dilution (2 and 20 copies/µl) were measured with 10 replicates in three separate runs. All other PCRs were carried out in duplicates.

Samples containing a very low amount of DNA and without positive *HhaI* detection were measured in a second PCR assay using the QuantiTect® Virus NR mastermix (Qiagen). This modified mastermix allows for an increased volume of sample DNA in the PCR reaction up to 10 µl and is useful for samples with low concentrations of target DNA. The reaction mixture contained 4 µl of 5x QuantiTect Virus NR mastermix (containing HotStar® Taq Plus DNA Polymerase, QuantiTect® Virus PCR buffer, dNTP mix and $MgCl_2$), 1 µl Primer Mix 20x and 0.5 ml QuantiProbe® solution 20x (Appendix I) in a 20 µl reaction. The profile on a RotorGene 6000 included an initial activation for 5 min with 95°C and 45 cycles with 15 sec at 94°C and 30 sec at 60°C. Fluorescence was acquired on the FAM channel during the annealing step at 60°C.

To exclude any inhibiting factors in the extracted DNA from plasma, a test for inhibition was done as described in 2.1.8.

To verify the DNA content in each sample, a PCR was done detecting the human *β-actin* gene (Gene ID: 60). The Mastermix contained 1X HotStar® Taq Polymerase buffer (Qiagen), 200 µm dNTP, 300 nM each of forward and reverse primers (Appendix I), 0,2 µl SYBR® Green (1:1000 diluted in DMSO, Roche, Mannheim, Germany), 2.5 units HotStar® Taq Polymerase, and 2 µl DNA in a 20 µl reaction. The gene was amplified in a Rotor Gene 3000 (Corbett Research, Sydney, Australia) using the following conditions: 1x15 min at 95°C, 35 cycles of 94°C for 15 sec, 58°C for 15 sec and 72°C for 15 sec. Fluorescence was acquired on the FAM channel. To exclude

unspecific SYBR® Green products, a melting curve from 62°C-99°C was performed after each run.

2.6.4 Plasma from *B.malayi* infected jirds

Jirds (*Meriones unguiculatus*) were infected with L3 (infective third stage larvae) of *B. malayi*. Eight weeks (56 days) after infection, plasma was collected and adult worm recovery was documented. No Mf could be found at this time point indicating a pre-patent status of the jirds. The patent period of *B. malayi* infected jirds usually starts between 75 and 110 days after infection [129]. DNA was extracted of 100 µl pelleted plasma with the QIAamp® DNA Mini Kit. The pellet was dissolved with 180 µl ATL buffer and proteinase K for one hour at 56°C. DNA was quantified as described in 2.6.3 using the QuantiTect Virus NR mastermix and the protocol with 10 µl sample DNA per reaction.

2.6.5 DNA extraction from plasma used as positive/negative controls

To test the specificity of the *HhaI* PCR assay plasma samples from ten *W. bancrofti* infected individuals, 15 patients with other parasitic infections and ten European blood donors without history of a parasitic infection were extracted according to the protocol described in 2.6.3. All volunteers had signed an informed consent. These samples were used as negative controls. Another 30 plasma samples from Mf positive patients and 9 from LE patients from Central Sulawesi were extracted as descibed and used as positive reference samples.

2.6.6 Determination of a threshold of active infections by PCR

A threshold limit of *HhaI* copy numbers was determined to differentiate most-likely "active" infections from simple exposure to infective larvae. For this the Mf loads of Mf positives (Central Sulawesi and South Sulawesi

transmigrants, n=49) and LE patients (Central Sulawesi, n=9) were correlated to *HhaI* copies/µl to calculate the Y-intercept from the linear regression. The results were further analysed by a Receiver-Operating Characteristics (ROC) curve analysis using a binormal mixture model [130, 131]. Individual samples were scored positive (Mf positive, LE) or negative (transmigrant samples of the ≤ 1 month and 2-4 months group) since the L3 to adult worm development requires about 8 months. The Area Under the Curve (AUC) for the ROC-curve was calculated to measure the discriminatory power of the PCR assay [132].

2.6.7 Statistical analyses

PASW 18.0 (IBM®, Somers NY, USA) was used to test the comparison of two binary variables with the Fisher`s exact test, to correlate between two continuous parameters using Spearman`s rank test and ROC-curve analysis. The Cochran-Armitage test for trend was calculated with SAS 9.2 (SAS, Cary, NC, USA) to compare the trend of *HhaI* copy numbers between the 0.1-3 year and the 4-6 years residents. This trend test modifies the chi-square test and is used in categorical data analysis when the aim is to assess the presence of an association between a variable with two categories and a variable with *k* categories and to incorporate a suspected ordering in the effects of these categories. In this study the two categories were individuals resident 0.1-3 years and 4-6 years; *k* = the intervals for *HhaI* copies/µl. $P<0.05$ was considered significant.

2.7 Genetic associations in brugian filariasis analyzed in patients from Alor Island, Indonesia

2.7.1 Study population

The participants in this case-control study were from Alor Island, Indonesia, who took part in a larger parasitological study of *B. timori* infection [133]. Alor Island is located north of Timor and has approximately 164,000 inhabitants. The highland village of Mainang was chosen for the detailed study and is located in a swampy valley, an excellent breeding site for *Anopheles* mosquitoes, the vector of *B. timori*. The study was approved by the Indonesian Ministry of Health and the University of Indonesia, Jakarta. Samples were collected from March to May 2001. The participants of the detailed survey in Mainang originated from three different residential areas of the village: Welai Selatan, Malaipea and Tominuku. A house-to-house survey was carried out in these quarters to determine the eligible population. All individuals having lived in the area for more than four years were asked to participate in the study. Inhabitants from Welai Selatan and Malaipea came voluntarily to the health center. To exclude a bias due to self-selection, all inhabitants of Tominuku who were present in the village were asked individually to take part in the study. About 90% of the selected persons participated in the survey. Other individuals who heard about the survey and asked for an examination were examined but excluded from the study population. Informed consent was obtained from all persons participating in the survey.

2.7.2 Phenotyping

After registration, all individuals were examined by experienced physicians for clinical signs of lymphatic filariasis, including enlarged or tender superficial inguinal and femoral lymph nodes, acute lymphadenitis and lymphangitis of the extremities, scars at the sites of previously ruptured lymph nodes or vessels, or LE of the extremities. LE of the limbs was classified from grade 1 (pitting, reversible LE) to grade 4 (elephantiasis, non-pitting edema with fibrotic and varicose skin changes) [134]. All participants were additionally asked for a history of clinical signs related to lymphatic filariasis. All persons with LE, including those with elephantiasis, were asked for a day blood collection on the next morning between 10:00 A.M. and noon.

To determine the status of microfilaremia, 1 ml of venous night blood was collected and filtered using 5 µm polycarbonate filters (Millipore, Eschborn, Germany). Filters were stained with Giemsa solution and examined with 100X magnification and the Mf were counted. Filarial-specific IgG4 antibodies to recombinant BmR1 antigen were assessed as described previously [135]. Additional blood was collected and diluted 1:1 with 8 M urea for later DNA extraction.

Patients with LE were introduced to hygienic methods to use on their affected legs and other procedures that may help stop progression of the disease. Following the survey, treatment using a combination of a single dose of DEC (6mg/kg of body weight) and of ALB (400 mg) was offered to all infected individuals.

2.7.3 Genotyping

In total the association of LF with 30 SNPs was analyzed (Appendix II).

From the original 586 participants of the parasitological study, we identified 122 unrelated cases and controls and genotyped them for IL-18 SNPs -607 (rs1946518) and -137 (rs187238) and IL-4R SNPs Ile50Val (rs

1805010) and +3111 3'UTR (rs1049631). Individuals were defined as related if the nearest common ancestor made them first-degree relations. For the IL-18 SNPs, cases for LE were defined as persons with LE stage 2-4 [134]. Controls were persons with no lymphatic pathology, but positive for IgG4 antibodies to filarial antigen. For the IL-4R SNPs, cases for microfilaremia were defined as unrelated persons positive for Mf while Mf negative persons that also did not have lymphedema, but were positive for IgG4 antibodies to filarial antigen, were controls. Levels of specific IgG4 antibodies decrease after loss of Mf and adult worms within a few months in brugian and bancroftian filariasis [60, 136]. The loss of IgG4 has been shown to correlate with the loss of antigen in bancroftian infections [137]. Therefore positivity in a non-quantitative dipstick assay of IgG4 is a reasonable indicator of ongoing infection. While not ideal, in the absence of an antigen test this is currently the only way, in addition to endemicity of filariasis to demonstrate LE due to brugian filariasis rather than another cause. On Alor, the prevalence of LE strictly follows the prevalence of brugian filariasis.

In the IL-18 SNP set, LE cases and controls totaled 39 and 83, respectively. For the IL-4R SNP set, samples size was 83, 41 cases and 42 controls.

Genomic DNA was isolated from 400 µl blood (stored diluted 1:1 with 8M urea at 4 ºC) using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). DNA was eluted from the column in 200 µl AE buffer. For the IL-18 SNPs, genotyping was performed using TaqMan-based assays that were purchased from Applied Biosystems (Foster City, CA). The PCR was performed with a GeneAmp PCR system, model 9700 (Applied Biosystems). Fluorescence levels of the PCR products were measured with an ABI Prism model 7900 HT sequence detector (Applied Biosystems). The IL-4R SNPs were detected using the pyrosequencing technique at the Cologne Centre for Genomics in Cologne, Germany as previously described [138]. The pyrosequencing was performed in a Biotage PSQ HS

96A SNP system (Pyrosequencing AB, Uppsala, Sweden), and the data were captured with PSQ HS SNP software as described by Ronaghi [138].

2.7.4 Statistics

For Hardy-Weinberg Equilibrium (HWE) calculations, including the inbreeding coefficient (Appendix II), the deFinetti online program from Strom and Wienker was used (http://ihg.gsf.de/cgi-bin/hw/hwal.pl). Baseline data were analyzed with the Stat View software (version 5.0) for Windows (SAS Institute Inc., Cary, NC, USA). Single-marker and haplotype analyses were done with the FAMHAP software (http://famhap.meb.uni-bonn.de/) as described previously [139]. In the single-marker analysis, an Armitage trend test (ATT) was used to test significant association of the single SNPs in cases and controls [140]. *P* values with $P \leq 0.05$ were considered to be nominally significant before adjustment for multiple testing. The expectation-maximization (EM) algorithm was used to estimate the maximum-likelihood haplotype frequencies [139, 141]. A likelihood-ratio test was used to assess significance of the haplotype distribution. To correct for multiple testing, the program performs simulations to obtain a *P* value that does not rely on asymptotic theory. To determine the corrected *P* value, FAMHAP performs an Omnibus statistic which permutes the disease status of the individuals for each simulated replicate such that the numbers of cases and controls are the same as in the original sample. Permutation with 200,000 simulated replicates was done to obtain a corrected *P* value (Global *P* value) [142].

3. Results

3.1 Treatment of 4 and 6 weeks doxycycline in onchocerciasis

3.1.1 Nodules

To evaluate the efficacy of doxycycline against *Wolbachia* endobacteria as antifilarial therapy in onchocerciasis patients a randomized placebo-controlled study was conducted.
Patients were treated for 6 weeks with 200 mg/day doxycycline, 4 weeks doxycycline followed by 2 weeks matching placebo or matching placebo for 6 weeks. The aim was to study in a placebo controlled manner the efficiacy of 4- and 6-week treatment with doxycycline followed by IVM, in order to define a minimum regimen needed to achieve *Wolbachia* depletion. Second aim was to assess a potential macrofilaricidal activity of doxycycline on *O. volvulus*.
To quantify the amount of *Wolbachia* in doxycycline and placebo treated patients, real-time PCR for the bacterial *ftsZ* gene was done. Since there is variation between nodules with regard to the amount of human tissue in relation to worms, the *Wolbachia* loads were normalized to *O. volvulus β-actin* as described previously [143]. Nodules without a *β-actin* signal were not considered for further analysis. Depletion of *Wolbachia* after doxycycline, demonstrated by quantitative PCR, was observed at all follow-ups. At 6, 20 and 27 months the nodules from both doxycycline groups showed *Wolbachia* levels that were almost 10-fold lower than nodules from the placebo-treated group (Table 1). This was sustained over the observation period, with no indication of an increase. The median *ftsZ*/*β-actin* ratio at 20 and 27 months in the 6-week group is clearly

lower than in the respective 4-week treatment group (not significant), indicating a better doxycycline activity with the longer treatment duration.

Table 1 Effect of doxycycline treatment on *Wolbachia* loads in worm tissue[a]: results of quantitative PCR

Treatment group	Time (months)	Number of patients/ nodules	Index *ftsZ* / actin[b] (10-90th percentiles)	*P* value[c]
Placebo	6	12 /31	3.24 (0.3-19.5)	-
	20	16 / 70	3.04 (0.2-27.0)	-
	27	7 / 19	1.85 (0.1-29.1)	-
Doxycycline (4 weeks)	20	7 / 13	0.47 (0.01-3.2)	0.0016
	27	5/ 15	0·20 (0.04-3.31)	0.0011
Doxycycline (6 weeks)	6	10 / 33	0.19 (0.02-4.4)	0.0005
	20	12 / 22	0.30 (0.04-6.9)	<0.0001
	27	5 / 11	0.13 (0.04-1.4)	0.0004

[a] DNA was prepared from parts of onchocercomas extirpated at the indicated time points, and the copy numbers of the *Wolbachia* single copy gene *ftsZ* and the nematode gene *β- actin* were determined by quantitative PCR.

[b] Median

[c] Significant differences in the respective doxycycline compared to the placebo group (Mann-Whitney-U test).

No significant differences between the two doxycycline groups were observed at any time point.

3.1.2 Skin samples

To determine the depletion of *Wolbachia* in skin Mf (PCR) and analyse the Mf content in the treated and untreated patients (microscopic analysis), skin biopsies near the site of the extirpated nodule were taken from all patients who were available for follow-up and underwent nodulectomy. Not all patients had enough skin material for both microscopic and PCR analysis. As IVM treatment affects skin Mf only patients who had taken IVM at 6 months after treatment were included for further analysis to have an identical treatment background.

Table 2 presents the skin Mf load at all follow-ups by microscopic analysis. The results showed that at month 20, in the placebo group that had received only IVM, Mf loads were reduced (Table 2). Except one patient, all were positive for skin Mf. In contrast, in both groups that had received doxycycline plus IVM, the median Mf level was highly reduced, being zero in the 6-week doxycycline group 20 months after treatment compared to placebo. In both doxycycline groups, the proportion of patients, who were still positive for skin Mf, was significantly lower than in the placebo group. At month 27, the significant and sustained reduction of skin Mf as compared to placebo was still maintained in the 6-week doxycycline group. There was less reduction of skin Mf in the 4-week group, while in the placebo group, there was a clear resumption of Mf production, confirming earlier reports which show that production of Mf starts a few months after the administration of IVM [144, 145].

To assess *Wolbachia* levels in Mf after treatment with doxycycline, real-time PCR measuring the amount of bacterial *ftsZ* and worm *β-actin* was performed. The PCR results showed that the remaining Mf contained almost 10-fold lower levels of *Wolbachia* at 20 months after treatment in the 4- and the 6-weeks group. In the 4-weeks doxycycline group at month 20, the ratio *ftsZ*/*β-actin* was significantly lower compared to the respective placebo group. This can not be maintained, as at month 27, there is a *ftsZ*/*β-actin* ratio similar to that found in the placebo group. A

different picture is seen in the 6-weeks doxycycline group, were at month 20 and 27 a significantly lower *ftsZ*/*β-actin* ratio can be observed (Table 3).

Table 2 Effect of doxycycline treatment on Mf loads in skin biopsies: results of microscopic analysis

Treatment group	Time (months)	Number of patients assessed	Mf levels[a] GMI (range)	Median	Number of patients with skin Mf (% total)
Placebo	6	16	14.3 (0-64)	27.6	15 (94%)
	20	18	4.4 (0-58)	4.3	17 (94%)
	27	15	5.5 (0-75)	3.0	12 (80%)
Doxycycline (4 weeks)	20	10	0.7 (0-6)	0.3	6 (60%)
	27	10	1.3 (0-23)	0.4	5 (50%)
Doxycycline (6 weeks)	6	16	14.7 (0.4-60)	25.2	16 (100%)
	20	14	0.1 (0-1)	0.0	3 (15%)
	27	10	0.8 (0-6)	0.6	7 (70%)

(Published by Hoerauf *et al.,* 2008)

[a] Geometric mean intensity (GMI), median values, and ranges of Mf/mg skin are shown. The GMI was calculated using the log (x+1) method according to Williams.

Histology examinations were done by S. Specht and D. W. Büttner as published [45].

Table 3 Effect of doxycycline treatment on *Wolbachia* loads in skin biopsies: results of quantitative PCR

Treatment group	Time (months)	Number of patients/ skin snips	Index *ftsZ* / actin[a] (10-90th percentiles)	*P* value[b]
Placebo	6	16/25	0.04 (0.0006-0.045)	-
	20	17/29	0.03 (0.0018-0.15)	-
	27	8/14	0.02 (0.005-0.51)	-
Doxycycline (4 weeks)	20	8/18	0.004 (0.0005-0.02)	0.0009
	27	7/14	0.02 (0.003-0.38)	0.6800
Doxycycline (6 weeks)	6	12/19	0.004 (0.0006-0.04)	0.0003
	20	13/17	0.006 (0.001-0.11)	0.0209
	27	5/15	0.005 (0.0005-0.03)	0.0009

[a] Median

[b] Significant differences in the respective doxycycline compared to the placebo group (Mann-Whitney-U test).

3.2 Treatment of 5 weeks doxycycline in onchocerciasis

3.2.1 Nodules

Previously, a treatment with 100 mg/day doxycycline administered for 6 weeks was studied [41]. The aim of the present study was to investigate the effects of a shortened regimen of only 5 weeks doxycycline treatment on the depletion of *Wolbachia* endobacteria from *O. volvulus*.

All palpable nodules of 20 doxycycline and ten untreated patients were excised and analyzed by histology, after excluding patients which could not take part in the study due to incomplete treatment or refusal of nodulectomy

Out of the 20 doxycycline treated patients, 12 patients had enough tissue material for PCR analysis at 20 months after treatment (45 nodules), whereas of these, 10 were able to give PCR samples at 27 months after treatment (22 nodules). There were 9 untreated control PCR samples (23 nodules). Compared to the untreated control group, at both follow-ups the nodules of the doxycycline treated group showed a significantly lower *ftsZ*/*β-actin* ratio (Table 4). There was also a significantly lower ratio at month 20 compared to month 27. These findings fit to the histology results. In doxycycline treated patients, 76% female and 62% male worms did not contain *Wolbachia*. Only 7% of the worms in the doxycycline treated group showed normal embryogenesis and 7% of the nodules had living Mf in the human tissue [46]. These Mf were found only adjacent to young productive *Wolbachia*-positive worms, which had probably been acquired after the end of treatment during the follow-up period [46, 146].

Table 4 Effect of 5 weeks doxycycline treatment on *Wolbachia* loads in worm tissue[a]: results of quantitative PCR

Treatment group	Time (months)	Number of patients/ nodules	Index *ftsZ* / actin[b] (10-90th percentiles)	*P* value[c]
Control	27	9/23	1.88 (0.33-6.92)	
Doxycycline (5 weeks)	21	12/45	0.08 (0.011-1.24)	<0.0001
	27	10/22	0.30 (0.054-1.57)	<0.0001

[a] DNA was prepared from parts of onchocercomas extirpated at the indicated time points, and the copy numbers of the *Wolbachia* single copy gene *ftsZ* and the nematode gene *β- actin* were determined by quantitative PCR.

[b] Median

[c] Significant differences in the respective doxycycline compared to the control group (Mann-Whitney-U test).

3.2.2 Skin samples

As in the placebo controlled study, the decrease of Mf levels was first monitored by microscopic analysis (Table 5). Skin Mf levels were determined at 21 and 27 months after doxycycline treatment. Some patients only attended one of the follow-ups, therefore the available skin Mf value was taken for the analysis and gives a combined result of month 21 and month 27. In the doxycycline treated group, 13 of the 21 patients had no Mf and the Mf load had decreased to 0.2 Mf/mg skin, indicating an interruption of Mf production by doxycycline alone since the last IVM intake was by then 31-43 months prior to the skin biopsy. As in the placebo controlled study, only patients who had not taken IVM more than two times prior to study onset were included.

Since transmission in this area was not interrupted (R.Garms, Bernhard-Nocht Institute, unpublished report, 2006), it is assumed that remaining

Mf are from female worms that are acquired after the end of doxycycline treatment.

Table 5 Effect of 5 weeks doxycycline treatment on *Wolbachia* loads in skin biopsies: results of microscope analysis

Treatment group	Time (months)	Number of patients examined	Number of Mf carriers	Mf levels[a]	
				GMI (range)	Median
Control	27	10	10	43 (5-597)	55
Doxycycline (5 weeks)	21 or 27	21	8	0.9 (0-58)	0.2

(Published by Hoerauf *et al*., 2009)

[a] Geometric mean intensity (GMI), median values, and ranges of Mf/mg skin are shown. The GMI was calculated using the log (x+1) method according to Williams

Histology examinations were done by S. Specht and D. W. Büttner as published [46].

The PCR results show a significant decrease in bacterial *ftsZ*/*β-actin* ratio in the doxycycline treated group compared to the untreated control group at month 21 (Table 6). At month 21 the remaining Mf in the treated group had increased *Wolbachia* levels. At month 27 the median of the ratio *ftsZ*/*β-actin* was much lower than the median in the control or even the doxycycline group at month 21, but the range in some samples was higher than the control samples. Therefore no significant difference between the 27 months samples and the control samples can be observed. When combining the doxycycline values for month 21 and month 27, there was still a significant decrease in the *ftsZ*/*β-actin* ratio compared to the placebo group (P=0.022).

Table 6 Effect of 5 weeks doxycycline treatment on *Wolbachia* loads in skin biopsies: results of quantitative PCR

Treatment group	Time (months)	Number of patients/ skin snips	Index *ftsZ* / actin[a] (10-90th percentiles)	*P* value[b]
Control	27	9/18	0.04 (0.004-0.15)	
Doxycycline (5 weeks)	21	11/12	0.01 (0.001-0.14)	0.004
	27	18/31	0.009 (0.002-0.56)	0.117

[a] Median

[b] Significant differences in the respective doxycycline compared to the control group (Mann-Whitney-U test).

3.3 Treatment of 6 weeks azithromycin in onchocerciasis

To analyse the efficacy of the antibiotic azithromycin to deplete *Wolbachia* endobacteria, a study with onchocerciasis infected patients was conducted. The azithromycin treatment was well tolerated by most participants. A few patients complained of mild side effects. Four had diarrhoea and three had abdominal pain. None of the patients complained or presented severe adverse reactions.

Beside histological examination of the extirpated nodules, some patients at 6 months after study onset had enough nodule material for an additional DNA extraction and PCR analysis for the *Wolbachia ftsZ* and the *O.volvulus β-actin* gene.

Table 7 Effect of Azithromycin treatment on *Wolbachia* loads in worm tissue: results of quantitative PCR

Treatment group	Time (months)	Number of patients/ nodules	Index *ftsZ* / actin[a] (10-90th percentiles)
Control	0	9/14	11.84 (1.87-62.8)
Azithromycin 250mg/day 6 weeks	6	4/5	16.59 (5.62-549.1)
Azithromycin 1200mg/week 6 weeks	6	2/4	31.08 (5.50-67.03)
	12	8/32	63.13 (6.97-749.4)

[a]Median

DNA was prepared from parts of onchocercomas extirpated at the indicated time points, and the copy numbers of the *Wolbachia* single copy gene *ftsZ* and the nematode gene actin were determined by quantitative PCR.

The PCR results did not show a significant decrease of the *Wolbachia ftsZ/*worm *β-actin* ratio compared to the control group, neither in the 250 mg/day for 6 weeks nor the 1200/week for 6 weeks group at any time point. Median levels of the *ftsZ/β-actin* ratio showed even higher levels as in the control group (Table 7).

Monitoring of *Wolbachia* expressed as *ftsZ/β-actin* ratio provided insight into the content after treatment at the molecular level. Histological results revealed that the *Wolbachia* loads of the worms were not different from those of the untreated group at 6 months after treatment. Also, the Mf in the skin biopsies taken 6 months after azithromycin treatmen still contained *Wolbachia*. In contrast, 12 months after administration of the 250 mg/day azithromycin regimen, the worms presented a significantly reduced *Wolbachia* load analysed by histology: only 65% of the living female filariae contained many (>50) endobacteria compared to 92% in untreated living females [147]. At this time point no samples for PCR were left for that group to confirm these results with PCR.

In conclusion, by histological analysis a reduction of *Wolbachia* after 6-weeks daily azithromycin treatment could be observed, but no depletion as confirmed by PCR. It is further concluded from the histological data, that 6-weeks azithromycin treatment does not reduce or interrupt the embryogenesis of *O.volvulus*.

3.4 Treatment of *W. bancrofti* infection with rifampicin and/or doxycycline

To evaluate the efficacy of the antibiotic substance rifampicin on *Wolbachia* depletion in LF with several treatment durations and dosages, *W. bancrofti* infected patients were included in an open trial.

Fourtyeight men were randomized and 39 patients completed the study. Of the 48 men, 12 were treated with 200 mg doxycycline/day for 4 weeks, 16 were treated with a combination of 200 mg doxycycline/day + 10mg/kg/day rifampicin for 2 weeks and 11 patients received placebo matching doxycycline. Nine patients did not complete the allocated intervention, three from the doxycycline 4 weeks arm, one from the doxycycline+rifampicin combination and five from the placebo group (Figure 15).

Table 8 illustrates the changes in microfilaremia and *Wolbachia/*Mf from baseline and at follow-up 4 months after treatment. Baseline measurements were similar between the allocated treatment groups, except Mf load which was higher in placebo group than in the two treatment groups. However, Mf loads between the 4-week doxycycline group and the 2-week doxycycline+rifampicin group were comparable (Table 8).

At 4 months post treatment, *Wolbachia ftsZ* copies/Mf were reduced by 94% (P=0.002, Wilcoxon Rank test) in the 4-week doxycycline group, followed by 86% reduction (P=0.034, Wilcoxon Rank test) in the 2-week rifampicin+doxycycline group. There was no significant change in the placebo group (P=0.138); rather, values had a trend to increase. At 12 and 24 months after treatment, Mf in the 4-week doxycycline group were only detectable via microscope in a few samples (1-4 Mf positive patients). Therefore we excluded PCR at these follow-ups because PCR results of only one patient would not be comparable to the other treatment groups.

Additional results [121]

At 12 months after treatment, the 4-week doxycycline treatment completely reduced the Mf load (P=0.012). Significant reductions in microfilaremia were also seen in the 2-week doxycycline+rifampicin (P=0.003) and placebo (P=0.043) regimens. However, at 18 and 24 months, while the 4-week doxycycline group and 2-week doxycycline+rifampicin regimens continued to maintain significantly low microfilarial loads, there was a rise in Mf loads in the placebo patients. Measurement of adult worm vitality with USG showed that at 12 months follow up, the 4-week doxycycline group had no change in worm vitality. The same trend could be seen in the number of FDS. While there was a decrease of FDS in the doxycycline and the combination group, there was rather an increase in the placebo group. At 18 months after treatment, this pattern remained the same with the 4-week doxycycline group having 100% loss of worm nests, the 2-week doxycycline+rifampicin group showing a 50% loss of worm nests while the placebo group remained unchanged. This trend was also observed at 24 months follow up but was less significant.

Table 8 Primary variables measured before and after doxycycline and rifampicin treatment

4 weeks Doxycycline	**Before treatment**	**4 months**
Number of **MF-positive individuals/all** (%)	12/12 (100%)	9/12 (75%)
P value[b]		*P*=0.515
Median of **Microfilaraemia**[a]	523 (154 - 1013)	307 (12 - 1068)
P value[b]		*P*=0.388
Median ***Wolbachia* load/Mf**[a]	167 (71 – 238)	11 (4 – 24)
P value[b]		*P*=0.002
2 weeks Rifampicin plus Doxycycline		
Number of **MF-positive individuals/all** (%)	12/12 (100%)	10/12 (83%)
P value[b]		*P*=1.0
Median of **Microfilaraemia**[a]	392 (122 - 460)	394 (39 – 703)
P value[b]		*P*=0.695
Median ***Wolbachia* load/Mf**[a]	121 (95 – 200)	18 (7 – 52)
P value[b]		*P*=0.034
4 weeks placebo matching Doxycycline		
Number of **MF-positive individuals/all** (%)	5/5 (100%)	5/5 (100%)
Median of **Microfilaraemia**[a]	2380 (1230 – 3680)	2585 (412 – 3738)
P value[b]		*P*=0.686
Median ***Wolbachia* load/Mf**[a]	133 (85 – 164)	358 (100 – 879)
P value[b]		*P*=0.138

[a] Median with 25th-75th percentile (Published by Debrah *et.al*, 2011)
[b] Wilcoxon Rank test

3.5 Retarded *O. volvulus* L1 to L3 larval development in the *Simulium damnosum* vector after anti-wolbachial treatment of the human host

3.5.1 Selection of *Simulium* capture side

In selecting a capture site, we determined the natural parous and infection rates by collecting and dissecting wild *Simulium* flies. A total of 1722 flies were dissected, 19.6% were parous and 0.001% infected.

3.5.2 Kinetics of larval development

To determine the skin microfilarial loads and recovery of *O. volvulus* larvae from flies that had fed on doxycycline or placebo treated individuals, skin biopsies and dissected flies were analyzed for developing larvae. Mf were counted and expressed as Mf per skin biopsy (Table 9). The total number of larvae (L1, L2, and L3) collected after dissection from the flies was calculated. The proportion of each larval stage that developed at each follow-up in each group was generated by dividing the larval number (L1+L2 or L3) by the total number of larvae for each time point (Figure 19). The proportions were plotted against the month of dissection (month post-treatment) to follow the kinetics of larval development in both groups. Starting 3 months post-treatment, differences in the median recovery of L1+L2 and L3 from flies fed on the doxycycline treated volunteers were seen. The proportions of L1+L2 larvae recovered from flies that had fed on doxycycline treated individuals were significantly higher ($P<0.05$, Mann-Whitney U test) at 3 and 5 months post-treatment compared to placebo (Figure 19).

Table 9 Median larval recoveries from skin and blood-fed black flies of doxycycline and placebo volunteers

Treatment	**Doxycycline n=7 (200mg/day 6 weeks)**					
Month of the study	0	1	2[a]	3	4	5
Mf/skin snip[b]	26 (13-62)	43 (32-98)	40 (8-78)	13 (7-35)	9 (2-111)	28 (4-70)
L1+L2 larvae stage[b]	52 (45-122)	29 (3-101)	130 (28-365)	678 (167-941)	297 (103-343)	426 (201-1177)
L3 larvae stage[b]	703 (509-949)	281 (150-554)	797 (211-1354)	709 (335-935)	318 (71-549)	53 (44-209)
	Placebo n=5					
Mf/skin snip[b]	25 (10-70)	83 (41-178)	114 (15-162)	27 (10-97)	63 (9-88)	63 (4-209)
L1+L2 larvae stage[b]	124 (71-147)	33 (7-67)	112 (42-244)	390 (90-761)	217 (118-531)	575 (388-926)
L3 larvae stage[b]	789 (396-923)	313 (226-469)	666 (385-855)	983 (487-1126)	406 (247-530)	240 (116-433)

[a] Median of larval recovery with range

[b] At 2 months post treatment only 6/7 doxycycline treated volunteers attended the follow-up meeting

Correspondingly, at 3 and 5 months post-treatment a significant decrease ($P<0.05$, Mann-Whitney U test) in the proportion of L3 that developed in flies that had fed on doxycycline treated individuals as compared to placebo treated individuals was observed (Figure 19).

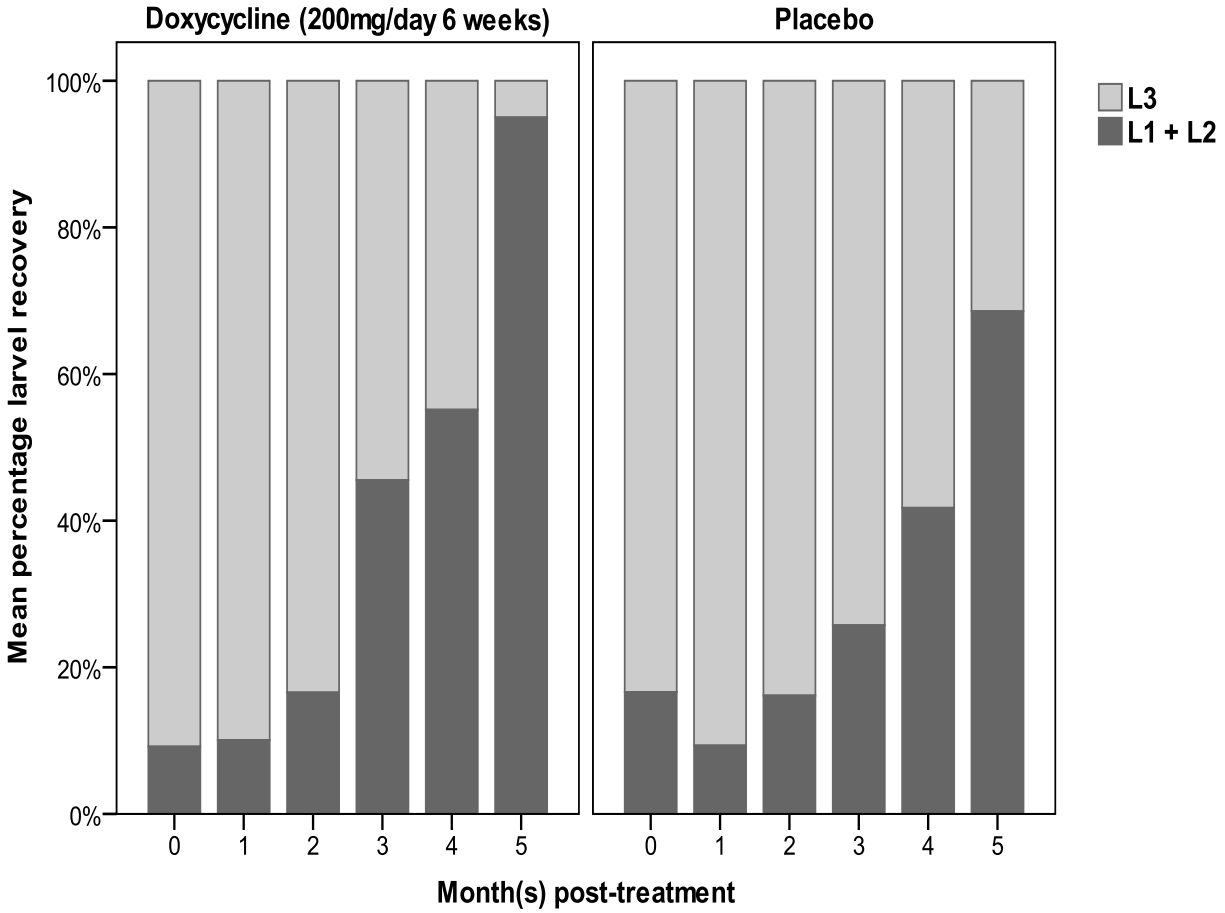

Figure 19 Larval proportions

The proportion of larvae at each stage was calculated by dividing the number of larvae (stage L1+L2; L3) by the total larvae collected from the captured blackflies after they had fed on the doxycycline (n=7) or placebo (n=5) treated volunteers. A) The proportion of L1+L2 larvae in flies that had fed on doxycycline treated individuals (black bars) was higher beginning at 3 months post-treatment compared to placebo.

BThe proportion of L3 larvae in flies that had fed on doxycycline treated volunteers (grey bars) was lower than that in the placebo volunteers at months 3,4 and 5 post-treatment. Proportions are given as percentages of median larval recovery. A 100% correspond to the total number of larvae recovered at the indicated timepoint.

3.5.3 Wolbachia depletion after treatment with doxycycline

To analyse the effect of antibiotic treatment on the bacterial endosymbionts of *O. volvulus* Mf from the skin and larvae isolated from the vector, a quantitative real-time PCR assay was established for the *O.*

volvulus Wolbachia ftsZ gene. For normalization to worm material in the individual sample, a second PCR assay for *O. volvulus β-actin* was done.
Compared to the placebo group, fewer samples were positive for *ftsZ* above the PCR detection limit in the Mf of doxycycline treated individuals at 2, 3, 4 and 5 months post-treatment. Analysis of the Mf that had emerged from skin biopsies showed a greater than 50% reduction in the number of *Wolbachia* one month post-treatment in the doxycycline treated group (Table 10). *Wolbachia* reduction further increased to 81% in the follow-up time points. At the two month follow-up only two of the six Mf samples (33%) gave a positive *ftsZ* signal in the PCR which further decreased to only one *ftsZ* positive sample (14%) at the 5 months follow-up. A similar pattern was seen in all larval stages collected from the vector. In contrast, in the placebo group, almost every larval stage at each time point produced a clear signal for bacterial *ftsZ*. Only two placebo samples (L1 at pre-treatment and L2 at month 2 post-treatment) did not have a positive signal above the detection limit for *ftsZ.* These samples also gave a weak signal for *actin* (data not shown), indicating a very low DNA content. For all samples, an inhibition test PCR with a mouse IFN-γ plasmid did not show any inhibition by the sample DNA. The negative *ftsZ* samples therefore did not result from any inhibitory factors in the DNA.

Table 10 Effect of doxycycline and placebo treatment on *Wolbachia* loads in larval worms: results of quantitative PCR

Doxycycline (200mg/day 6 weeks)	**Number of *Wolbachia ftsZ*positive volunteers for the indicated larval stages** (median *ftsZ/β*-actin ratio)			
Month of the Study	MF skin	L1 (Mf vector)	L2	L3
0 (pre-treatment)	**7/7** (0.06)	**7/7** (0.058)	#	**6/6** (0.026)
1	**6/7** (0.024)	**4/7** (0.002)	#	**3/7** (0.008)
2	**2/6** (0.068)	**0/6**	**1/6** (0.007)	**2/6** (0.011)
3	**2/7** (0.131)	**2/7** (0.06)	**4/7** (0.029)	**4/7** (0.059)
4	**3/7** (0.085)	**0/7**	**3/7** (0.002)	**2/7** (0.036)
5	**1/7** (0.167)	**3/7** (0.001)	**1/7** (0.002)	**3/6** (0.017)
Placebo				
0 (pre-treatment)	**5/5** (0.02)	**4/5** (0.03)	#	**5/5** (0.05)
1	**4/4** (0.05)	**5/5** (0.06)	#	**5/5** (0.03)
2	**5/5** (0.22)	**5/5** (0.11)	**4/5** (0.06)	**5/5** (0.05)
3	**4/4** (0.70)	**5/5** (0.41)	**5/5** (0.16)	**5/5** (0.19)
4	**5/5** (0.30)	**4/4** (0.32)	**4/4** (0.16)	**5/5** (0.43)
5	**5/5** (0.56)	**3/3** (0.08)	**4/4** (0.09)	**5/5** (0.12)

L1 and L2 counts were combined at months 0 and 1 of the study

3.6 Detection of the *HhaI* gene in infection with *B. malayi* by real-time PCR

3.6.1 PCR validation

To establish a method for detection of parasite DNA in human infection with *B. malayi*, especially cryptic (pre-patent or latent infections), PCR for *HhaI* was used to measure parasite DNA from plasma. Using the Quantitect® Probe master mix and a plasmid standard dilution series, the efficacy of the reaction was >85%. In 100% of the samples, 200 copies/µl could be detected. In 85% of the samples 22 copies could be detected. This was set as the detection limit of the PCR with 2 µl sample DNA (Figure 21). For samples which were below the detection limit for *HhaI* in the 2 µl assay, a second PCR with the QuantiTect® Virus mastermix using 10 µl of DNA was done. A plasmid standard dilution series showed that the sensitivity with the 10 µl assay could be decreased down to 2 copies/µl and the reaction efficacy reached 100% (Figure 22). Each PCR replicate used 10 µl of diluted plasmid as DNA target in a final 20 µl reaction volume with the QuantiTect® Virus NR Master Mix. At the lowest dilution (2 copies/µl) 50% of the samples were detected. 100% of the samples were detected beginning with the 20 copies/µl.

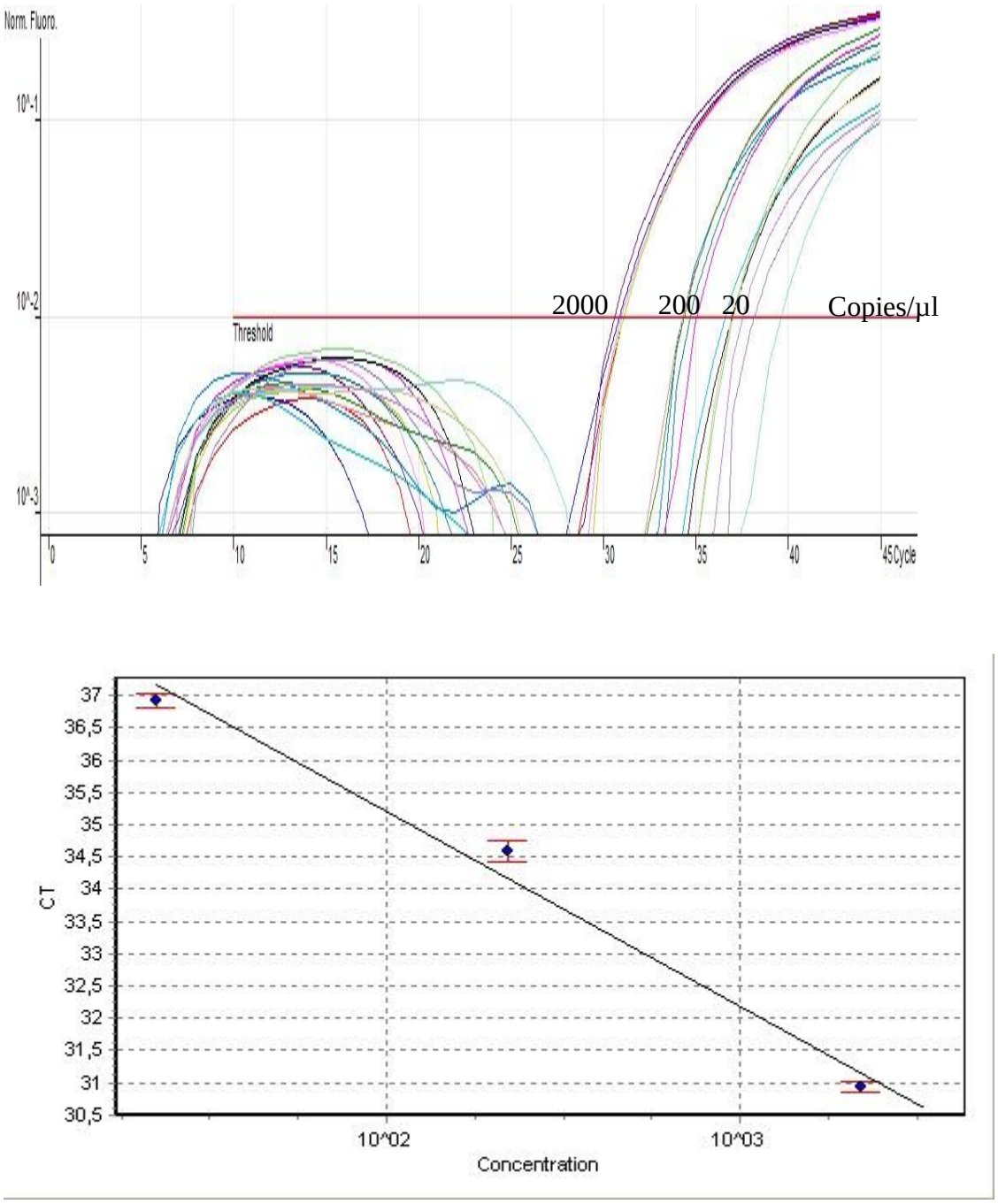

Figure 21 Detectionlimit *HhaI* PCR 2 µl Assay

The detection limit of the *HhaI* PCR was determined with a plasmid dilution series using 2-2000 copies/µl. Each sample contained 2 µl of diluted plasmid in a final reaction volume of 20 µl using the QuantiTect® Probe PCR Master Mix, Qiagen. In the lowest dilution (2 copies/µl), no signal was detected. In the second lowest (20 copies/µl) 85% of the samples gave a positive signal. With the 200 copies/µl dilution, 100% of the plasmid samples were detected. The PCR reaction efficacy was 85%.

The standard curve is shown with standard error of mean.

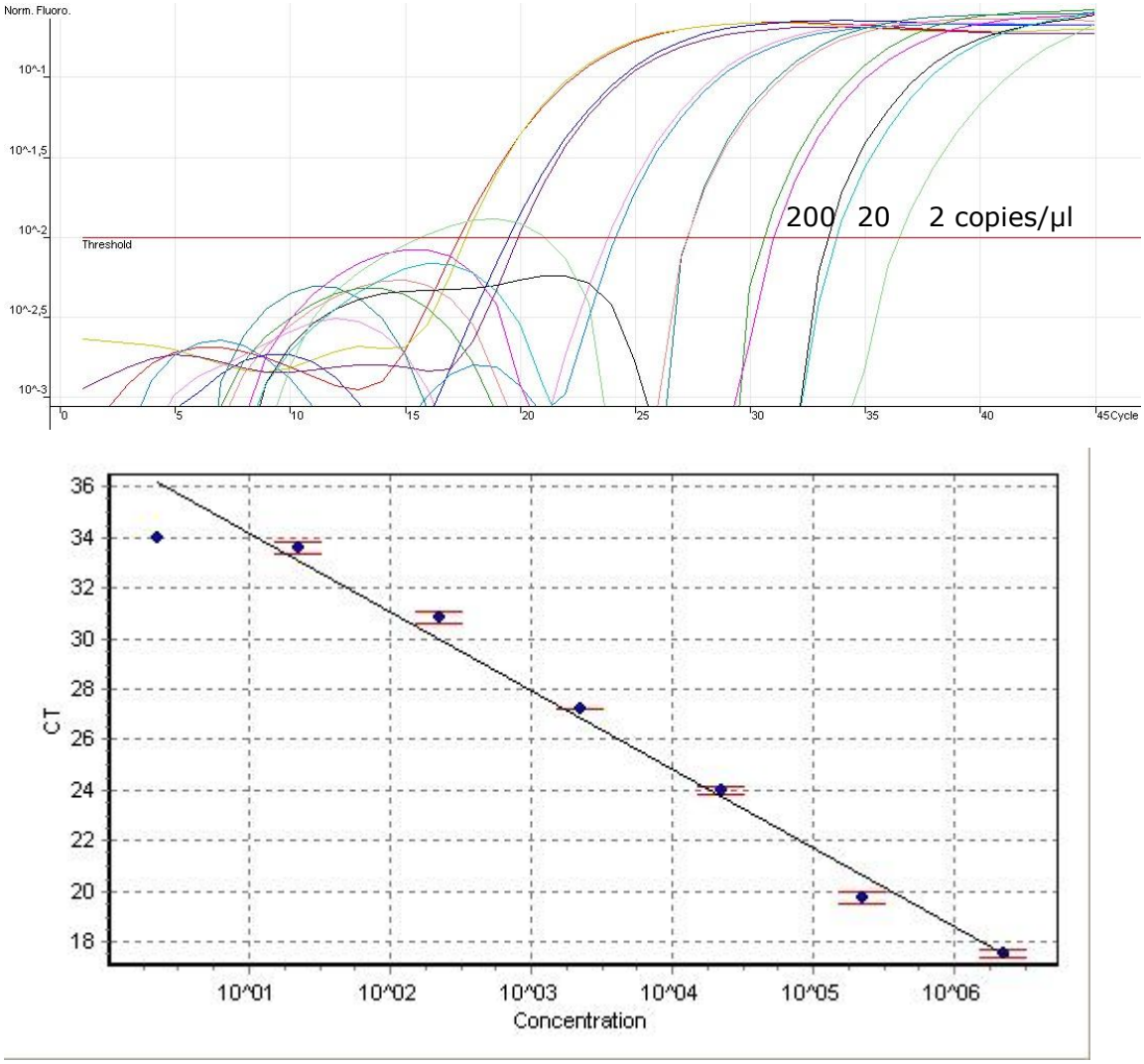

Figure 22 Detection limit *HhaI* PCR 10 µl Assay

Each PCR replicate used 10 µl of diluted plasmid as the DNA target in a final 20 µl reaction volume. At the lowest dilution (2 copies/µl) 50% of the samples were detected. 100% of the samples were detected beginning with the 20 copies/µl.

The standard curve is shown with standard error of mean.

3.6.2 Detection of the *HhaI* gene in *B. malayi* infected jirds by real-time PCR

To validate the *HhaI* PCR assay used for detection of *B. malayi* infection in plasma, the real-time QuantiTect® PCR assay was used on plasma taken eight weeks after infection from nine infected and nine naive jirds. No Mf could be found at this time indicating a pre-patent status. Patent B. malayi infections in jirds can usually be detected 75-110 days after infection [129]. All infected jirds were confirmed to have adult worms (Median=9, range=9-76) when the animals were necropsied.

Table 12 *HhaI* detection in plasma from pre-patent jirds (56 days post infection)

Animal group	*HhaI* positives	Adult worm recovery[a]
infected jirds	9/9	40 (9-76)
uninfected jirds	0/9	0

[a] Median (range)

The *B. malayi HhaI* PCR assay detected a positive signal in all infected jirds, but was negative in plasma samples from uninfected jirds (Table 12). The results confirmed the sensitivity of the *HhaI* PCR in pre-patent, amicrofilaremic samples.

3.6.3 DNA extraction from plasma used as negative/positive controls

To validate the PCR, 30 Mf positive and 9 elephantiasis (latent infected) samples from patients from Central Sulawesi were measured (Table 13). In the nine elephantiasis patients, only one was Mf positive. Additionally, 10 plasma samples of European blood donors, 19 samples with different parasite infections and 10 plasma samples from another study in Ghana

with *W. bancrofti* infected patients were also tested with the *HhaI* PCR 2 and 10 µl assays (Table 14).

Table 13 Positive control validation *HhaI* PCR Sulawesi, Indonesia

Mf positive patients	PCR positive 2 µl[a]	%	PCR positive 10 µl[b]	%
30/30	30/30	100	30/30	100
Elephantiasis patients				
Mf+ n=1 Mf- n=8	7/9	78	9/9	100

[a] 2 µl of sample DNA was used in the PCR

[b] 10µl of sample DNA was used in the PCR

Table 14 Negative control validation

	2 µl[a]	10 µl[b]	%
W. bancrofti Mf positive patients	0/10	0/10	0
Toxocara spec.	0/4	0/4	0
Schistosoma spec.	0/5	0/5	0
Strongyloides stercoralis	0/4	0/4	0
Dirofilaria repens	0/2	0/2	0
European blood donors	0/10	0/10	0

[a] 2 µl of sample DNA was used in the PCR

[b] 10µl of sample DNA was used in the PCR

In all Mf positive samples, detection of the *HhaI* gene was successful. In the elephantiasis group, first PCR with the 2 µl assay gave 100% positive detection, although only one patient was Mf positive with one Mf/ml. In a second PCR only 7/9 were positive in the PCR (Table 13). This is due to the low amount of parasite DNA in the sample, which is in a range were the rate of detection is 85% in the 2 µl assay. In the following 10 µl assay, all samples of the elephantiasis group were positive. In the samples used as negative controls (Table 14) there was no positive detection of the *HhaI* gene, confirming the specificity of the assays.

3.6.4 Plasma from *B.malayi* infected patients from Indonesia and threshold determination

After validating the assays, the real-time PCR was used to detect parasite DNA in a transmigrant population, compared to life-long residents in a region endemic for *B. malayi*. There were 229 plasma samples of a transmigrant population in South Sulawesi and 105 plasma samples of life-long residents (LLR) measured for the *HhaI* gene of *B.malayi* (Table 15). All of the 19 Mf positive patients were positive by the *HhaI* PCR assay, whereas only 17/19 (89.5%) had previously been detected by the IgG4 ELISA test (Figure 23).

Compared to the IgG4 antibody tests, which are based on an IgG4 ELISA using crude extract with adult worm antigen or BmR1 antigen, the *HhaI* PCR showed a higher level of detection at all time points, even in transmigrants that had been in the endemic area only a few months at the time of sampling (Table 15).

Conversely, there were 42 *HhaI* negative samples of which 19 were IgG4 positive, arguing for previous exposure to *B. malayi* and against latent infection (Figure 23 and 24).

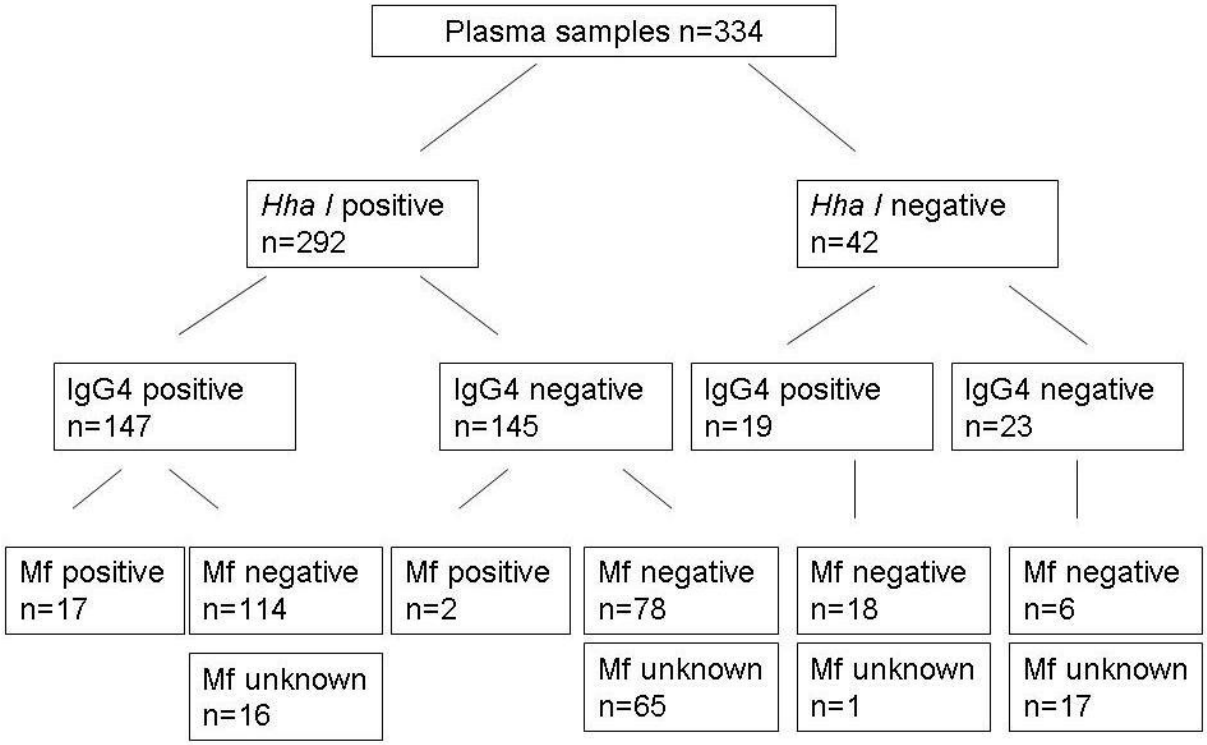

Figure 23 Flow Chart HhaI and IgG4 ELISA

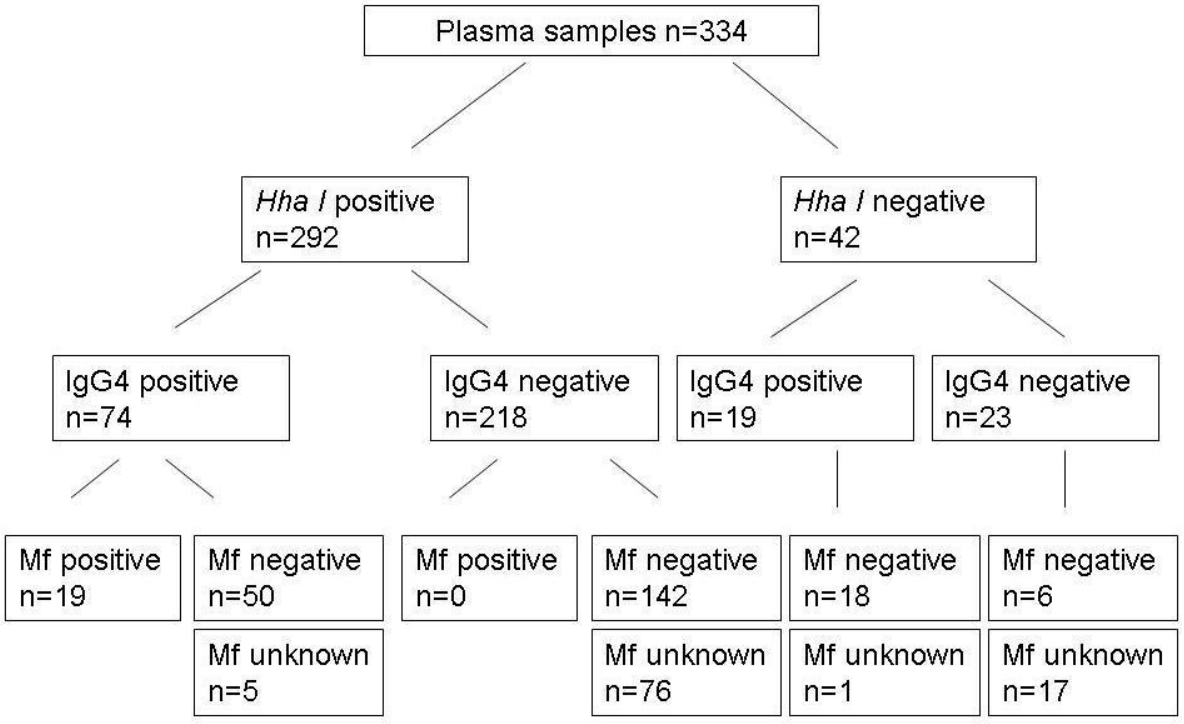

Figure 24 Flow Chart HhaI and IgG4 dipstick BmR1

Table 15 Detection of *B. malayi HhaI* in transmigrants and life-long residents in South-Sulawesi, Indonesia

Length of residence	Cases	Children/ Adults[a]	Mf prevalence	*HhaI* prevalence (%)	IgG4 prevalence (%) SWA-ELISA	IgG4 + SWA-ELISA/ *HhaI*+ [b]	IgG4 prevalence (%) BmRI	IgG4 + BmR1/ *HhaI* + [b]
≤ 1 month	17	3/14	- [c]	14/17 (82)	0/17 (0)	0/14	0/17 (0)	0/14
2-4 months	33	10/23	- [c]	25/33 (76)	3/33 (9)	2/25	0/33 (0)	0/25
3 years	67	34/33	0[d]	58/67 (87)	18/67 (27)	18/58	5/67 (7)	5/58
4 years	52	19/33	0[e]	50/52 (96)	24/52 (46)	22/50	6/52 (12)	6/50
5 years	19	8/11	0	16/19 (84)	11/19 (58)	9/16	8/19 (42)[f]	7/16
6 years	41	20/21	5	34/41 (83)	23/41 (56)	19/34	17/41 (41)[g]	16/34
LLR	105	71/34	14[e]	95/105 (90)	87/105 (83)	77/95	42/105 (40)	40/95
Total	334	165/169	19					

[a] Children ≤ 15 years, adults 16 years+
[b] match of IgG4 (SWA/BmR1) to *HhaI* PCR results
[c] for logistic reasons blood was collected during daytime in 2 villages, therefore Mf counts are absent
[d] Mf prevalence in the 3-year residents could be determined in 20 individuals only
[e] one patient with unknown Mf status was included
[f] 3 individuals were positive for IgG4 BmR1 and *HhaI* PCR, but negative for SWA-ELISA
[g] 4 individuals were positive for IgG4 BmR1 and *HhaI* PCR, but negative for SWA-ELISA

Defining Mf+ (Central Sulawesi and transmigrants) and LE samples as having/having had an active infection, the Mf numbers significantly correlated with *HhaI* copies/µl (r=0.52, P<0.001, Spearman rank correlation). The linear regression line from the correlation crossed the Y-axis at 47.31 *HhaI* copies/µl (SD=1.43) (Figure 25). The threshold of active infections was calculated to be 53.03 *HhaI* copies/µl (Y-intercept plus 4 SD) [148].

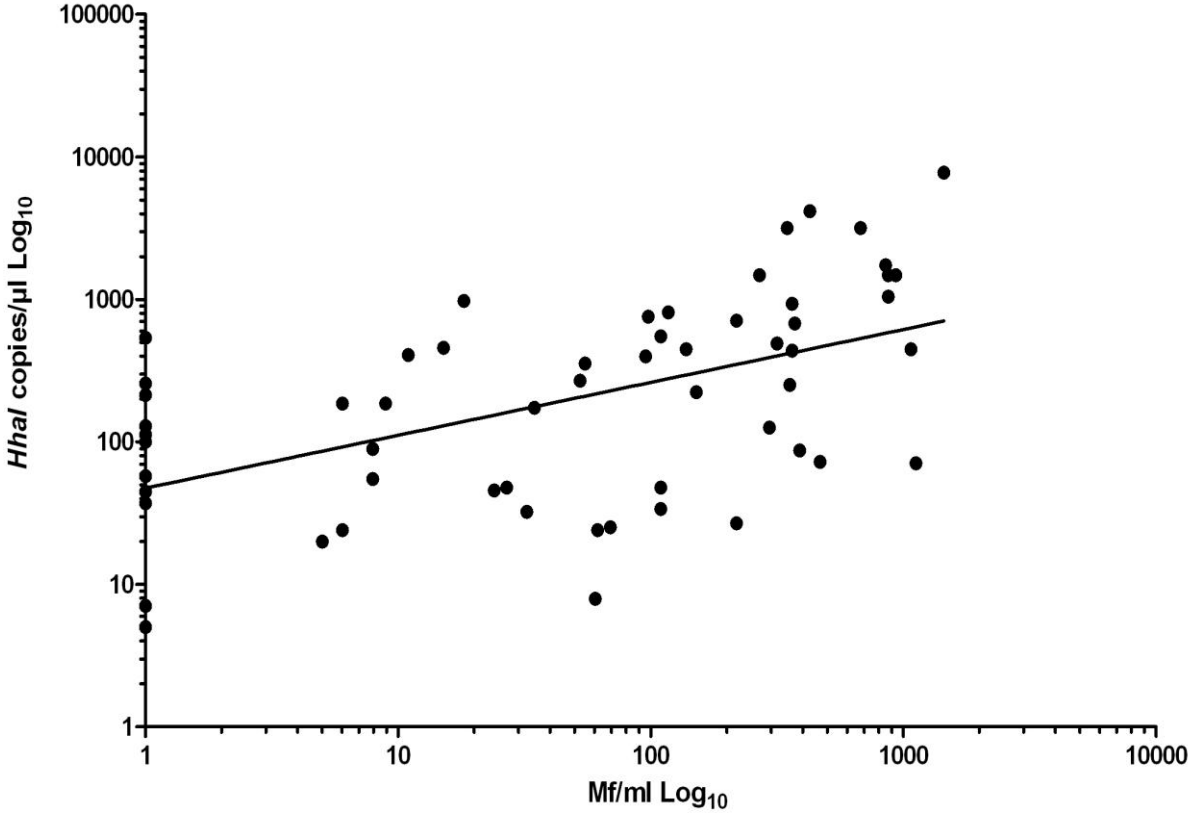

Figure 25 Determination of a threshold for active infection

Mf loads from Mf+ and LE patients were correlated to *HhaI* copies/µl (n=58). Linear regression was performed to calculate the Y-intercept and standard deviation of the line. The threshold was defined as: Y-intercept (47.31) + 4xSD (1.42). Spearman Rank correlation resulted in r=0.52 with *P*<0.001.

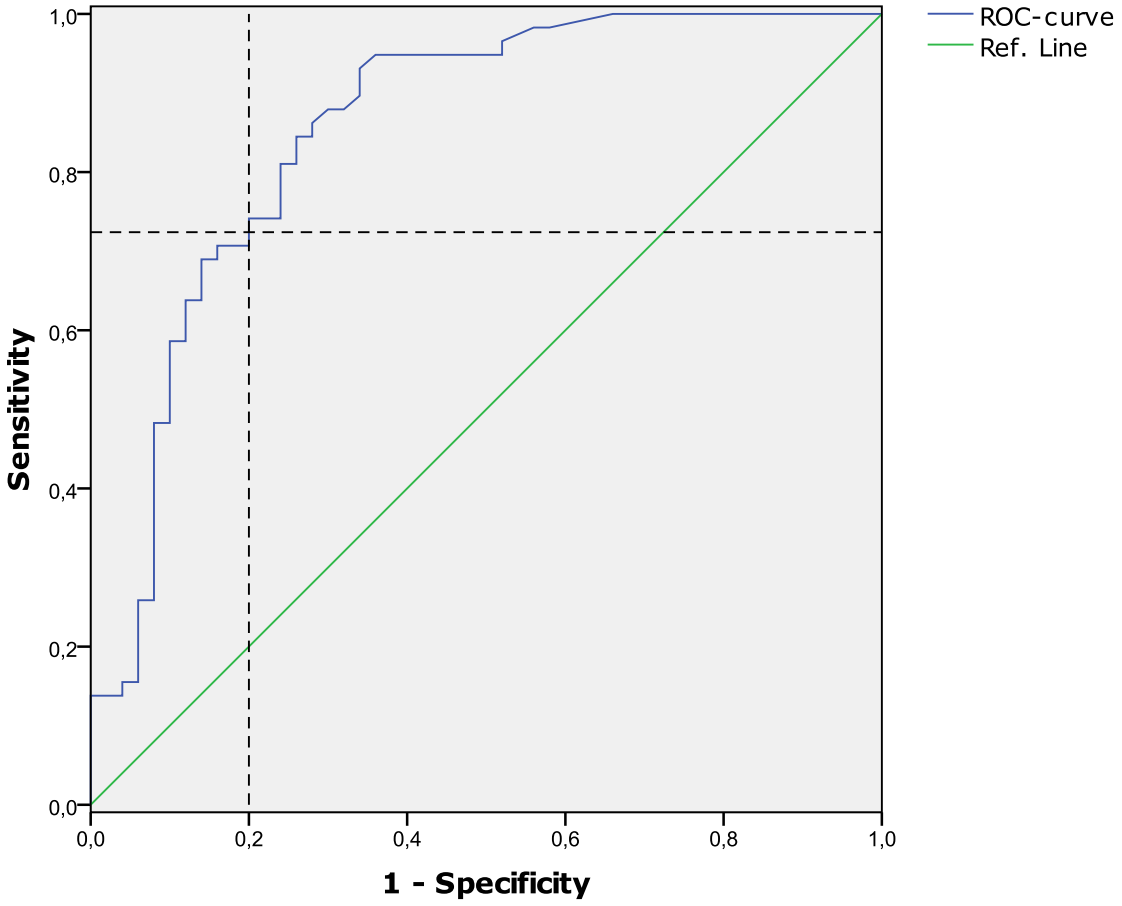

Figure 26 ROC-curve

Individual samples were scored positive (Mf positive, LE) or negative (transmigrant samples of the ≤ 1 month and 2-4 months group). The blue line represents the ROC-curve with sensitivity on the Y-axis and 1-Specificity on the X-axis. Dashed lines represent the intercept on the curve when 53 *HhaI* copies are used as threshold.

ROC-curve analysis confirmed that this threshold had good discriminatory power (AUC=0.854) with asymptotic significance (P<0.001). The AUC value represents the propability that the assay result for a randomly chosen positive case (active infection) will exceed the result of a randomly choosen negative case (larval exposure). Using this criterion, assay results of >53 *HhaI* copies/µl are classified as positive (active infection), which leads to a sensitivity of 0.741 and 1-specificity of 0.20 (Figure 26, Table 16). Thus approximately 74.1% of all samples with active infections would be correctly identified as such (Sensitivity), and 20% of all samples with larval exposure would be incorrectly identified as active infections.

Table 16 Coordinates of the ROC-curve

HhaI Threshold	Sensitivity	1 - Specificity
43,50	,81	,24
45,50	,79	,24
47,00	,78	,24
50,00	,74	,24
53,50	,74	,20
56,00	,72	,20
58,50	,71	,20
61,00	,71	,18
66,00	,71	,16
70,50	,69	,16
72,00	,69	,14
80,00	,67	,14
88,00	,66	,14
90,00	,64	,14
95,50	,64	,12
106,00	,62	,12
119,00	,60	,12
126,50	,59	,12
128,00	,59	,10
150,50	,57	,10
179,50	,55	,10
200,50	,52	,10

We therefore used 53 *HhaI* copies as threshold to differentiate patients with an active infection (persons with adult worms) from those who only had been exposed to infective larvae.

To better understand what the real-time PCR was detecting and to use the calculated threshold to discriminate between active infections and those who were exposed to larvae, a more detailed analysis was performed by quantifying the *HhaI* gene in the transmigrant samples and sorting by time in the endemic region. In the group present in the endemic area for less than one month, 85.7 % of the *HhaI* positive individuals had <53 *HhaI* copies/µl. Similar levels were seen in the 2-4 month group (68%) (Table 16, Figure 27).

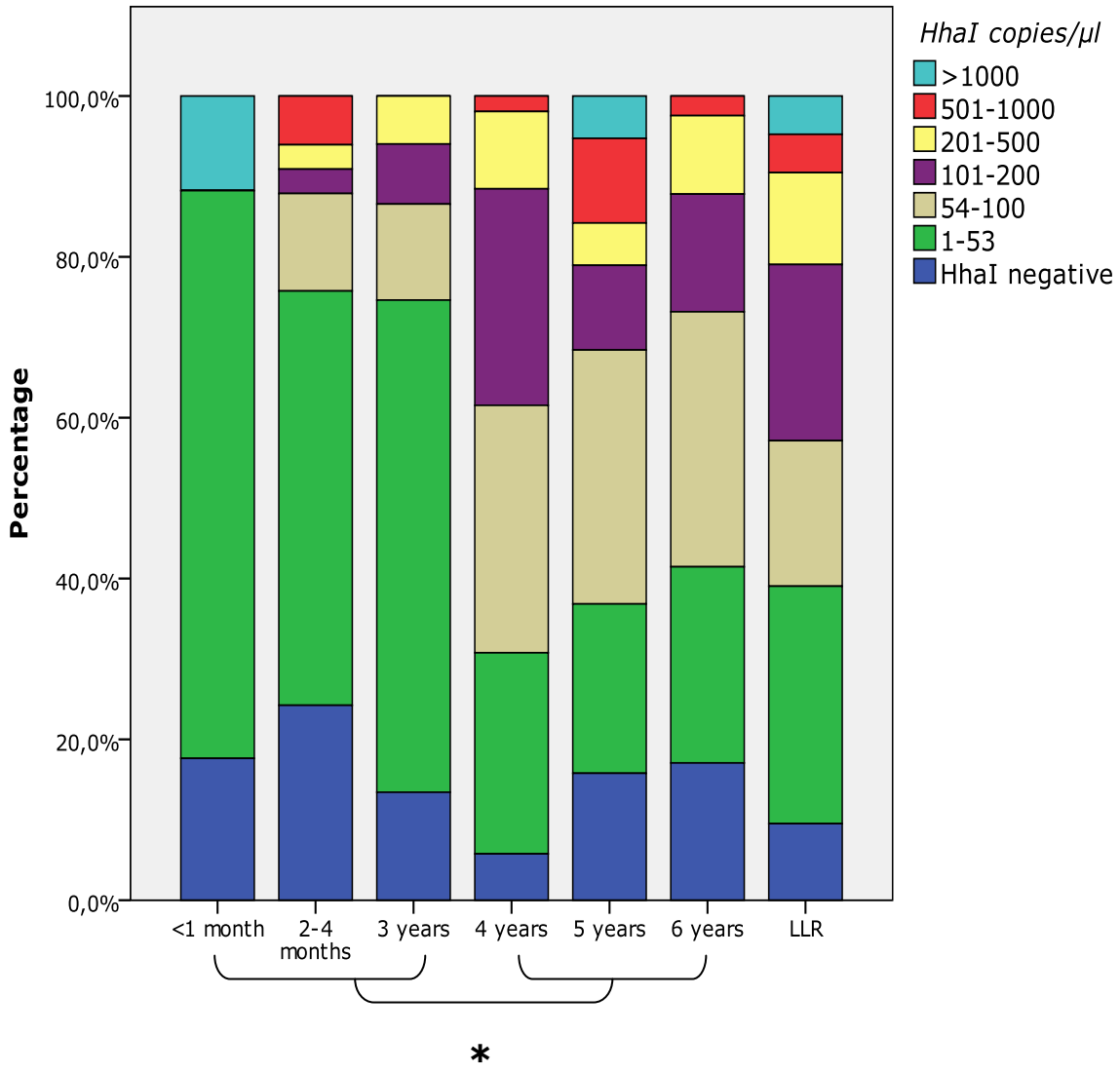

Figure 28 Stratification of *B. malayi HhaI* copies/µl in transmigrants and life-long residents.

Transmigrants resident <3 years had significantly fewer *HhaI* copies/µl than those resident >4 years, which had *HhaI* loads similar to LLR.

*Cochran-Armitage test for trend *P*<0.001

Comparing the transmigrants resident in the endemic area less than 4 years to those resident for 4-6 years, there was a significant increase in *HhaI* copy numbers in the latter group (Cochran-Armitage test for trend *P*<0.001) (Figure 28). The percentage of transmigrants resident in the endemic area for at least 3 years with >53 *HhaI* copies/µl was increased (54-100 copies/µl: 14% at 3 years to 32% at 4 years; 101-200 copies/µl: 9% at 3 years to 28% at 4 years) (Table 17).

Table 17 *B. malayi HhaI* copies/µl in transmigrants and life-long residents

Time of residence (*HhaI* copies/µl median, range)	*HhaI* copies/µl							total
	negative	≤53	54-100	101-200	201-500	501-1000	> 1000	
≤ 1 month 10 (0-1476)	3	12	0	0	0	0	2	17
IgG4 ELISA positive	0	0	0	0	0	0	0	0
IgG4 BmR1 positive	0	0	0	0	0	0	0	0
2-4 months 9 (0-988)	8	17	4	1	1	2	0	33
IgG4 ELISA positive	1	1	0	1	0	0	0	3
IgG4 BmR1 positive	0	0	0	0	0	0	0	0
3 years 18 (0-500)	9	41	8	5	4	0	0	67
IgG4 ELISA positive	0	12	2	2	2	0	0	18
IgG4 BmR1 positive	0	3	1	0	1	0	0	5
4 years 84 (0-515)	2	14	16	14	5	1	0	52
IgG4 ELISA positive	2	5	8	7	2	0	0	24
IgG4 BmR1 positive	0	2	2	1	1	0	0	6
5 years 67 (0-3057)	3	4	6	2	1	2	1	19
IgG4 ELISA positive	2	3	3	1	0	1	1	11
IgG4 BmR1 positive	1	2	2	1	1	1	0	8
6 years 60 (0-808)	7	10	13	6	4	1	0	41
IgG4 ELISA positive	4	5	9	3	2	0	0	23
IgG4 BmR1 positive	1	2	7	3	3	1	0	17
LLR 82 (0-4137)	10	31	19	23	12	5	5	105
IgG4 ELISA positive	10	27	14	18	10	4	4	87
IgG4 BmR1 positive	2	13	6	8	8	2	3	42

The number of individuals with >53 copies/µl was significantly higher in the individuals resident for 4-6 years (P<0.001 Fisher's exact test) compared to the group resident fewer than 4 years (Table 18).
The IgG4 positive rate in the first three years tested by SWA-ELISA was 18% and 4.3% by dipstick BmR1, whereas the PCR detected 83% of samples (Table 18).

Table 18. Transmigrant population divided into two groups by length of residence in the *B. malayi* endemic area – positive and negative individuals for *HhaI* compared to IgG4 results

	Time of residence in the *B. malayi* endemic area			
	0-3 years n (%) n=117		4-6 years n (%) n=112	
	positive	negative	positive	negative
HhaI	97 (82.9)	20 (17.1)	100 (89.3)	12 (10.7)
IgG4 (SWA)[a]	21 (17.9)	96 (82.1)	58 (51.8)	54 (48.2)
IgG4 (BmRI)[a]	5 (4.3)	112 (95.7)	31 (27.7)	81 (72.3)

[a] IgG4 0-3 years vs. 4-6 years *P*<0.001 (Fisher's exact test)

Of the 82% SWA-ELISA and 95.7% BmR1 IgG4 negative samples, >80% were positive for *HhaI* in the first three years. The number of IgG4 positive individuals had significantly increased (51.8% in SWA-ELISA; 27.7% in BmR1) for patients resident in the endemic area longer than four years, while the detection of *HhaI* positive samples remained high (89.3%) (Table 18). These data indicate that in Mf negative individuals, the PCR has a higher rate of detection than IgG4 serology in transmigrants.

3.7 Genetic associations in brugian filariasis analyzed in patients from Alor Island, Indonesia

3.7.1 Baseline

In a previous parasitological study in the village of Mainang, 586 persons (37% of the population) were examined for pathology and blood was collected to determine microfilaremia and for genotyping. No significant differences in the rate of microfilaremia or lymphatic pathology were seen between those persons that were asked to participate during a house-to-house visit or those that voluntarily came to the central health center when requested by local health workers [133]. Of those taking part in the survey, 157 (27%) patients were Mf positive (Table 19) and 77 (13%) had LE pathology (Table 20). In total, 30 SNPs were analyzed for association to different phenotypes in LF (Mf/LE) (Appendix II). Four SNPs, two in the IL-18 gene and two in the IL-4R gene were found to have an association with LF and were further analyzed.

In the Mf dataset, the number of males and females with Mf was 80 and 77, respectively (Table 19). The mean number of Mf per ml blood was 194 Mf/ml (median 118 Mf/ml, range 0-4700 Mf/ml) in males and 173 Mf/ml (geometric mean 160 Mf/ml, range 0-6028 Mf/ml) in females, and was not significantly different (Mann-Whitney U test, P=0.238) [133]. After excluding patients with LE and absent IgG4 titer, only Mf-positive (cases) as well as Mf-negative but IgG4 positive patients (controls) were genotyped for the IL-4R SNPs. The numbers of males and females with Mf were 28 and 13, respectively. Males had a mean microfilarial level of 872 Mf/ml (median 303, range 100-4700) and females had a mean microfilarial level of 1231 Mf/ml (median 1100, range 201-3964). The difference in Mf/ml between males and females was not significant (Mann-Whitney U test, *P*=0.146).

Table 19 The age and sex distribution of microfilaremic and control patients genotyped

Group	No.of patients	Males	Females
examined for pathology[1]	586	240	346
Microfilaremic (Mf+) patients[1]	157	80	77
Mean No. of Mf/ml (median; range)[1]		194 (118; 0-4700)	173 (160; 0-6028)
Inclusion of LE negative, IgG4 positive	83	50	33
Microfilaremic patients (Mf+)	41	28	13
Mean No. of Mf/ml (median; range)	1225 (100-4700)	872 (303; 100-4700)	1231 (1100; 201-3964)
Amicrofilaremic patients (Mf-)	42	22	20

[1]published by Supali *et al.,* 2002

In the LE dataset, the number of males and females with LE was 28 and 49 respectively (Table 20) [133]. After excluding related individuals and people negative for filarial specific IgG4 antibodies, only patients with LE pathology (cases, n=39) and patients without LE pathology, but IgG4 positive (IgG4+, controls, n=83) were genotyped for the IL-18 SNPs. In both groups, Mf positive and Mf negative patients were included.

There were 22 females and 17 males with LE stage 2-4 (Mann-Whitney *U* Test, *P*=0.944) (Table 22).

Calculation of the inbreeding coefficient F (deviation from HWE) for the IL-18 and the IL-4R SNPs did not give evidence for first-degree relations in the case or the control groups (Table 21 and 22).

Both SNP datasets passed quality control parameters in FAMHAP including a missing rate below 5 %.

Table 20 The age and sex distribution of LE and control patients genotyped

Group	No.of patients	males	females
examined for pathology[1]	586	240	346
Patients with LE pathology[1]	77	28	43
Inclusion of unrelated LE and IgG4 positive	39	17	22
LE negative IgG4 positive	83	50	33

[1]published by Supali *et al.,* 2002

3.7.2 Single Marker analysis for IL-4R

Polymorphisms in the IL-4R gene were compared between the Mf positive and the Mf negative phenotype in LF. Two SNPs in the IL-4R gene (rs 1805010 and rs1049631) were analyzed using the FAMHAP program performing a single marker analysis to calculate *P* values with an Armitage trend test (Table 21). Of the 41 Mf positive patients, two samples failed in the sequencing procedure leaving 39 samples for the association analysis. The ancestral genotype for Ile50Val SNP (rs 1805010) is A/A, coding for the aminoacid isoleucine. An A to G missense mutation results in the aminoacid valine at this position and has been associated with reduced IL-4R activity [111]. In our study population, 17 Mf positive patients (43%) carried the G/G genotype coding valine, 16 patients (41%) were heterozygous and 6 (15%) carried the ancestral A/A genotype coding isoleucine. In the control group, the homozygous G/G genotype was detected in 9 individuals (21%), 22 (52%) were heterozygous and 11 (26%) were homozygous for the ancestral A/A genotype. The genotype differences between Mf positive cases and Mf negative controls was significant P=0.0395 with frequency of allel G being 0.64 in Mf cases compared to 0.48 in the control group (Table 21). The Odds ratio for the G allele was 2.37 (CI: 1.12-5.02).

Table 21 Statistical results of Ile50Val SNP (FAMHAP and deFinetti)

	MF cases n=39		Control (Mf-) n=42		
Genotype	frequency[1] (%)	f_al G[2]	frequency[1] (%)	f_al G[2]	*P* ATT[3]
G/G	17 (43)	0.64	9 (21)	0.48	0.0395
G/A	16 (41)	+/-0.057	22 (52)	+/- 0.053	
A/A	6 (15)		11 (26)		
F (inbreeding) [4]	0.10		-0.05		
P (Pearson 1df) [5]	0.49		0.74		

1 Genotype frequency

2 Frequency of allele G (FAMHAP)

3 Armitage's trend test (FAMHAP)

4 Inbreeding coefficient, deviation from HWE (deFinetti)

5 Pearson's goodness-of-fit chi-square (degree of freedom=1) (deFinetti)

For the +3111 SNP in the 3'UTR region the genotype frequency between cases and controls was significantly different *P*=0.0145 (Table 22). In the Mf positive group, 26 patients (66%) carried the ancestral G/G genotype and 13 patients (33%) were heterozygous. In the control group, 18 individuals (42%) were homozygous G/G and 21 individuals (50%) showed the heterozygous genotype (Table 22). The frequency of the G-Allel was 0.83 in Mf cases compared to 0.68 in the Mf negative control group with Odds ratio for the G allele being 2.36 (CI: 1.17—5.02). Although both groups were in HWE, the A/A genotype was found only in 3 control samples (7%). No clear association of the genotype with the MF phenotype could be determined.

Table 22 Statistical results of 3'UTR +3111 SNP IL4R (FAMHAP and deFinetti)

	MF cases n=39		Control (Mf-) n=42		
Genotype	frequency[1] (%)	f_al G[2]	frequency[1] (%)	f_al G[2]	*P* ATT[3]
G/G	26 (66)	0.83	18 (42)	0.68	0.0145
G/A	13 (33)	+/-0.038	21 (50)	+/-0.047	
A/A	0 (0)		3 (7)		
F (inbreeding) [4]	-0.20		-0.14		
P (Pearson 1df) [5]	0.21		0.34		

1 Genotype frequency

2 Frequency of allele G (FAMHAP)

3 Armitage's trend test (FAMHAP)

4 Inbreeding coefficient, deviation from HWE (deFinetti)

5 Pearson's goodness-of-fit chi-square (degree of freedom=1) (deFinetti)

3.7.3 Single Marker analysis for IL-18

Two polymorphisms in the IL-18 gene were compared between Mf positive patients (Mf phenotype, no LE) and patients with LE pathology.

The HWE statistics showed that both SNPs were in HWE as deviation of HWE was not significant (Table 23 and 24).

For the two IL-18 promoter SNPs, a single-marker analysis using the FAMHAP program was done as described in 3.7.2. For SNP IL-18 -137, two samples in the control group failed the sequencing procedure, leaving 39 cases and 81 control samples for the association analysis. In the cases group, 92% (n=36) carried the ancestral G/G genotype, whereas only 75% (n=61) in the control group had this genotype. The heterozygous state was more frequent in the control group with 25% (n=20) carrying G/C, whereas only 8% (n=3) of the cases had this genotype. The genotype C/C was seen in neither of the groups. A significant difference of the IL-18 -137 genotypes were found between cases and controls using the ATT (*P*=0.0267) (Table 23) with Odds Ratio for the G-allele being 3.32 (CI: 1.01-12.23).

Table 23 Statistical results of the IL-18 -137 SNP

	LE cases n=39		Control (Mf+) n=81		
Genotype	frequency[1] (%)	f_al G[2]	frequency[1] (%)	f_al G[2]	*P* ATT[3]
G/G	36 (92)	0.96	61 (75)	0.88	0.0267
G/C	3 (8)	+/-0.021	20 (25)	+/-0.024	
C/C	0 (0)		0 (0)		
F (inbreeding) [4]	-0.04		-0.05		
P (Pearson 1df) [5]	0.80		0.74		

[1] Genotype frequency

[2] Frequency of allele G (FAMHAP)

[3] Armitage's trend test (FAMHAP)

[4] Inbreeding coefficient, deviation from HWE (deFinetti)

[5] Pearson's goodness-of-fit chi-square (degree of freedom=1) (deFinetti)

Additionally the IL-18 -607 SNP was analyzed for association to LE pathology. Of the 122 patients, one case and one control sample failed the sequencing procedure, leaving 38 case and 82 control samples. In the case group, 71% (n=27) carried the G/G genotype, 21% (n=8) were heterozygous G/T and 8% (n=3) were T/T. In the control group, 46% (n=38) carried the G/G or the G/T genotype while 7% (n=6) were homozygous T/T (Table 24). The ancestral allele of the IL-18 -607 SNP is the T allele. No significant association of the IL-18 promoter with LE was found, although the second SNP showed a trend to association (*P*=0.051) with Odds Ratio for the G-allele being 1.94 (0.995-3.79).

Table 24 Statistical results of the IL-18 -607 SNP

	LE cases n=38		Control (MF+) n=82		
Genotype	frequency[1] (%)	f_al G[2]	frequency[1] (%)	f_al G[2]	*P* ATT[3]
G/G	27 (71)	0.82	38 (46)	0.70	0.0515
G/T	8 (21)	+/-0.051	38 (46)	+/- 0.034	
T/T	3 (8)		6 (7)		
F (inbreeding) [4]	0.299		-0.05	-0.093	
P (Pearson 1df) [5]	0.064		0.74	0.398	

[1] Genotype frequency

[2] Frequency of allele G (FAMHAP)

[3] Armitage's trend test (FAMHAP)

[4] Inbreeding coefficient, deviation from HWE (deFinetti)

[5] Pearson's goodness-of-fit chi-square (degree of freedom=1) (deFinetti)

3.7.4 Haplotype analysis IL-4R

To assess whether the observed associations to Mf pathology were attributable to the IL50Val polymorphism alone or together with the +3111 SNP in a haplotype, a haplotype frequency estimation with the FAMHAP software was done. Single haplotype frequencies were estimated performing a likelihood ratio test (X^2, 1df). To get a corrected *P*-value an Omnibus statistic with 200,000 simulations was done (Table 25). All four possible haplotypes were present in the groups of Mf cases and controls, where the Ile50Val SNP is analyzed in the first and the +3111 SNP in the second position. Haplotype A G was less common in both groups, whereas haplotype G A was equally distributed between cases and controls. Haplotype with A A was significantly less frequent in Mf cases (*P*=0.0145), whereas haplotype G G was significantly dominant in Mf cases (*P*=0.0378). Allele G in the IL50Val SNP, coding for valine in conjunction with allele G in the 3'UTR region shows a frequent haplotype in Mf cases, whereas the controls carry the A A genotype more frequently. The corrected *P*-value analyzed with the omnibus statistic which corrects for multiple testing effects, remained highly significant (*P* = 0.026), which strengthens the results of the single haplotypes.

Table 25 Haplotype analysis IL-4R using FAMHAP

Haplotype	Frequency		
1 2[1]	Cases	controls	*P*-value X^2
A G	0.032	0.031	
A A	0.131	0.289	0.0145
G G	0.610	0.444	0.0378
G A	0.225	0.233	

Corrected *P*=0.026

[1]At position 1 is SNP Ile50Val (rs1805010) and at position 2 is SNP +3111 (rs1049631).

3.7.5 Haplotype analysis IL-18

The haplotype analysis to find an association of the three possible haplotypes in the cases and controls with LE while simultaneously correcting the data for multiple testing was done as described in 3.7.4. A significant association for haplotype G G in the cases was seen, with an uncorrected *P*=0.036 that remained significant after correction for multiple testing (*P*=0.043). The haplotype, where SNP -137 has G and -607 has G, which are both associated with higher promoter activity and higher IL-18 protein expression [101, 102] was significantly more frequent in patients with LE pathology, whereas the haplotype C T, responsible for lower IL-18 levels, had a significantly lower frequency in the cases (Table 26).

Table 26 Haplotype analysis IL-18 using FAMHAP

Haplotype	Frequency		
1 2	cases	Controls	*P* value X^2
G G	0.811	0.691	0.036
G T	0.148	0.183	
C T	0.039	0.125	0.022

Corrected *P*=0.043

At position 1 is SNP -137 (rs187238) and at position 2 is SNP -607 (rs1946518).

4. Discussion

Two of the major filarial infections, onchocerciasis and LF, affect >150 million people. More than 1 billion people are living in LF endemic areas and >1 million people live in *O. volvulus* endemic areas and therefore are at risk of infection [2, 5]. Public health programs to control these infections have existed for years and have evolved from activities driven by the WHO into global programs with public-private partnerships. Currently, these programs use yearly mass application of drugs that mainly kill Mf with the aim to prevent uptake by transmitting insect vectors and thus, to block transmission and reduce infections to such levels that in 15-30 years from their beginning, the diseases will no longer pose a public health problem.

It has been known for more than 35 years that filarial nematodes contain endosymbiotic bacteria of the genus *Wolbachia* of the order Rickettsiales [149]. These endosymbiotic bacteria are found in the hypodermis of male and female worms, ovaries, oocytes, embryos and all developing larval stages [150]. As in many animal filarial species, these endobacteria are present in human filariae including *W. bancrofti*, *Brugia* species and *O. volvulus*. The principle of anti-wolbachial chemotherapy of filariasis is based on earlier findings in animal models as well as in human onchocerciasis and LF that depletion resulting in a more than tenfold reduction of the *Wolbachia* endobacteria in adult female worms, precedes female worm sterility [41, 120] and worm death [42, 43, 45]. The discovery of the essential role of *Wolbachia* in worm fertility and survival has resulted in the development of an antifilarial chemotherapy with doxycycline, which depletes *Wolbachia* from the worms and leads to long-term worm sterility [44, 47] and macrofilaricidal activity in bovine onchocerciasis [151] and human LF and onchocerciasis [42, 49].

PCR based assays have become the preferred methods for the diagnosis of many parasitic infections in diagnostic laboratories [67]. In recent years, real-time PCR has increasingly replaced C-PCR having the potential

to be used as high-throughput diagnostic tool for the screening of DNA extracted from blood and tissue. To improve diagnostic methods beside microscopic and histological analysis in filarial infections with *O. volvulus*, *W. bancrofti* and *Brugia malayi*, several real-time PCRs were established to: 1) monitor *Wolbachia* depletion in studies with humans looking at the efficacy of different dosages and treatment times for doxycycline and alternative anti-wolbachial drugs, 2) analyse the effect of *Wolbachia* depletion by doxycycline on larval development in the insect vector and 3) establish a more sensitive method of identifying amicrofilaremic Brugian infections. To these, the *Wolbachia ftsZ* single copy gene and the *Brugia malayi HhaI* gene were quantified by real-time PCR.

The second arm of the thesis project was a genetic association study in LF patients. Studies in humans have shown that susceptibility to infection, parasite load and lymphatic pathology cluster in families but only a few studies have looked for genes associated with LF. In total 30 SNPs were analyzed and genotypes were compared between LF patients with MF or LE phenotypes. Two SNPs of the IL-18 gene and two SNPs of the IL-4R gene showed an association to LF phenotypes in a group of patients from Indonesia infected with *B. timori*.

Quantitative PCR

4.1 Onchocerciasis

4.1.1 Drug studies

Drug study with doxycycline **200** mg/day **4** and **6** weeks

To monitor the success of antibiotic treatment on *Wolbachia* depletion in *O. volvulus* infections, a real time qPCR for the *O. volvulus Wolbachia ftsZ* gene was established. The PCR was very sensitive with a detection limit of 30 ftsZ copies/µl. The use of PCR was an advanced monitoring tool beside microscopic and histological analyses.

One aim of the study was to define the minimum regimen of antibiotic treatment needed to achieve *Wolbachia* depletion and complete sterilization of adult female worms, leading to a reduction of skin Mf over a longer period to decrease the transmission potential. It was previously shown that a 6-week administration of 100 mg/day in onchocerciasis patients led to a depletion of *Wolbachia* from adult worms [41]. Two and three weeks of doxycycline treatment were not sufficient in previous studies (unpublished data). Assessment of a macrofilaricidal activity was a second objective.

To achieve the first objective, we undertook a placebo-controlled, randomized study with 200 mg/day of doxycycline, administered for 4 and 6 weeks, to onchocerciasis patients. The PCR results confirmed almost a tenfold depletion of *Wolbachia* with the expected interruption of embryogenesis that led to a cessation of production of new Mf at 20 and 27 months after the onset of treatment. Histology results also revealed a macrofilaricidal activity of more than 60% in the 6-week treatment arm at these points of time. After subtraction of those worms that had been newly acquired in the interval between doxycycline treatment and the 20-27 months follow-up analysis and thus had not been treated, the

macrofilaricidal rate would have been approximately 70% after substracting new infections. This was confirmed recently from our group, reanalysing the data of doxycycline treatment on onchocerciasis patients [146].
The sustained absence of embryogenesis, of skin Mf and the macrofilaricidal activity was greater after 6 weeks compared to 4 weeks of doxycycline administration.

Drug study with doxycycline **100** mg/day **5** weeks

To analyse the efficacy of doxycycline alone without administration of IVM an open trail with 5 weeks treatment of doxycycline 100 mg/day in onchocerciasis patients was undertaken. In addition qPCR analysis of *Wolbachia* depletion, assessment of a macrofilaricidal activity and long-term sterilizing effect were made.
In previous studies, a 6-week course of doxycycline 100 mg/day resulted in long-term (>24 months) sterilization of female worms and an absence of skin Mf but no macrofilaricidal effect could be seen [41, 44, 47].
Three new findings were observed with the 5 weeks doxycycline treatment: (1) *Wolbachia* depletion and interruption of embryogenesis was achieved after reduction of the treatment duration from 6 to 5 weeks at 100 mg/day of doxycycline. (2) *Wolbachia* depletion and sterility were observed for a longer time of 21 and 27 months than previously seen. (3) this Doxycycline regime showed a macrofilaricidal activity [46].
At 21 months after treatment, the *ftsZ*/*β-actin* ratio was decreased almost 10 fold. At 27 months after treatment, the difference to the control group was not significant anymore, even if the median ratio was as low as at month 21. Ongoing transmission leads to the acquisition of new worms after the administration of doxycycline, some of which in our study have already become fertile during the 21-27 month observation period. This was confirmed be reanalysis of the data by our group [146]. The female worms harbouring many *Wolbachia* showed the characteristics of young

worms that had been newly acquired after the end of doxycycline treatment. These newly acquired worms also produce new Mf that are detected by PCR of the skin biopsies.

Drug study with azithromycin

Azithromycin has good activity against intracellular bacteria such as *Chlamydia* spp. (which are related to *Wolbachia*) and *Mycoplasma* spp.. It was administered to onchocerciasis patients in different dosages for 6 weeks with the aim to study effects of azithromycin on the *Wolbachia* of *O.volvulus* and on Mf production. The advantage for including azithromycin into anti-wolbachial chemotherapies is that it can be given to children, thus closing one of the gaps left by doxycycline. In addition, the weekly regimen of 1,200 mg of azithromycin that is used in HIV-infected individuals as prophylaxis against infections with atypical mycobacteria [52], azithromycin would potentially provide an easier application in areas with rural health standards.

As in previous studies with doxycycline, real-time PCR was used to monitor the effects of the antibiotic treatment on the *Wolbachia* depletion. The bacterial *ftsZ* gene was quantified after the azithromycin treatment and compared to the control group.

Unfortunally, the weekly regimen showed no efficacy for *Wolbachia* depletion analyzed by qPCR, confirming the histological analysis. A recent study with treatment of onchocerciasis patients for five days with rifampicin or azithromycin or with both drugs showed that neither of this regimens caused depletion of *Wolbachia*, a reduction of Mf in the skin nor degeneration of adult worms 9 months after treatment [152]. The authors concluded that short courses with these antibiotics will not clear *Wolbachia* from *O. volvulus*. Our data suggest that longer regimens with azithromycin alone were also not effective. Histological data found that there was a significant reduction in the proportion of worms with many bacteria (“many bacteria” was defined as the presence of worms with

many bacteria in several sections of the same worms with at least one section showing more than 50 and usually more than 100 bacteria) 12 months after administration of 250 mg/day azithromycin [119], suggesting an effect of the drug on some worms. However, 53% of living female worms still displayed normal embryogenesis, and 68% of nodules from the group at 12 months had Mf in the nodular tissue. These proportions were similar to those of the untreated group, the 6-months follow up and the 1,200 mg/week regimen.

The PCR results additionally showed that a *Wolbachia* reduction could not be achieved with daily or weekly treatment. The reduction of the *Wolbachia* content at 12 months seen with histology could not be confirmed by PCR. This is may due to remaining DNA of degenerating bacteria which were still detectable with PCR but not seen in histology examination of the worms. In conclusion, a 6-week treatment with 250 mg/day or 1,200 mg/week with azithromycin alone is not suitable for treatment of human onchocerciasis. It may be worthwhile however to investigate the *Wolbachia*-reducing capacity of azithromycin in combination therapies together with other anti-*wolbachia* drugs.

Summary drug studies in Onchocerciasis

In summary, anti-wolbachial chemotherapy of human onchocerciasis with doxycycline can be orally applied for special indications to sterilize the female filariae and reduce the adult worm load. It cannot replace IVM for mass treatment in areas with ongoing transmission. Its use in community based control is compromised by the logistics of the length of treatment and contraindications in children and pregnant women.

Even a 4- and 5-week treatment with doxycycline is too long for use as MDA and the appearance of newly acquired worms after doxycycline in a focus with ongoing transmission show clearly that IVM remains the drug of choice for the areas which are currently covered by the African Programme for Onchocerciasis control [22].

However, a recent report on community-directed delivery of doxycycline for the treatment of onchocerciasis indicated that a successful delivery of doxycycline for six weeks is practicable. The therapeutic coverage and the compliance treatment rate achieved in this study coupled to the known efficiency of doxycycline showed that mass administration may be used in selected problem areas (i.e. loiasis co-endemic areas) as an additional measure to control onchocerciasis [153].

In addition, the new chemotherapeutic principle is beneficial to the treatment of individual patients, who have left transmission areas and thus can achieve a strong reduction of the adult worm load and a clearance of Mf. The new regimen may also be considered in areas where there has been evidence for a sub-optimal performance of IVM. Worm populations that might have acquired some degree of resistance may thus be eliminated with an existing drug to prevent resistance from further spreading. Doxycycline without IVM may also be considered for treatment in areas with co-endemicity of onchocerciasis and loiasis [154].

4.1.2 Vector monitoring

Retarded *O. volvulus* L1 to L3 larval development in the *Simulium damnosum* vector after anti-wolbachial treatment of the human host

In previous studies much work has been done showing the requirement of *Wolbachia* for oogenesis, embryogenesis and adult worm survival in infections with the filarial parasite *O. volvulus* [41, 44]. Other studies have analyzed the effects of tetracycline on larval development in the mammalian host. It was seen that tetracycline treatment in a mouse model with *L. sigmodontis*, leads to significant growth retardation in worms [38] when administered concomitantly with the start of infection. Oral treatment with tetracycline inhibited *Brugia pahangi* and *Brugia malayi* development from L3 to adult worms in jirds and gerbils [155-158]. Different to animal models, little is known in human infection about the effects of an antibiotic treatment of the mammalian host on the larval development in the insect vector. Therefore the focus of this study was to analyse the biology of the *Wolbachia-O. volvulus* symbiosis regarding the effect of *Wolbachia* depletion on larval development in the insect vector. It was investigated whether Mf from onchocerciasis patients that had been treated with doxycycline, and thus had reduced or absent *Wolbachia* levels, could still develop into the infective L3 in the *Simulium* vector. *Simulium* flies were allowed to bite onchocerciasis volunteers and were then captured and raised in an insectarium. The capture site was selected based on the results of dissection of wild *Simulium* flies to determine the natural parous, infection and infective rates. The river site picked to allow volunteers to be bitten by blackflies had very few infected flies (infection rate of 0.001%). Therefore, all flies captured on the volunteers after the bloodmeal should only contain Mf from the volunteers.
After 7 days, the *Simuliae* were dissected and the L3, L1 and L2 larvae counted.

Our results showed that Mf treated with doxycycline, and therefore depleted of their *Wolbachia*, developed into L3 at a considerable lower rate than Mf from placebo treated patients during the observation period. This was most apparent as a decrease of L3 with a corresponding increase of L1 and L2 was seen in the doxycycline group at 3, 4 and 5 months post-treatment. Whether the few remaining L3 with low numbers of *Wolbachia* might be able to establish an infection in humans remains an open question that is technically and ethically impossible to answer for *O. volvulus* infections.

Although not significant, within the placebo group the number of L3 larvae recovered from the *Simulium* decreased over time post-treatment. One explanation for this could be the drop in temperature (from 28°C to 24°C) in the area at these time points. It is likely that due to seasonal differences, the capacity of the *Simuliae* to support the infective L3 is reduced at these time points. However, this would need to be confirmed in further experiments. Nevertheless, there is a significant difference in the L3 reduction observed between the doxycycline and the placebo treated volunteers. Inhibition of larval development after antibiotic treatment has been observed in several other studies. In a murine model with *L. sigmodontis*, significantly fewer L3 developed in the intermediate host if Mf were depleted of their *Wolbachia* endobacteria with tetracycline treatment [159]. Furthermore, infection of jirds with *Wolbachia*-depleted L3 resulted in the development of very few female worms, while male worm numbers were unchanged. The few female worms that were able to develop from *Wolbachia*-depleted Mf had endobacteria levels equivalent to control worms. It was postulated that these worms developed from Mf with a *Wolbachia* threshold level at or above the minimum that allows for successful development of the larvae, and that different thresholds appear to exist for male and female worms.

For the current study, equal conclusions for the L3 that had successfully developed can be drawn. The L3 apparently resulted from L2 containing the minimum threshold of *Wolbachia* endosymbionts needed for

development into L3. To prove the dependence of larval development on *Wolbachia,* the study included quantitative analysis of the endobacterial content in the different larval stages. The depletion of the endobacteria was verified by a quantitative PCR assay for the *Wolbachia ftsZ* gene, which allowed for quantification of fewer than 30 DNA copies (30 *Wolbachia*) in one reaction. The PCR results showed a clear reduction of *Wolbachia ftsZ* in the Mf after one month of antibiotic treatment (52% compared to placebo), with Mf from fewer than 50% of volunteers having a detectable *ftsZ* signal at 2, 3, 4 and 5 months post-treatment. This was concomitant with a clear reduction of the *ftsZ/β-actin* ratio in larval samples from the blackfly vector in the doxycycline group, with many samples being *ftsZ* negative. As there were still remaining L3 with a positive *ftsZ* signal in the PCR, we assume that the depletion of *Wolbachia* was suboptimal and therefore resulted in a slower depletion of the endobacteria. It is also possible that *Wolbachia* DNA from damaged/degraded larvae is detected with the PCR. It was previously shown in a *in-vitro* study with *Wolbachia* infected insect cells, that *Wolbachia* tend to further decrease even after antibiotic withdrawal [160] These results were consistent with *in vivo* studies with the *O.volvulus* related parasite *O. ochengi* in cattle [123]. This could explain why there is no total decline of *Wolbachia* as measured with the *ftsZ* gene directly after the treatment (1 month post-treatment). Due to the small sample size a statistical test for significant differences between the two groups was not possible. However, these results show that *Wolbachia* are not only essential for *O. volvulus* oogenesis and embryogenesis, but they are also needed for proper development of the larvae in the insect vector.

A couple of studies considered that the antibiotic treatment might directly affect the larval development independently of the loss of endobacteria [156, 158]. In the current study the larvae were not exposed to doxycycline while in the insect vector since the treatment occurred before the blackflies had fed on the infected volunteers. Because doxycycline rapidly blocks embryogenesis by *Wolbachia* depletion [114] , the Mf taken

up by the flies during the blood meal should only be those present at the time of doxycycline treatment. From this it can be concluded that *Wolbachia* depletion alone causes the impairment of larval development and is not a direct effect of the antibiotic on the larvae. Because L2 larvae were able to develope in the flies, we also conclude that many of the Mf from the doxycycline patients were still alive, indicating that Mf do not rely on their endosymbionts, but still need a threshold number of *Wolbachia* in order to further develop in the insect host.

The possible role of *Wolbachia* during filarial development and the underlying molecular mechanisms are still unknown. The bacteria might function as a source of heme for enzymes participating in the biosynthetic pathways of hormones or other metabolites needed for moulting [40, 161]. It is known that in *Dirofilaria immitis* and *B. pahangi*, moulting from L3 to L4 *in vitro* is dependent on heme requiring hormones like ecdysone [162, 163]. The annotated genome of *B. malayi* lacks the genes for heme synthesis but could be found in the *Wolbachia* genome [36, 40]. Therefore *Wolbachia* could serve as a source of heme and benefit the parasite larval development.

This is the first study analysing the effects of antibiotic treatment in human onchocerciasis on larval development in the insect vector. The results clearly show that depletion of *Wolbachia* not only affects the larval stages and adult worms in the mammalian host, it has also a negative effect on larval development in blackflies. These findings have epidemiological implications. The decrease in L3 production, even though it is not 100%, could reduce the transmission potential of black flies following treatment of communities with doxycycline to control onchocerciasis. Thus anti-wolbachial therapy for filarial infections would have an additive effect of interrupting transmission by 1) blocking embryogenesis 2) macrofilaricidal activity and 3) reduction of the number of L3 that can be transmitted by black flies that have fed on infected persons before the Mf have been cleared from the skin.

As mentioned earlier in the discussion (4.1.1) a report on community-directed delivery of doxycycline for the treatment of onchocerciasis is achievable and showed that mass administration may be used in selected problem areas such as in areas coendemic for loiasis to control onchocerciasis [153].

4.2 Lymphatic filariasis

4.2.1 Drug studies

Drug study with rifampicin and/or doxycycline in *W. bancrofti* infection

Lymphatic filariasis (LF) is a common cause of morbidity in humans, and of the presently used anti-filarial drugs to combat it, only DEC (which is not used in African countries co-endemic for onchocerciasis) has moderate macrofilaricidal activity, whose mode of action is not fully understood [8, 164]. Previous trials with doxycycline targeting the *Wolbachia* endobacetria indicated that either a 4- or 6-week course of 200 mg/day leads to gradual and sustained loss of Mf from host blood and has strong macrofilaricidal activity in infections with *W. bancrofti* [42, 43, 51]. However, the previously published doxycycline regimens are long, and therefore a shorter regimen is desirable to complement classical anti-filarial drugs [154]. Rifampicin is a safe drug that can be given to children and has already shown good activity against *Wolbachia* in experimental trials [53, 54]. In the current study, a combination of doxycycline and rifampicin was analyzed in a 2-week treatment and compared to 4-week treatment with doxycycline alone in *W. bancrofti* infected individuals.

The depletion of endobacteria was analyzed using a real-time PCR assay for the *ftsZ* gene of the *Wolbachia* in *W. bancrofti*. In the group treated for 2 weeks with doxycycline+rifampicin, a 86% *Wolbachia* depletion and a moderate macrofilaricidal effect (50%) indicated by the loss of worm nests (FDS) could be observed. In contrast, in the placebo patients the worm nests remained stable throughout the follow up examinations.

A very low microfilaremia at 12 and 18 months after doxycycline+rifampicin treatment confirmed the effect of the combined antibiotics. The present trial confirmed a previous report that a 4-weeks doxycycline treatment results in more than 80% macrofilaricidal effect

[165]. The second and novel result of this study was that the combination of rifampicin with doxycycline had an anti-wolbachial effect on *W. bancrofti* in humans and this effect occured with a shorter (2 weeks) course of treatment. It is of interest to note that even though 3 weeks doxycycline alone in a previous trial had 86% *Wolbachia* depletion [50], it did not show a macrofilaricidal effect. In the group treated for 2 weeks with the combination of rifampicin+doxycycline with a similar degree of *Wolbachia* depletion (86%) a moderate macrofilaricidal effect was shown. The reason for this difference may be that, in addition to its anti-wolbachial effect, rifampicin may have some anti-filarial activity as known in animal models [54, 166, 167]. Hence, a longer regime such as 3 weeks may show a stronger macrofilaricidal effect. The advantage of a macrofilaricidal drug is that it targets the adult worms that are the inducers of lymphatic pathology and thus can bring direct relief to individual patients that seek treatment, e.g. in outpatient clinics, without having to rely on the indirect means of reduction of infection pressure due to transmission control by microfilaricidal drugs. However, the previously published doxycycline regimens are long, and therefore a shorter regimen is desirable to complement classical anti-filarial drugs [154].

This calls for a larger study to test other regimes of longer duration with rifampicin in LF. A current field study of our group with different regimens of doxycycline and rifampicin will determine whether a longer duration will have a better macrofilaricidal effect or not. Such a regimen with stronger macrofilaricidal effect, especially with rifampicin alone will be beneficial to infected children in endemic areas who cannot take doxycycline but can take rifampicin.

It is likely that 2 weeks doxycycline+rifampicin could have stronger macrofilaricidal activity than the 50% observed in this study when patients are observed for a longer period. However, due to high transmission rate in this area, longer follow-ups are not possible because of re-infection. Notwithstanding, the study showed that 2 weeks doxycycline+rifampicin had a macrofilaricidal effect and is encouraging in

the sense that it serves as further proof that anti-wolbachial therapy as a treatment against LF may be possible with a shorter regimen if anti-wolbachial drugs are combined. The results of the combination of 3 weeks doxycycline and rifampicin in an ongoing trial will provide more information.

4.2.2 *Brugia malayi* Diagnostic PCR

Detection of *HhaI* gene in infection with *B. malayi* by real-time PCR

To date, LF has been mainly diagnosed by direct microscopic demonstration of Mf in peripheral night blood of infected people. However, this method does not detect those individuals who have adult worm infection but are amicrofilaremic or have a pre-patent infection and are therefore cryptic infections. For *W. bancrofti* infections, assessment by the determination of antigenaemia using ICT® or TropBio ELISA test kit is available to detect amicrofilaremic infections, which in addition offers the convenience of "any-time-of-day testing" [168]. It could be shown in bancroftian filariasis, that approximately twice as many individuals are CFA positive compared with being microfilaremic, so that a proportion equal to the Mf positives harbours cryptic infections. The use of USG is an additional tool to diagnose infections in LF because of *W. bancrofti* and is able to detect adult filarial worms by their typical movement pattern (FDS) [6]. The worm nests are stable over time in the scrotal area of men [7, 49]. In brugian filariasis, which accounts for 10% of the 120 million infections of LF worldwide, no antigen (CFA) test is available and early attempts to detect adult filariae with USG examinations failed [169]. In a study by our group validating USG for detection of brugian filariasis it was possible to detect adult *B. malayi* in rodents [170] and worm nests in single human cases, but the worm nests were not stable, i.e. they were not visible for longer than 10-12 min [9]. It is assumed that the adult *B. malayi* change their location within the lymphatic vessels of the human

body. Thus, the use of USG is limited for the monitoring of brugian infections.

Several recombinant *B. malayi* antigens have been used in ELISAs or rapid-format assays to detect filarial-specific IgG4 antibodies [168]. A disadvantage of antibody detection is that it is an indirect diagnostic approach which relies on the host's immune response. Since the antibodies persist even after the worms have died and have been cleared from the host, a positive test results do not necessarily indicate current infection. Nevertheless, anti-filarial IgG4 antibodies have been correlated with the presence of Mf in the peripheral blood as the antibody levels decline rapidly after microfilaricidal treatment [63]. Several studies tested the sensitivity of SWA-ELISA and BmR1-ELISA vs. BmR1 dipstick assay [124, 171]. The ELISA format using SWA showed higher sensitivity than the BmR1 dipstick assay, whereas the BmR1 ELISA was less sensitive compared to BmR1 dipstick test. It was suggested that due to the mixture of filarial antigens used in SWA, which may recognise antibodies produced by exposed but not infected individuals, and/or by individuals who have cleared the infection, this ELISA detects more IgG4 positive individuals. The combined results of two multicenter evaluations of the BmR1 dipstick test using *B. malayi* sera from three endemic countries and using sera from individuals with non-lymphatic filarial infections revealed a sensitivity of 97% and a specificity of 99% [172]. The results are based on Mf positive samples, whereas the number of infected but Mf negative individuals cannot be evaluated with the current diagnostic procedures. In our study using quantitative PCR, we observed similar results for the two IgG4 test, as the SWA-ELISA matched to PCR in 50.1%, whereas the BmR1 dipstick results only matched in 33.5%. Notably, the SWA-ELISA was unable to get a positive result for all Mf positive individuals (2 of 19 individuals were negative for SWA-ELISA), whereas all 19 Mf positive samples gave a postivie BmR1 dipstick result. This is in agreement with the suggestion that the BmR1 format is more senstive for active fiarial

infections, whereas the SWA antigen mixture also detects IgG4 antibodies of cleared infections or exposed but not infected individuals.

Filariasis experts have requested the development of tests that can detect infection in individuals with ultra low Mf densities or cryptic infections [173]. To meet this request the *HhaI* QuantiTect® PCR, previously developed by Rao *et al*. [128], was optimized and validated with a *B. malayi* animal model and a transmigrant population in Indonesia. In the current experiments, the QuantiTect® PCR assay was sensitive at detecting *Brugia* DNA in pre-patent amicrofilaremic animals, Mf positive patients from Central Sulawesi and amicrofilaremic patients from South Sulawesi.

The 320-bp *HhaI* tandem repeat sequence of *B. malayi* has been used as a target for *B. malayi* PCR assays for many years [174]. Rao *et al*. found that detection of the *Brugia HhaI* repeat was as sensitive as microscopic detection of Mf. Additionally the real-time PCR assay showed higher sensitivity compared to conventional PCR and TaqMan PCR assays and detected DNA of nocturnally periodic *B. malayi* in day blood samples. This represented a significant improvement over previously available diagnostic methods in brugian filariasis. The *HhaI* repeat is specific for brugian filariasis and was not detected in *W. bancrofti* infections*,* which is also endemic in South East Asia.

To get a more detailed view about the sensitivity of the *HhaI* assay, particularly in amicrofilaremic infections, a large number of individuals that had moved from a non endemic area to a *B. malayi* endemic area were analyzed. Particularly in the group of individuals who were in the endemic area less than three years, the results showed a very high *HhaI* positive rate (up to 87%), whereas in the first months, none of these individuals were IgG4 positive. After four years, *HhaI* copy numbers increased concomitant with more individuals tested IgG4 positive and reached similar levels of LLR.

The uniform detection rate for *HhaI* (>80%) in many transmigrants in their first three years of residence might be explained by high exposure to

infective larvae but not yet adult worms, as indicated by the low *HhaI* copy numbers detected (72% had <53 *HhaI* copies/µl). This could explain the discrepancy of PCR and IgG4 results in the first years of living in an endemic area. The parasite burden is probably not high enough to produce detectable IgG4 antibodies, whereas PCR is able to detect parasite DNA including individuals that are exposed to infective larvae. Notably there were individuals with high *HhaI* copies/µl who were IgG4 negative. Based on serology they would have been diagnosed as *Brugia* negative even though it is likely they had active infections. Conversely, however, there were also 19 IgG4 positive individuals that were diagnosed as PCR-negative, indicating previous but not current exposure to filarial worms or larvae.

The PCR results showed that *HhaI* copies/µl increased with length of time in the area and probably indicate active infections. For monitoring *Brugia* infections using day samples a threshold associated with active infection was set to >53 *HhaI* copies/µl using 10 µl of plasma DNA with the optimized QuantiTect® Virus NR Kit protocol, based on ROC-curve analysis. This analysis predicts 80% sensitivity for detecting an active infection in amicrofilaremic individuals with a corresponding false positive rate of 20% ("1-Specifity").

Two individuals (children) resident in the area <1 month had >1000 *HhaI* copies/µl. Repeated PCR runs and sequencing verified the PCR product and *HhaI* copy numbers. These two outliers may have been highly exposed to infective larvae or they may have been infected by larvae of *B. timori* or *B. malayi* prior to settlement in the area.

Introduction of the CFA test for *W. bancrofti* infections showed that 50% of the population in an endemic area had a latent infection. In our study with quantitative real-time PCR we observed a similar level of latent infections with *B. malayi* not detected by IgG4 serology or microscopic analysis. Due to possible development of severe LF pathology such as LE, the ability to determine the status of infection, especially in amicrofilaremic patients, as early as possible could lead to prevention of

disease progression in individuals of endemic areas. In the absence of an antigen test for brugian infections the optimized *B. malayi HhaI* PCR can be used as a surrogate to identify infected, Mf negative individuals. Real-time PCR can be used to screen human samples to identify and map areas of endemicity and should also be useful for assessing the success of MDA in the framework of the Global Program to Eliminate Lymphatic Filariasis.

4.3 Genetic associations in brugian filariasis analyzed in patients from Alor Island, Indonesia

Host genetics can be the basis for understanding an individual's disease status. One of the first reports of a specific SNP associated with the variations seen in filarial pathologies showed an association of the Arg110Gln variant in the IL-13 gene and severe skin pathology in onchocerciasis [83]. Previous studies have shown the association between polymorphisms in various host genes [e.g., human leucocyte antigen (HLA), Chitotriosidase (CHIT1), mannose-binding lectin (MBL), vascular endothelial growth factor A (VEGF-A) and Toll-like receptor 2 (TLR2)] and susceptibility as well as clinical types of LF [84-90]. Additionally, a variant in the transforming growth factor-beta 1 (TGF-β-1) gene in LF patients was found to be associated with the lack of Mf and differential Mf loads in blood [91].

The objective of the present study was to analyse 30 SNPs for association to LF pheotypes. After analysing the genotyping results with FamHap, two SNPs in the IL-18 gene and two SNPs in the IL-4R gene showed association in lymphatic filariasis patients.

4.3.1 IL-18

Both IL-18 SNPs are known to be associated with up-regulation of IL-18 levels in several inflammatory diseases. These include development of atopic dermatitis-like inflammatory skin lesions [175] and collagen-induced inflammatory arthritis [176] in mice; Crohn's disease [177, 178], atherosclerosis [179], rheumatoid arthritis [97, 180], Systemic Lupus Erythematosus [102, 181, 182] and sarcoidosis [103] in humans.

IL-18, earlier known as IFN-γ and TNF-α inducing factor, is a pro-inflammatory cytokine which plays an important role in the development of both T_h1 and T_h2 cells. The major source is macrophages, although many other cell types are also capable of producing IL-18. It is produced as an inactive precursor molecule, which is processed by caspase-1 whereby biologically active molecules are released [183]. Its production and activity is regulated by caspase-1, IL-12 and nitric oxide. Although production of IL-18 is affected by many factors, individual differences in levels of this cytokine are also caused by genetic polymorphisms [101].

The influence of the -137 and -607 SNPs on IL-18 mRNA levels were first described in autoimmune diseases [101]. Higher IL-18 mRNA levels in multiple sclerosis (MS) patients homozygous for G at position -607 and G at position -137 were observed. The authors also found a correlation to IL-18 and IFN-γ expression in patients which carried the G variant. In the promoter region of the IL-18 gene, there is a cAMP response element (CRE) sequence near position -607. When activated by cAMP, the cAMP response element binding protein (CREB) binds to this region of the promoter to activate transcription [184]. A change from nucleotide G to nucleotide T disrupts the CRE sequence. Therefore, the G/G genotype results in higher transcription activity than the other genotype, with a corresponding expression of higher levels of the IL-18 protein.

In patients with sarcoidosis, a multisystem disorder characterized by granulomas in lungs or lymph nodes, the G allele is significantly more frequent compared to controls [103]. SLE is an autoimmune disease

characterized by production of high titers of autoantibodies and clinical manifestations that include arthritis, vasculitis and nephritis. In SLE patients, who have elevated IL-18 levels, the frequency of genotype G/G at position -607 was significantly higher, whereas the heterozygous genotype G/T was significantly decreased [102].

The same picture was seen the current study in patients with lymphedema (LE), where the homozygous G allele at position -607 was more frequent (71% cases, 46% controls), whereas the heterozygous G/T was less frequent (21%) compared to controls (46%).

The SNP at position -137 is in an H4TF-1 nuclear factor-binding site [101]. It was postulated that the C allele at position -137 would alter binding of the H4TF-1 nuclear factor and would therefore result in lower IL-18 mRNA expression. In the study patient group we did not detect any LE cases homozygous for C, and even the heterozygous genotype was decreased in the cases group (8%), although HWE was not disturbed. Thus, cases were more frequently homozygous for the G allele (92%), which is associated with higher IL-18 levels. The lack of homozygous C at -137 is not novel. In the context of a cluster report in 2004, Cox and Patil (Perlegen Science) analyzed 42 samples of Chinese descent for the -137 SNP and found the homozygous G/G genotype in 81% of the group while 19% were heterozygous C/G. The C/C genotype was not detected in this analysis (Cluster report ss24320556; http://www.ncbi.nlm.nih.gov/).

The pathology of LE was attributed to the death and destruction of adult worms within the lymphatic vessels. Clinical studies have also implicated a role for secondary opportunistic infections along with other cofactors in progression of LF pathologies. Chronic inflammation was characterized by recurrent attacks of acute filarial lymphangitis [75]. Asymptomatic microfilaremic individuals have low (suppressed) immune responses to parasite antigens, whereas those with pathology have inflammatory reactions to the parasite that respond strongly to such antigens [185]. With respect to lymphocyte proliferation to filarial antigens, several studies documented that individuals with chronic pathology have elevated

levels of IgE, IgG1-3 and IFN-γ [186, 187], which can all be induced by IL-18. Asymptomatic microfilaremic individuals produced relatively low levels of IgG 1-3, IgE, IFN-γ and GM-CSF. Instead they produced high levels of IgG4, IL-10 and TGF-β, which are associated with immuno-suppression. The response to parasite antigens is: 1) the production of pro-inflammatory mediators of the immune system (T_h1 cytokines) and 2) the elimination of the worm by a T_h2 mediated response. IL-18 can promote both T_h1 and T_h2 immune responses [92, 188]. In the current study an association of genotypes responsible for up-regulation of IL-18 in individuals with chronic LE pathology was found. Thus, patients with this genotype may be more at risk of developing LE due to their genetic predisposition to produce high levels of IL-18 as part of their immune response.

4.3.2 IL-4R

IL-4 and IL-13 are two cytokines which play key roles in the development of T_h2 cells, a hallmark of many worm infections. Both cytokines use the same receptor alpha chain and seem to share many physiological functions. Chronic human helminth infection in onchocerciasis, especially the rare sowda form, is characterised by a strong T_h2 phenotype with very high IgE levels compared to the immunotolerant form (generalized onchocerciasis) [189, 190].

The Arg110Gln variant of IL-13, proposed to have higher bioreactivity resulting in higher levels of serum IgE provided information on the different degrees of skin pathology seen in patients infected with *O. volvulus* [83]. Analysing the IL-13 SNP in onchocerciasis patients, it was seen that a variant in this SNP is a significant risk factor for the development of sowda and higher IgE levels were found in this group of patients.

The Ile50Val SNP in the IL-4R gene is associated with different receptor activity and IgE levels. To test whether the Ile50Val variant promotes

dysregulation of IgE synthesis, Mitsuyasu *et al*. conducted a genetic association study for serum IgE levels in a Japanese population [111]. A significant difference in the genotype frequencies was seen between control and atopic subjects; Ile 50 was associated with atopic asthma and raised total serum IgE levels. Further experiments with mouse and human B-cell lines showed almost 3 times greater cell growth and expression under the control of the IgE promoter with the Ile 50 variant compared to Val 50 transfected cells (15.4-vs 5.4-fold increase, respectively).

The C-terminal ends of the IL-4R and IL-13R subunits interact with tyrosine kinases of the JAK-family, leading to interaction with STAT6, which binds to consensus sequences in the promotors of IL-4 and IL-13 regulated genes [107, 108]. Thus, it was also confirmed that the Ile 50 variant augmented STAT6 activation 1.8-fold compared with the Val 50 variant in mouse and human B-cells [111]. These data suggested that the Ile 50 variant significantly up-regulates the receptor response to IL-4, resulting in increased activation of STAT6, and hence increased cell proliferation and increased IgE production.

In murine filariasis with *L. sigmodontis* infection it was seen that IL-4 knockout mice have an up to 100-fold-higher and a significantly prolonged microfilaremia compared to wild-type BALB/c mice as well as 20 times more Mf in the thoracic cavity, the site of infection [106]. It confirmed that IL-4 is essential for Mf containment and a limiting factor on Mf survival, whereas the adult female fertility is enhanced and larval development is unaffected. Another study with IL-4 and IL-4/IL-10 knockout mice confirmed that in the IL-4 knockout mice the worm recovery is increased after 42 and 65 days p.i. [105]. With mice lacking the IL-4R-alpha-chain (unable to respond to IL-4 or IL-13) it could be shown that they had the same Mf loads as IL-4-deficient mice and thus excluded that IL-13 could compensate for the lack of IL-4 with regard to Mf containment [191]. Adult worms were affected in the opposite direction, if the IL-4R is knocked out. Volkmann *et al*. and other experiments from Maizels *et al*. observed a lower recovery of adult worms

in IL-4R knockout mice compared with IL-4 knockout and wild-type [192]. It is assumed that the extremely strong induction of fibrosis (probably mediated by IL-13 and the IL-13R2) that these mice display also extends to encapsulating worm nests and thus leads to lower recovery of adult worms. Because of the importance of IL-4 and IL-4R as modulators of the immune response and their importance in limiting Mf loads in murine filariasis, two SNPs in the IL-4R loci were studied.

More than 40% of the Mf patients (cases) were homozygous for the G allele coding for the amino acid valine at position 50 (rs 1805010). The ancestral allele A coding for the amino acid isoleucine is homozygous in only 15% of the cases, whereas in the control group, the A allele is more frequent (homozygous 26% and heterozygous 52%). As the isoleucine variant is associated with elevated receptor activity and therefore increased STAT6 and IgE levels, it seems likely that the valine variant stays for contrary effects. This would result in low IL-4 and IL-4R activity in Mf patients which fits to previous results mentioned above, where the important role of IL-4 and the IL-4R on Mf containment was described.

The results are strengthened by a haplotype analysis with another SNP in the IL-4R. This SNP is positioned in the 3'UTR region (+3111 mRNA position) and showed no positive association in Mf patients in the single-marker analysis. Together with the Ile50Val SNP, the haplotype G G was more frequent and significantly associated with Mf patients compared to controls.

Patients who have elevated levels of Mf and no LE development show decreased IgE levels [74]. This could be due to a diminished IL-4R activity, as the majority of these patients in the current study did not have the genotype for elevated IL-4R activity and increased STAT6 and IgE levels. They carried the valine genotype which was thought to have a significantly lower IL-4R activity and therefore lower STAT6 activity and the resultant lower IgE production. The reduced signalling by the IL-4R and the decreased IgE levels would support the conclusion that more Mf are able to circulate in the host.

Summary Genetics in LF

Until now, very little is known about specific SNPs and their influence in brugian LF. The immunological perspective of filarial disease would suggest that it is the character of the immune response that determines the host's clinical outcome of infection. It is likely that the genetic background of an infected individual influences the immune response and therefore the disease phenotype. The results of our association analysis of IL-18 and IL-4R have provided further evidence for an association of specific SNPs in immune regulating genes with LE and microfilaremia in brugian filariasis. Knowledge of an individual's genetic propensity to be microfilaremic could be used for individual therapy. Patients could be screened using an IL-4R epitope antibody to identify patients with the 50 Val SNP who could then theoretically be treated to increase IgE levels. This could lead to a greater portion of the affected population being amicrofilaremic, which would then help to halt further spread of the disease.

The results of association of IL-18 and LE pathology provides more insight to the genetic basis of associated immune regulators previously found to be correlated with the outcome of pathology in infected patients. The longstanding assumption of a genetic predisposition and the outcome of the disease were confirmed based on associated SNPs.

Summary

In the first part of this thesis, the aim was to establish real-time qPCR assays to complement histological analysis by quantifying the depletion of *Wolbachia* endobacteria after several different treatment regimens with doxycycline alone, in combination with rifampicin, or with azithromycin in onchocerciasis and LF patients. This was achieved by using the bacterial *ftsZ* gene normalized to worm *β-actin* and was found to be a reliable method to monitor anti-wolbachial treatment in filarial infections. *Wolbachia* depletion after treatment with 200 mg/day doxycycline for 6 weeks was most effective and showed macrofilaricidal activity in onchocerciasis. A 4-week treatment with 200 mg/day or a 5-week treatment with 100 mg/day can be used if interruption of embryogenesis is the desired outcome in onchocerciasis infections. Although it must be noted that a 49% macrofilaricidal activity after the 5-weeks dosage was also seen. Azithromycin currently tested on its own did not show anti-wolbachial activity in onchocerciasis. More trials to investigate the use of combinations are under way.

Treatment should always be complemented with IVM to eliminate Mf from the skin and bring relief from any dermitis suffered by those with infection. However, the duration for doxycycline treatment is still longer than that desired for new antifilarial therapies suitable for mass application. It is noteworthy that a recent study in Cameroon demonstrated that even a 6-week treatment with doxycycline can be transferred to the responsibility of a village through community-directed treatment methods. These options should be followed with doxycycline treatment in filariasis endemic countries which require treatment options beyond available MDA, e.g. in areas with non-responsiveness of the parasite to MDA drugs and in areas with co-endemicity with loiasis.

Treatment of bancroftian filariasis with doxycycline in combination with rifampicin is promising and was sufficient to mediate long-time reductions in Mf and also showed a moderate macrofilaricidal activity.

Doxycycline was also useful when looking at the insect vector of onchocerciasis. This was analyzed as part of this thesis with *Wolbachia* depleted Mf that could not develop into infective L3 in *Simulium* flies in the same proportions as in the placebo group after doxycycline treatment. The results demonstrate that doxycycline treatment can also decrease transmission rates by blocking/delaying larval development in the insect vector.

Further results of this thesis complement establishment of PCR as a promising tool for detection of *Brugia* DNA in human plasma samples. This was the first study analyzed by real-time PCR for the *HhaI* gene including a large population in an endemic area with predominantly amicrofilaremic individuals. Definition of a threshold for *HhaI* measurement associated with active infection and the discrimination to larval exposure was determined based on PCR results. The results showed a high positive rate of samples with PCR which was highly sensitive even in Mf and IgG4 negative samples.

Finally a genetic association in brugian filariasis was shown by demonstrating an association of IL-18 and IL-4R SNPs with LE and microfilaremia, respectively. These analyses were just the beginning of a larger project analysing more than 90 SNPs in 1800 African patients infected with *W. bancrofti*, which just recently started. The knowledge of genomic associations in the different phenotypes and pathologies of LF could help to identifiy individuals at risk and lead to preventional interventions to avoid outcome of severe pathology.

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APPENDIX I

Primer Sequences

Gene	Sequence 5'-3'
O. volvulus β-actin forward	GTGCTACGTTGCTTTGGACT
O. volvulus β-actin reverse	GTAATCACTTGGCCATCAGG
O. volvulus ftsZ forward	AGGAATGGGTGGTGGTACTG
O. volvulus ftsZ reverse	CTTTAACCGCAGCTCTTGCT
O. volvulus ftsZ hybridization probe	CCTTGCCGCTTTCGCAATCAC 5'-Modification: 6-FAM 3'-Modification: Tamra
murine IFN-γ forward	TCAAGTGGCATAGATGTGGAAGAA
murine IFN-γ reverse	TGGCTCTGCAGGATTTTCATG
W. bancrofti ftsZ forward	GCTTGGGCTTGAAGAATTACAAA
W. bancrofti ftsZ reverse	TCATTGGCAACCCTAAACAAGTT
W. bancrofti ftsZ hybridiazation probe	ACGTAGACACGCTTATTGTCATTCCAAACA 5'-Modification: 6-FAM 3'-Modification: Tamra
W. bancrofti β-actin forward	CACTGGTGATGGTGTTACGC
W. bancrofti β-actin reverse	GATTGCACCACAATTTCACG
human *β-actin* forward	GATGAGATTGGCATGGCTTTA
human *β-actin* reverse	AACCGACTGCTGTCACCTTC
Brugia HhaI forward	GCAATATACGACCAGCAC
Brugia HhaI reverse	ACA*TTAGA*CAAGGAAATTGGTT * indicates modified nucleotide
Brugia HhaI QuantiProbe	TTTTTAGTAGTTTTGGC 3'-Modification: 6-FAM 5'-Modification: Eclipse™ Dark Quencher 5'-Modification: minor groove binder moiety

APPENDIX II

In total 30 SNPs were analyzed for an association in LF.

SNP	rs Nummer
VEGF -634	2010963
VEGF -460	833061
VEGF -634	2010963
VEGF -1154	1570360
Endothelin-1	5370
TNFa -308	1800629
IFNg	1861494
INFg -3810	2069720
IL-4R	**1805010**
IL-4R	**1049631**
IL-6 -174	1800795
IL-6 -572	1800796
TGFb Arg25Pro	1800471
TGFb C509T	1800469
TGFb Leu10Pro	1800470
TGFb -G800A	1800468
TLR2 Arg733Gln	5743708
TLR4 Thr399Ile	4986791
TLR4 Arg229Gly	4986790
IL-18 -137	**187238**
IL-18 -607	**1946518**
VEGFR3 P1114L	na
TLR2 R677W	na
FLT4 -G857R	na
FLT4 -H1035R	na
FLT4 -L1044L	na
FLT4 -R1041P	na
IL-12 p40	na
IL-13	na
IL-12B	na

na: not available in the public databases

APPENDIX III

Calculation of the inbreeding coefficient F

Calculation of the inbreeding coefficient F in a population is the analysis of a deviation from Hardy-Weinberg Equilibrium. The Hardy–Weinberg equilibrium (also termed as RUG, random union of gametes) states that both allele and genotype frequencies in a population remain constant; that is, they are in equilibrium from generation to generation unless specific disturbing influences are introduced. Those disturbing influences include non-random mating, mutations, selection, limited population size, "overlapping generations", random genetic drift and gene flow. It is important to understand that outside the lab, one or more of these "disturbing influences" are always in effect. This states that Hardy–Weinberg equilibrium is impossible in nature. Genetic equilibrium is an ideal state that provides a baseline to measure genetic change.

Static allele frequencies in a population across generations assume: random mating, no mutation (the alleles don't change), no migration or emigration (no exchange of alleles between populations), infinitely large population size, and no selective pressure for or against any traits.

In the simplest case of a single locus with two alleles: the dominant allele is denoted **A** and the recessive **a** and their frequencies are denoted by p and q; freq (**A**) = p; freq (**a**) = q; p + q = 1. If the population is in equilibrium, then we will have freq (**AA**) = p^2 for the **AA** homozygotes in the population, freq (**aa**) = q^2 for the **aa** homozygotes, and freq (**Aa**) = 2pq for the heterozygotes.

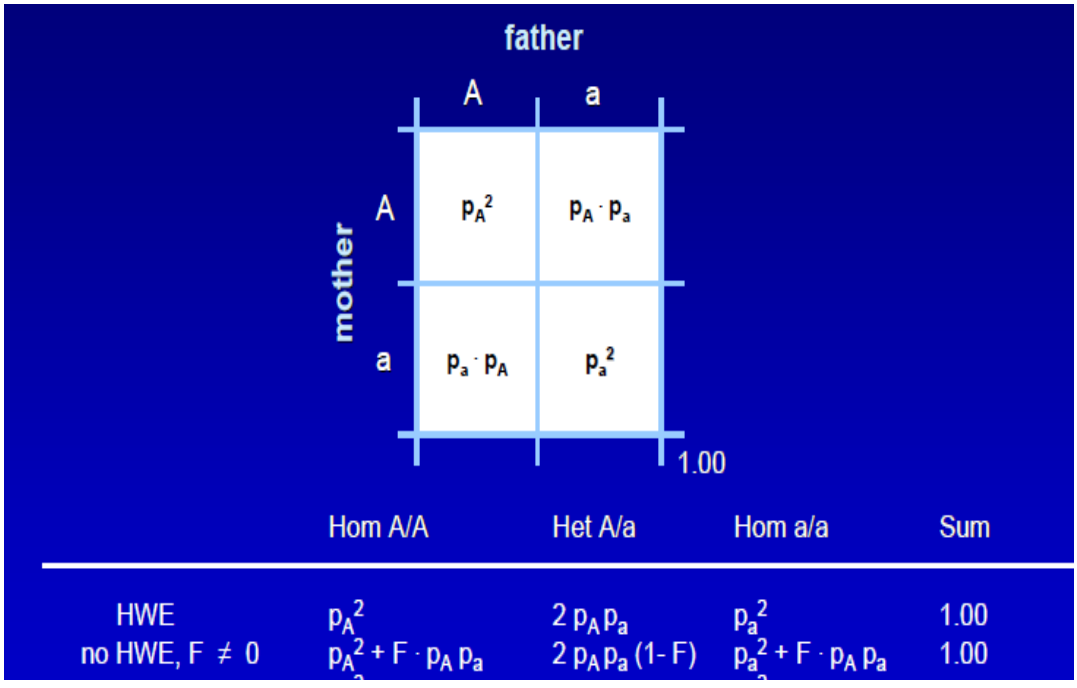
F (inbreeding coefficient) stays for the deviation from HWE (RUG) and is zero if a population is in equilibrium. If F>0, there is a heterozygous deficit, if F<0 a heterozygous overage.

Test of the inbreeding coefficient is important when looking at populations where HWE is likely to deviate from being in equilibrium. This is common in little populations where inbreeding occurs within family generations.

F	**relationship**
1	identical twins
0.5 (½)	full siblings
0.5 (½)	parent-offspring
0.25 (¼)	half siblings
0.25 (¼)	Uncle/Aunt-Niece/Nephew
0.25 (¼)	grandparent-grandchild
0.125 (⅛)	cousins

If F is 1 when looking at the genotypes of the analyzed population, blood sib ship or selection are the common explanations. For a genotype analysis only unrelated ancestors should be included.

Figure A2.1 HWE-Equilibrium (RUG)



P=genotypes p=allel frequencies © T.F. Wienker

In the following is shown a manual test example for the calculation of F in a fictive population. This can also be done by using the deFinetti software created by Strom and Wienker (http://ihg.gsf.de/cgi-bin/hw/hwal.pl).

In the example the analyzed population includes 157 individuals (N=157). The possible genotypes are:

AA Aa aa

Results of genotyping of the individuals show the numbers of genotypes (n) in the population:

n AA=87 n Aa=50 n aa=20 N=157

The genotype frequency (P) can be calculated:

P AA= n AA/N =87/157=0.554

P Aa =n Aa/N =50/157=0.318

P aa =n aa/N =20/157=0.127

∑=1

These are the observed frequencies of the single genotypes in the population.

With these numbers the allele frequency (p) can be calculated. This is the number (m) of allele a and allele A in the population.

m A=2*n AA +n Aa

m A=2*87+50 = 224

m a=2*n aa +n Aa

m a=2*20+50 = 90

m a + m A =2 N (2*157)=314

allele frequencies (p):

pA= mA /2N = 224/314 = 0.713
pa= ma /2N = 90/314 = 0.287
∑=1

With the allele frequencies, the genotype frequency expectation in HWE (RUG) can be calculated.

p A= m A /2N =0.713 m A=2*n AA +n Aa
p a= m a /2N =0.287 m a=2*n aa +n Aa

P AA = p A2
P Aa = 2 p A* p a =2 m A /2N * m a /2N → $\frac{2N_{AA}+ n_{Aa}}{2N} * \frac{2n_{aa}+ n_{Aa}}{2N}$
P aa = p a2

Expected genotype in HWE (RUG) = 0.4093

Observed genotype frequency: 0.3180
Expected genotype frequency: 0.4093
} factor: 1-F = **k**

The difference between the observed genotype frequency and the expected genotype frequency in HWE (RUG) is described as factor **k**.
Factor **k** is calculated by dividing the observed genotype frequency by the expected genotype frequency.

k = 0.318 / 0.4083
k = 0.0776

Calculating the inbreeding coefficient F (deviation from HWE (RUG)), **k** is substracted from one.

F = 1-**k**
F = 1-0.0776
F = 0.2231

F=0 → RUG
F>0 → heterozyg. deficit
F<0 → heterozyg. overage

A heterozygous deficit is the usual situation found in nature. A heterozygous overage could account for selection or lab mistakes. A negative F value can account for an experimental error, if it is significant. Next step is to calculate if F (deviation from HWE (RUG)) is significant. If *P* is > 0.05, a scatter coincidence occurred.

For testing the significance of the deviation from HWE (RUG), a X^2 test (Pearson goodness-of-fit-chi-square) with one degree of freedom (1 df) is used.

Table A 2.1 X^2 test in the fictional population

Allelfrequencies: p A = 0.713 p a = 0.287	N_{AA}	N_{Aa}	N_{aa}	Σ
observed frequencies	87	50	20	157
Expected frequencies **N**	79.8	64.25	12.93	156,98
Deviation Difference	79.8-87 = - 7.2	64.25-50 = 14.25	12.93-20 = - 7.07	0
Difference ^{2}	51.84	203.06	49.98	
Difference ^{2} / Exp.Freq. **N**	51.84/79.8= 0.649	203.06/64.25= 3.160	49.98/12.93= 3.865	**7.674**

Calculation of the expected genotype frequency **N**:

$\mathbf{N}_{Aa} = N^* P_{Aa} = N * 2 * P_A * P_a$

$= 157 * 2 * 0.713 * 0.287$

$= 64.25$

$\mathbf{N}_{AA} = N * P_{AA} = N * P_A^2$

$= 157 * 0.713^2$

$= 79.8$

$\mathbf{N}_{aa} = N * P_{aa} = N * P_a^2$

$= 157 * 0.287^2$

$= 12.93$

With the expected genotype frequencies, the difference to the observed genotype frequency is calculated. The squared difference divided by the expected genotype frequency results summarized for each genotype in the X^2 value (here 7.674).

The *P* value with 1 Df for this X^2 value is >0.005 and <0.01.

(http://de.wikibooks.org/wiki/Mathematik:_Statistik:_Tabelle_der_Chi-Quadrat-Verteilung)

In this fictive example, the deviation from HWE (RUG) is therefore significant. Selection, inbreeding or mutation could influence the population.

With this appendix, I would like to acknowledge and thank Prof. Dr. T.F. Wienker from the Institute for Medical Biometry, Informatics and Epidemiology, University Clinic Bonn. He kindly invited me for a private lesson in Hardy-Weinberg statistics and taught it to me in an understandable way for non-mathematician. He accepted a promise from me to show this simple calculation in my thesis and demonstrate the understanding besides using the software.

Declaration/ Erklärung

Die vorliegende Arbeit wurde in der Zeit von Mai 2006 bis März 2011 im Institut für Medizinische Mikrobiologie, Immunologie und Parasitologie unter der Leitung von Herrn Prof. Dr. Achim Hörauf angefertigt.

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst, andere als die angegebenen Quellen und Hilfsmittel nicht benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.
Ferner versichere ich, dass ich diese Dissertation an keiner anderen Universität eingereicht habe, um ein Promotionsverfahren zu eröffnen.

Publications:

Hoerauf, A., Specht, S., Büttner, M., Pfarr, K., Mand, S., Fimmers, R., Marfo-Debrekyei, Y., Konadu, P., Debrah, A.Y., Bandi, C., Brattig, N., Albers, A., Larbi, J., Batsa, L., Taylor, M.J., Adjei, O., Büttner, D.W., 2008.
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Planned Publications in 2011:

Am. J. of Trop. Med. and Hyg. 2011

The Ile50Val variant of the Interleukin-4 Receptor alpha gene is associated with microfilaremia in Brugian lymphatic filariasis

Anna Albers, Alex Debrah, Tim Becker, Mohammad Reza Toliat, Peter Fischer, Peter Nürnberg, Kerstin Fischer, Taniawati Supali, Kenneth M. Pfarr, and Achim Hoerauf

Am. J. of Trop. Med. and Hyg. 2011

IL-18 promoter single nucleotide polymorphisms -137 and -607 are associated with Lymphedema development in Brugian lymphatic filariasis

Anna Albers, Alex Debrah, Tim Becker, Mohammad Reza Toliat, Peter Fischer, Peter Nürnberg, Kerstin Fischer, Taniawati Supali, Kenneth M. Pfarr, and Achim Hoerauf