ANTI-WOLBACHIA TREATMENT OF LYMPHATIC FILARIASIS AND GENETIC ANALYSIS OF THE PATHOLOGY OF LYMPHEDEMA AS A CLINICAL MANIFESTATION OF THE DISEASE

A THESIS

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Declaration

I hereby declare that this submission is my own work towards the PhD and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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Abstract

Lymphatic filariasis (LF) caused by *Wuchereria bancrofti* is a disease of considerable public health and socio-economic burden in the tropics. The recommended drugs (ivermectin and albendazole) for the control of lymphatic filariasis are only microfilaricidal. The regimen of the standard treatment with doxycycline which has proven macrofilaricidal is 200mg/d for 4 weeks. This is considered a long period and dissuades complaince by patients receiving treatment. Therefore reducing the duration or the dosage from the current 200mg/d to 100mg/d would be a better option and will also increase compliance.

In search of a more effective drug to complement the existing ones, in an area endemic for bancroftian filariasis in Ghana, 261 adult worm positive men were recruited for a double blind placebo-controlled study in the Ahanta West District of Ghana. Six groups of patients were treated with the gold standard (4 weeks 200mg/d doxycycline), 5 weeks and 4 weeks 100mg/d doxycycline, 3 and 2 weeks combination of 200mg/d doxycycline and 10mg/kg rifampicin and 5 weeks placebo.

The effect of the treatment on *Wolbachia* depletion was assessed at 4 months after treatment; adult worm vitality assessed at pre-treatment, 12, 18 and 24 months, and microfilarial depletion assessed at pre-treatment, 4, 12, 18 and 24 months follow up time points. In accordance with the national mass drug administration programme, all the study participants were given 150mg/kg ivermectin and 400mg albendazole four months after treatment.

The treatment drugs were well tolerated with no serious adverse effects in both the treated and the placebo groups. There was significant *Wolbachia* depletion at 4 months time point in the standard group ($p=0.001$), 5 weeks 100mg doxycycline ($p=0.019$), 4 weeks 100mg doxycycline ($p=0.03$) and 3 weeks combination treatment ($p=0.028$).

However there was no significant *Wolbachia* depletion in the 2 weeks combination group as well as the placebo group ($p>0.05$).

Microfilarial assessment at 12, 18 and 24 months follow up time points showed a significant depletion in the standard group, 5 weeks and 4 weeks 100mg doxycycline groups as well as the 3 weeks combination of 200mg plus rifampicin group but not in the 2 weeks and placebo groups ($p>0.05$).

The macrofilaricidal activity was significant at 12, 18 and 24 months in the standard and the 5 weeks groups. In the 4 weeks group, it was significant at 18 and 24 months time point and in the 3 weeks group it was significant at 18 months follow up time point, but no significant difference was observed for the 2 weeks and the placebo groups.

On the other hand, there is the need to know the genetic markers associated with LF. Such knowledge will be beneficial in terms of diagnosis and possible therapy of various forms of the pathology. For this reason, a cross-sectional study of unrelated Ghanaian volunteers were designed to genotype single nucleotide polymorphisms (SNPs) in 266 lymphedema patients as cases and 691 infected patients without pathology as well as 346 endemic controls.

Out of the 147 chosen SNPs that were genotyped, 11 SNPs in eight genes were found to be associated with lymphedema. The associated SNPs were in the vascular endothelial growth factor receptor 3 (VEGFR3), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (NFKB- inhibition alpha), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1), tissue inhibitor of metallopeptidase 2 (TIMP) genes, interleukin 10 and two SNPs in insulin like growth factor-1 (IGF-1) and matrix metallopeptidase 2 (MMP-2). A SNP in the interleukin 17 gene revealed a trend but there was significant haplotype association.

In conclusion 5 weeks, 4 weeks and 3 weeks combination regimens of doxycycline were effective in treating LF infections, and SNPs in the angiogenic pathway were found to be associated with the pathology of lymphatic filariasis.

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CHAPTER 1

1.0 General Introduction

Filarial nematode infections affect approximately 150 million people worldwide (Hoerauf *et al.*, 2003a) and they are still a major cause of morbidity in the tropics. The major diseases caused by human filariae are onchocerciasis, loaisis and lymphatic filariasis (Taylor and Hoerauf, 2001).

Onchocerciasis, commonly known as river blindness, is caused by the filarial worm *Onchocerca volvulus*. It is contracted through the bite of a *Simulium* fly and it is an important cause of blindness and severe dermatitis (WHO, 1987). It affects nearly 37 million people in 34 countries and is most common in Africa (Taylor *et al*., 2010).

Another human filarial disease, loasis, is caused by the nematode worm, *Loa loa* (Jaksche *et al*., 2004). It is contracted through the bite of a Chrysops fly (Padgett and Jacobsen, 2008) and an estimated 12-13 million people are infected (Boussinesq, 2006). The disease can cause red itchy swellings below the skin called "Calabar swellings". Loiasis is restricted to the rain forest and swampy forest areas of West Africa, being especially common in Cameroon (Padgett and Jacobsen, 2008; Boussinesq, 2006).

Lymphatic filariasis (LF) on the other hand is known to infect an estimated 120 million people with at least 40 million people being seriously incapacitated and disfigured both physically and psychologically (WHO, 2000; Ottesen, 2000). About 90% of the estimated 120 million cases are caused by *Wuchereria bancrofti* and *Brugia spp.* (*B. malayi and B. timori*) accounts for the remainder (Mak, 1987).

In lymphatic filariasis (LF) endemic communities, 7% of the adult population tends to suffer from lymphedema and 30-50% suffers from hydrocele (Tisch *et al*., 2001; Ottesen, 2000). About 1.3 billion people representing 20% of the world's population are at risk of acquiring the infection in more than 81 endemic countries (WHO, 2010; Chu *et al.*, 2010). These include many parts of Africa, Asia, the Pacific and the Americas (WHO, 2006; 2007a; 2010).

In Africa alone, about 50 million people are already affected with lymphatic filariasis and 512 million people are at risk of being infected in Sub-Saharan Africa (WHO, 1994; Michael *et al*., 1996; Michael & Bundy, 1997; Ottesen *et al*., 1997). The disease is known to be transmitted by mosquitoes of the genus *Anopheles, Culex, Aedes* or *Mansonia* (Bockarie *et al.*, 2009).

The clinical manifestation in the endemic population is influenced by several factors including; (i) the anatomic location of adult worms,

(ii) the presence or absence of microfilaria and (iii) immunogenetics of the host and (iv) secondary bacterial infections (Mahanty *et al*., 1997).

Early symptoms consist of intermittent fever and enlarged tender lymph nodes (Pfarr *et al.,* 2009). The lymphatic vessels that drain into the lymph nodes and those which harbour the developing and adult worms also become inflamed and painful. This condition is termed acute dermatolymphangioadenitis (ADLA) [Dreyer *et al*., 1999]. This state is later worsened by concomitant streptococcal superinfection resulting in debilitating pathology which is linked to the progression of lymphedema to chronic state of elephantiasis *(*Pani and Srividya, 1995*).*

Hydrocele is also a chronic presentation of lymphatic filariasis which results from the accumulation of fluid in the tunica vaginalis of the affected scrotum (WHO, 2005). Approximately 30-50% of infected individuals develop hydrocele with (WHO, 2006; 2007a).

The international task force for disease irradication in 1993 identified lymphatic filariasis as one of the diseases that is eradicable (Dreyer *et al* 1997). The goals for the disease eradication are (i) to reduce microfilaraemia levels, by using filaricidal drugs to a level that is too low to sustain transmission of filarial parasites to humans and (ii) to reduce the morbidity associated with chronic filarial disease (Cox, 2000).

The recommended drugs for treatment were either diethylcarbamazine (DEC) with albendazole or in Afrca, ivermectin with albendazole for about 6 years (Molyneux and Zagania, 2002; Molyneux and Taylor, 2001; Ottesen *et al*., 1997). Ttreatment options for filarial nematodes are limited by costs, and side effects, with no single drug being

effective for the clinical manifestations such as hydrocele and lymphedema (Hise *et al.*, 2004). Drugs currently being used do not have strong macrofilaricidal effect or does not have sterilizing effect on the adult worms which are responsible for the pathologies of the disease. More effective drugs are therefore needed to complement the actions of the existing ones.

Wolbachia endosymbiotic bacteria originally identified in 1977 (Kozek, 1977) emerged recently as a target for the treatment with antibiotic (doxycycline) which eventually leads to the sterility of the adult female filariae (Hoerauf *et al.*, 2001; 2003a) and has been shown to have macrofilaricidal effect in *Onchocerca volvulus* and *Wuchereria bancrofti* (Taylor *et al.*, 2005; Debrah *et al.*, 2007a). A more recent discovery is the ability of doxycycline to cause amelioration of lymphatic dilation in microfilaraemic patients (Debrah *et al.*, 2006). The lymphatic dilation is known to be caused by proinflammatory cytokines such as TNF, IL-6 and IL-1B. *Wolbachia* is known to induce these proinflammatory cytokines which in turn induce lymphangiogenic factors such as Vascular endothelial growth factor A (VEGF-A), VEGF-C and soluble receptor (VEGF-R3) and it is the over expression of these lymphagiogenic molecules, produced by endothelial cells that cause lymphatic dilation (Debrah *et al.*, 2006).

These data on anti-*Wolbachia* chemotherapy is an important criterion of the WHO drug research program against filariasis which was acknowledged at the International Conference on Filariasis at the Bernhard-Nocht Institute, Hamburg, Germany in September 2001 and the International Meetings of the Scientific Working Group (SWG) on Lymphatic Filariasis in December 2003 in Philadelphia (WHO, 2004) and in May 2005

in Geneva (WHO, 2005) where potential preliminary indications for the use of doxycycline were formulated.

Nevertheless, despite these promising results with doxycyline, there is still the need for an additional drug since the treatment with doxycyline for 6 weeks is still long and so a drug with shorter duration would be more desirable (Debrah *et al*., 2006).

Studies carried out both *in vitro* and *in vivo*, indicate that besides doxycycline, other already registered antibiotics, especially rifampicin, also reduced *Wolbachia* from adult worms and damaged developing embryos and, at higher concentrations, adult worms were killed (Townson *et al*., 2000; Rao and Weil , 2002; Volkmann *et al*., 2003). In fact, in murine filariasis using *Litomosoidess sigmondontis,* administration of rifampicin either alone or in combination with doxycycline for a period of 14 days was sufficient to deplete *Wolbachia,* inhibited embryogenesis and led to filarial sterility and worm development, whereas treatment with doxycycline alone for 21 days led only to a modest reduction of *Wolbachia,* filarial growth retardation, worm viability and infertility (Volkmann *et al*., 2003). This shows that rifampicin might even be a shorter effective regimen and have a better *Wolbachia* depletion capacity and a higher macrofilaricidal activity than doxycycline.

A pilot study by Debrah *et al*. (2011) showed that rifampicin when combined with doxycycline leads to a partial macrofilaricidal activity. However, the sample size was too small for definitive conclusions to be drawn.

Based on these promising results from animal experiments and the pilot work, and also given that rifampicin is already a registered drug, the first part of the present study was to assess the effect of combination therapy of rifampicin with doxycycline.

Filarial infections also have a characteristic distribution in populations living in endemic areas. It presents a spectrum of clinical states with two major poles (Maizels *et al.*, 1995). One pole is represented by microfilaraemic patients with high parasite numbers and down-regulated cell-mediated responses, and the other by patients with elephantiasis and hydrocele, who typically have few or no parasites and vigorous specific immune reactions (Maizels *et al.*, 1995). The prevalence of overt clinical manifestations among adult residents of endemic areas is usually between 10-30% despite the fact that most individuals are presumably inoculated with infective larvae throughout life. This heterogeneity in infection and disease has been attributed to differences in both inflammatory processes that are immune-mediated (Taylor *et al.*, 2000b), secondary bacterial infections superimposed on the lymphatic dysfunction (Mahanty *et al.*, 1997) and the immunogenetics of the host (Hoerauf *et al.*, 2002, Debrah *et al*., 2007b).

Epidemiological studies in areas where filariasis is endemic have revealed differential susceptibilities to infection, both within entire populations as well as within families (Subrahmanyam *et al.*, 1978).

Since there have been relatively few investigations of host genetic contributions to acquisition and outcomes in human filarial infections, there is the need for further studies utilizing a candidate gene approach to identify genetic markers of the host to differential

susceptibilities to the infection. Identification of such genetic markers in high risk members of filarial pathology (lymphedema and hydrocele) could facilitate the identification and management of environmental factors that influence the expression and severity of lymphedema.

Elucidation of candidate genes of lymphedema and hydrocele may also be beneficial in terms of both diagnosis and possible therapy of various forms of the pathologies associated with the disease.

1.1 Aims and Objectives of the Studies

- To authenticate the paramount treatment regimen with doxycycline alone or combination of doxycycline and rifampicin in lymphatic filariasis through randomized placebo-controlled double–blinded clinical trial.
- To ascertain whether a reduction of the daily dosage of doxycycline from 200 mg/d for 4 weeks to 100 mg / d given for 4 weeks or for 5 weeks could lead to a long-term sterilizing and / or macrofilaricidal effect.
- To identify the genetic biomarkers of risk in lymphedema development using genotyping technique.

CHAPTER 2

2.0 Literature Review

2.1 The Parasite and Disease Transmission

The principal carriers of *W. bancrofti* are *Culex* species in urban and semi-urban environments and *Anopheles* in most rural areas of Africa and elsewhere (Sasa, 1976). However, there has been recent report of *Mansonia africana* and *Mansonia uniformis* as vectors for the transmission of *W. bancrofti* in Ghana (Ughasi *et al*., 2012).

The vectors ingest microfilariae (MF) during blood meals. In the insect, over a period of about 12 days, the microfilariae progress through two stages to an infective stage called the L3 larvae (WHO, 2000). These L3 larvae are deposited in the skin of the humans during subsequent blood meals. The larvae enter the body through the wound made by the insect and undergo two more moults to develop into adult worms, completing the cycle (Taylor, 2010) [Plate1]. Adult filarial parasites are sexually dimorphic and reside in the lymphatic vessels. The adult worms are long lived and can reproduce for 5-8 years producing millions of first stage larval or microfilarial stages that migrate from the lymphatic system into the blood (Taylor, 2010).

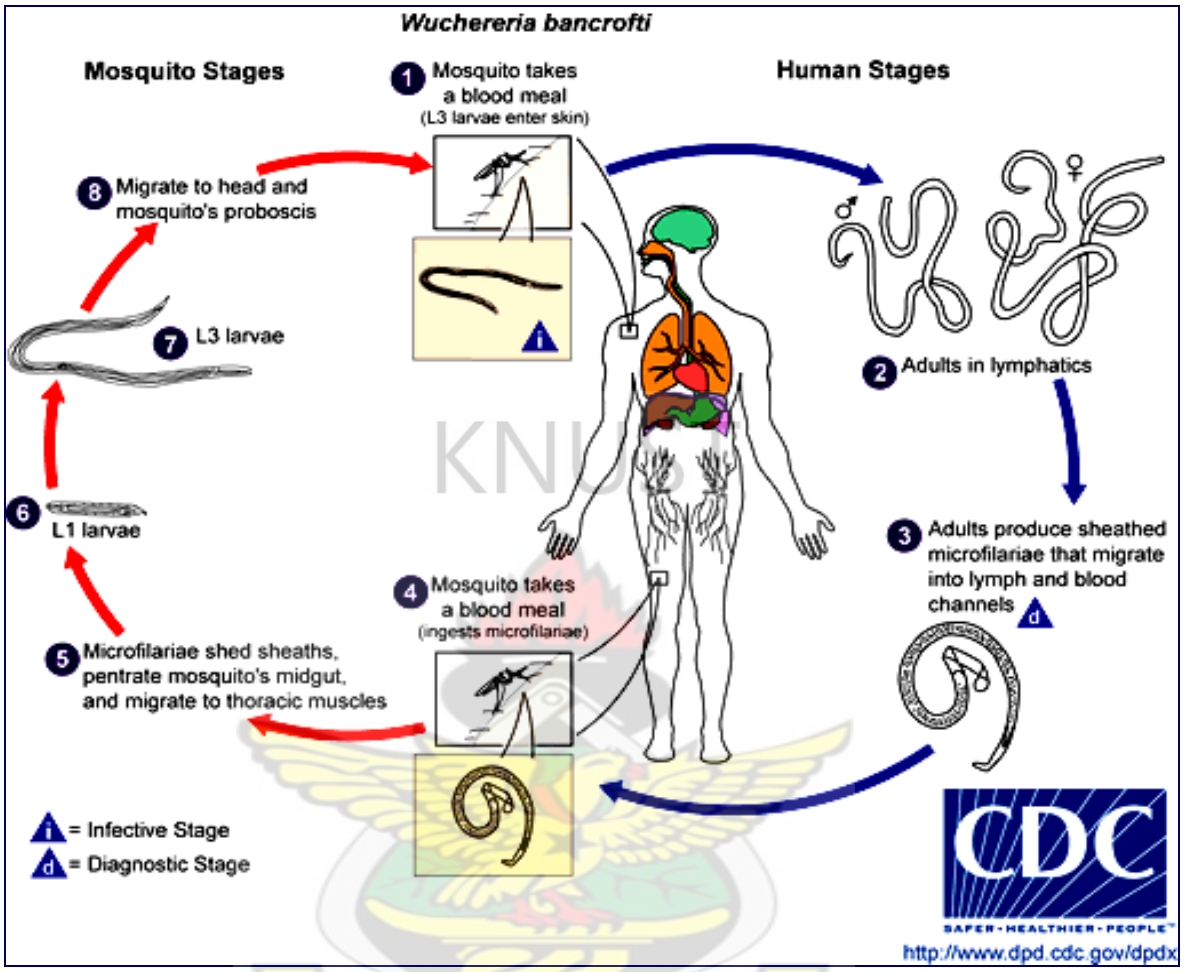


Plate 1: Life cycle of *Wuchereria bancrofti*

2.2 Pathological Manifestations

Lymphatic filariasis is so called because the site of the adult worm parasite is in a dilated nest (lymphangiectasia) within the lymphatic vessels, most commonly in the extremities and male genitalia (Plate 2) [Pfarr *et al*., 2009]. About 40 million people (a third of those infected) have clinical disease making lymphatic filariasis the second cause of permanent and long term disability worldwide (Hassan *et al*., 2004; Nuchprayoon, 2009). Findings from sensitive antigenaemia prevalence studies show that children are often infected within the first few years of life but exhibit subclinical pathological features of the lymphatics, with only occasional overt disease before 10 years of age which increases in frequency after puberty (Witt and Ottesen, 2001).

2.2.1 Acute and chronic disease manifestations

Lymphatic filarial nematodes cause acute and long-term chronic infection with a few infected individuals developing overt disease (Pfarr *et al*., 2009). Normally individuals with millions of vigorously motile MF often show no obvious clinical signs of the disease (Pfarr *et al*., 2009). Asymptomatic infections are characterised by several immune regulatory processes driven by living parasites, thought to promote their long-term survival (Hoerauf *et al*., 2005; Babu *et al*., 2000; 2009).

The chronic infection is as a result of chronic immune stimulus caused by incoming larvae that develop into adult worms, dying adult worms (be it immune mediated or just intrinsic worm lifespan), degenerated embryos and the release of parasite antigens or *Wolbachia* (Turner *et al.*, 2009 ; Brattig *et al*., 2004; Taylor *et al*., 2000a).

These stimuli then trigger the innate and adaptive immune cascades with associated angiogenesis and lymphangiogenesis of parasitized vessels induced by vascular endothelial growth factors. Pathologies of the disease develop progressively (Bennuru and Nutman, 2009; Bennuru *et al*., 2010; Debrah *et al*., 2006; 2009).

2.2.1.1 Hydrocele

Hydrocele is the most common clinical manifestation of bancroftian filariasis in endemic regions (Noroes and Dreyer, 2010; Noroes *et al*., 2003). It manifests clinically as swelling of the peritoneal lining that surrounds each of the testicles. Usually clear straw-coloured hydrocele fluid accumulates in this closed sac as a result of blockage in the lymphatic draining in the retroperitoneal and sub diaphragmatic areas (WHO, 1992).

Hydrocele is known to involve dilation of lymphatic vessels and extravassation of fluid from the vessels into surrounding tissues (Mand *et al*., 2010). Rarely the fluid has milky appearance caused by the presence of lymph, a condition known as chylocele. The adult *Wuchereria* are often lodged in the lymphatics of the spermatic cord causing scrotal damage and swelling (Mussner *et al*., 1997)

In advanced cases it can become very large (grape fruit or coconut size). Increase in size coincides with number of attacks of funiculitis (WHO, 1992; Evans *et al*., 1993). Hydrocele may result in altered testicular function or decreases spermatogenesis (Bayne *et al*., 2008) although no direct effect of hydrocele on fertility has been shown (Politoff *et al*., 1990).

The main clinical characteristics of hydrocele are headache, fever, swelling of the scrotum, pain and tenderness of the scrotum. The scrotal area can become very hot and in some instances there could be leakage of lymphatic fluid through the scrotal skin (Gyapong, 2000).

Hydrocele is rarely fatal and patients are not often in pain but it may carry grave social and economic consequences for those affected (Evans *et al.,* 1993).

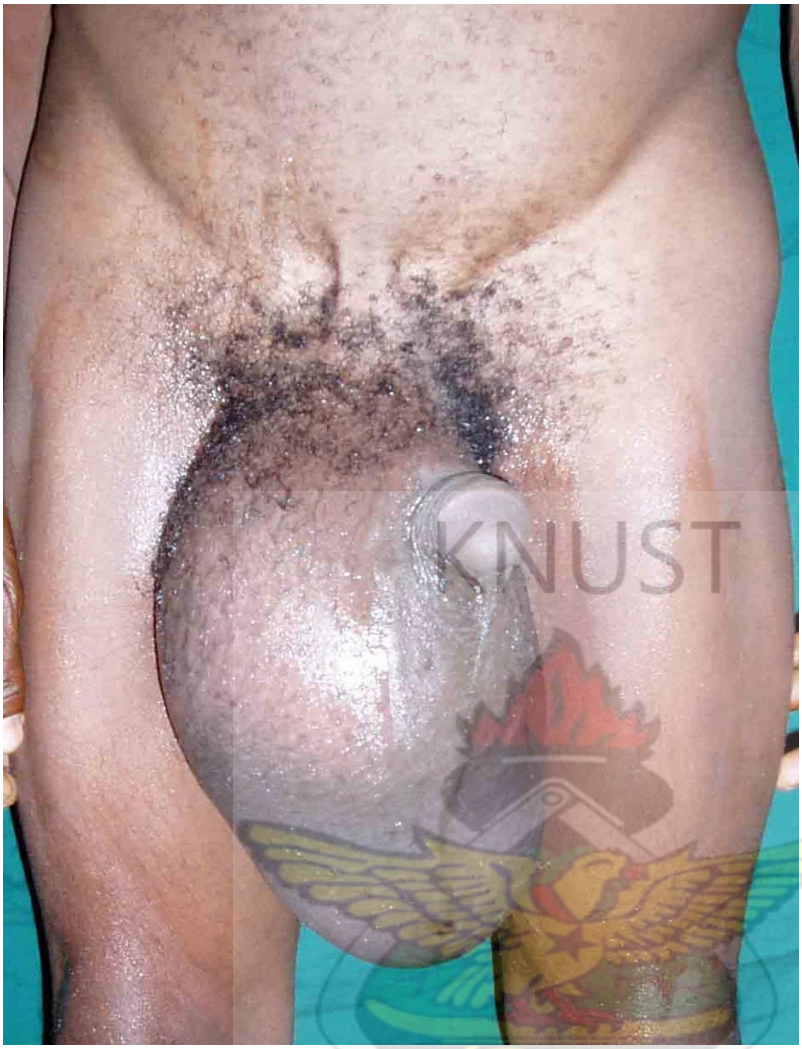


Plate 2: Clinical presentation of hydrocele (own source)

2.2.1.2 Lymphedema

Elephantiasis begins as lymphedema which is the first permanent sign of chronic lymphatic filariasis. The legs, scrotum, arms, penis, vulva and breasts are affected usually in that order of decreasing frequency (WHO, 2000). In some endemic areas such as Indonesia and the Pacific Area and Sri Lanka the swelling may remain below the knee but in Africa it may affect the whole leg (WHO, 2000). In the initial stages the swelling can best be observed around the ankles which gradually spread to the back of the foot, about three times the original size. Elephantiasis is usually preceded by periodic attacks of adenolymphangitis (Pfarr *et al.*, 2009). The hall mark of elephantiasis is thickening of the skin and fibrosis of the underlying tissue (Plate 3) [Connor *et al.*, 1986], which results from lymphatic obstruction. Affected tissues first become oedematous and then develop proliferative dermal changes with subsequent derma and subcutaneous fibrosis (Pfarr *et al.*, 2009). The external genitalia if affected may have varicose changes of the epidermis.

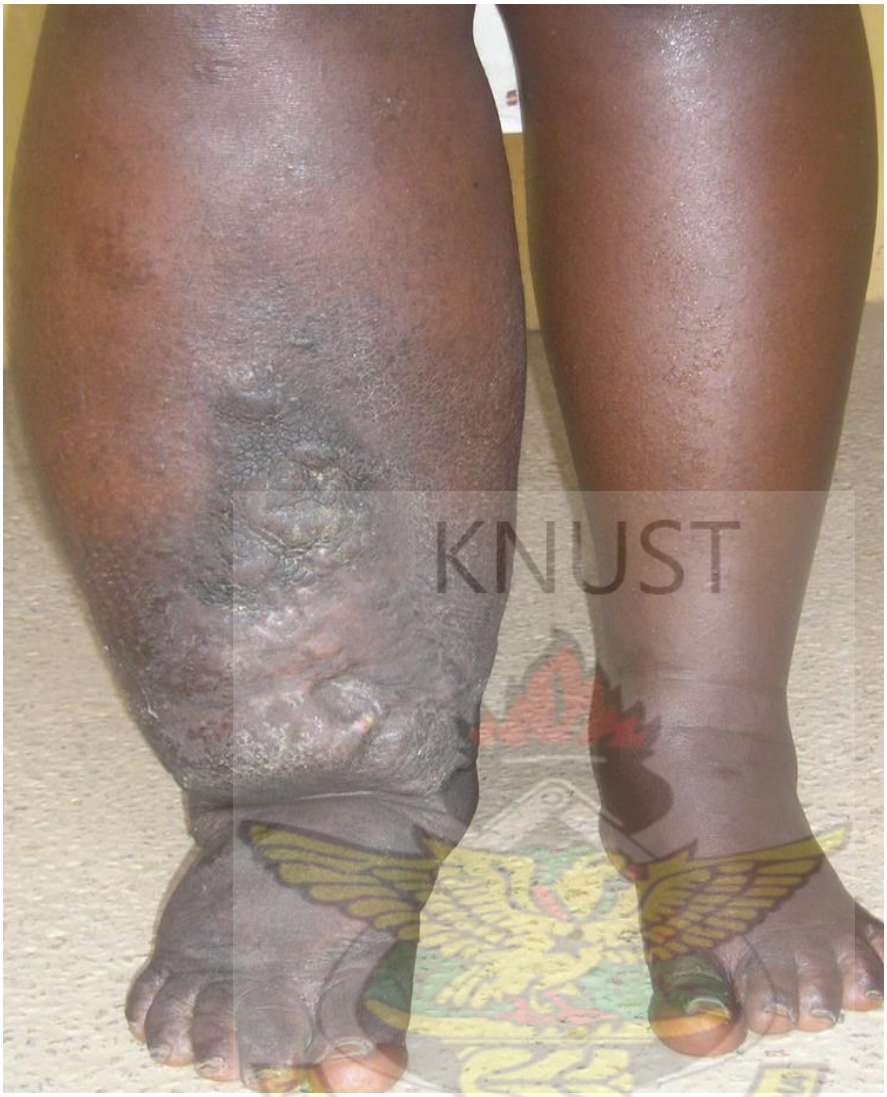


Plate 3: Clinical presentation of lymphedema (own source)

2.3 Chemotherapy

Drugs used to treat or control filariasis include diethylcarbamazine, ivermectin and albendazole. For lymphatic filariasis, diethylcarbamazine or ivermectin in combination with albendazole is used as the basis for the global programme to eliminate lymphatic filariasis (Global Alliance to Eliminate Lymphatic Filariasis: http://www.filariasis.org).

2.3.1 Ivermectin

Ivermectin (Mectizan®) is a fermentation product of *Streptomyces avermitis* and it is a semi- synthetic derivative of avermectin B1 (WHO, 1987; Campbell, 1991). Ivermectin is an antihelmintic drug introduced for human use since 1987 (Campbell, 1991) and has since been used for the treatment of onchocerciasis (WHO, 1995), strongyloidiasis, ascariasis, trichuriasis, and enterobiasis (Fox, 2006). In lymphatic filariasis it is an effective and well tolerated drug at doses of 100–200 μg/kg for reduction of microfilaraemia (Gyapong, 2005). Ivermectin is known to act by hyperpolarisation of glutamate-sensitive channels (Omura and Crump, 2004). A block of the contractile activity of the excretory/secretory vesicle as shown by Moreno and others may lead to compromising of the immune system of the patient leaving the microfilaria undefended in the lymph nodes (Moreno *et al* ., 2010).

Ivermectin when given four times every year for four consecutive years can have a moderate macrofilaricidal effect (Duke, 2005). However, most adult worms are not killed and as such, when individuals are treated to relieve their symptoms, it should be given for the adult worms' lifespan (5-8 years).

Combination treatment with DEC is not possible in Africa where there is coendemicity of onchocerciasis because of the severe adverse effects associated with such treatments (Molyneux *et al.*, 2003).

2.3.2 Diethylcarbamazine citrate (DEC)

Diethylcarbamazine (DEC) has been the drug of choice for treating lymphatic filariasis since its discovery in 1948. DEC has been given since 2007 as a single dose of 6 mg/kg to patients infected with *W. bancrofti* and *B. malayi* or *B. timori* (Gyapong *et al.*, 2005). It has been shown to have a strong and sustained killing of microfilaraemia for at least one year, and this has been the basis of mass drug distribution by the global programme to eliminate lymphatic filariasis in areas without co-endemic onchocerciasis (Ramzy *et al.*, 2006; Tisch *et al.*, 2005; Weil *et al.*, 2008).

Though DEC is seen to have great microfilaricidal effect, not all the microfilariae are cleared in an infected person and not all adult worms are killed (Gyapong *et al.*, 2005).

DEC has been shown to have low toxicity and safe to large-scale use even in circumstance of limited medical supervision (Ottesen and Ramachandran, 1995; Meyrowitsch and Simonsen, 1998). But, it is known to inflict severe adverse effect in those regions of Africa where *Onchocerca volvulus and Loa loa infections co-exist with Wuchereria bancrofti infection* (Ottesen and Ramachandran, 1995; WHO, 1995). This makes its usage unaceptable in those areas.

2.3.3 Albendazole

Albendazole is a benzimidazole derivative with broad-spectrum antihelmintic activity (WHO, 1992). Albendazole inhibits the polymerization of worm b-tubulin and microtubule formation (Hoerauf *et al*., 2011). A dose of 400mg is widely used for control of intestinal helminthiasis (WHO, 1996; Horton, 2002). Albendazole alone administered as 400mg twice daily for three weeks was reported to have more dramatic and statistically significant clearance of *Wuchereria bancrofti* (Jayakody *et al.,* 1993). However such multiple high dosages are not suitable for community based control and furthermore induce intolerable adverse effects (Jayakody *et al.*, 1993). Although it has been suggested for a combination treatment with either ivermectin or DEC, studies conducted on this combination give equivocal results (Pani *et al*., 2002; Critchley *et al*., 2005a; Critchley *et al*., 2005b).

2.3.4 Doxycycline

Doxycycline is a member of the tertracycline antibiotics group and is commonly used to treat a variety of infections (Jacquerioz and Croft, 2009; Duteil 2002). It is a semisynthetic tetracycline invented and clinically developed in the early 1960s by Pfizer inc. Doxycycline was then marketed under the brand name vibramycin and it is a broad spectrum antibiotic.

As in all members of the tetracycline group, doxycycline is frequently used in treating a host of diseases such as prostatitism, sinusitis, syphilis, *Chlamydia* infection, pelvic inflammatory disease and rickettsial infections (Johnson *et al*., 1993).

It is used as prophylasis against malaria and many bacterial infections (Jacquerioz and Croft, 2009; Duteil 2002).

The effect of tetracycline against filarial nematodes was first observed in studies on *Brugia pahangi* showing that tetracycline could inhibit the development of third stage larvae (L3) to adults and establishment of microfilaremia (McCall *et al.,* 1999). In 1999, these observations were linked to the presence of *Wolbachia* (an endosymbiotic bacteria, essential for the worm's survival) by Hoerauf who demonstrated that the loss of bacteria from worms resulted in the inhibition of development of infective larvae (L3) of *Litomosoides sigmodontis* leading to filarial infertility (Hoerauf *et al*., 1999; Bandi *et al*., 1998).

In humans, doxycycline was shown in 2003 to kill symbiotic *Wolbachia* bacteria in the reproductive tracts of filarial worms rendering them sterile. Further field trials even showed that treatment of bancroftian filariasis with a course lasting 4, 6, or 8 weeks of a 200 mg per day dose of doxycycline resulted in long-term sterility and eventual death of adult worms. (Debrah *et al*., 2006; 2007a; Taylor *et al*., 2005). Investigators have shown that individuals treated with anti-*Wolbachia* therapy show substantial improvements in lymphatic pathological features and decreased severity of lymphedema and hydrocele (Debrah *et al*., 2006; 2009). The benefits of doxycycline therapy have been suggested to extend to lymphedema patients without active infection, and it is better than the current morbidity management based on hygiene and self-care (Mand *et al.,* 2012).

2.3.5 Rifampicin

Rifampicin is a bactericidal antibiotic drug of the rifamycin group (Masters *et al*., 2005). It is a semi synthetic compound derived from *Amycolatopsis rifamycinica* (formally known as *Amycolatoppsis mediterranei* (Sensi *et al*., 1959). Rifamycin is intensely red solid and the small fraction which reaches body fluids is known for imparting harmless red orange color to the urine of user for a few hours after a dose.

Rifampicin is a licensed and well known drug used for long term treatment of several diseases such as tuberculosis (Fox, 1999). Rifampicin has been shown to have macrofilaricidal effect in a murine filariasis model *Litosimoides sigmodontis* after a 14 days treatment (Volkmann *et al*., 2003). In humans, partial macrofilaricidal activity has also been observed in a pilot study with 14 days rifampicin treatment (Debrah *et al*., 2011).

2.3.6 Adverse reactions to anti-filarial drugs

Ivermectin, DEC and albendazole are the presently used drugs for the treatment of filarial infections (WHO, 1995). Adverse reactions after filarial infections including onchocerciasis and lymphatic filariasis have been reported.

Adverse reactions to DEC in onchocerciasis can be so severe that the drug is no longer recommended for this disease (WHO, 1995; Taylor and Hoerauf, 1999).

Ivermectin, the present drug of choice in Sub-Saharan Africa is also associated with significant adverse reactions that affect compliance with the necessary repeated treatment rounds (Cooper *et al.,* 1999). Serious adverse event associated with mass distribution of

ivermectin in Cameroon where there is Loa loa infection has also been reported Gardon *et al*., 1997). The major side effects of ivermectin are fever, pruritus, prostration and postural hypotension (Awadzi *et al.*, 1990). Many of these adverse effects are characterised by progressive neurologic decline and encephalopathy within few days of taking the drug (Twum-Danso, 2003). Long-term periodic treatments with ivermectin have also been found to be associated with an increased prevalence of allergen skin test reactivity.

With Albendazole, there has not been much repoted cases of adverse effects due to its intake however, incidence such as acute hepatitis in patients with continous use of the drug have been reported (Amoruso *et al.,* 2009).

Adverse reactions due to these antifilarials have been found to be associated with the rapid killing of worms (microfilaria) and this can impede the community participation in control programmes. In heavily infected individuals it can be severe and even fatal (Taylor *et al*., 2000b). Though the precise mechanism is not clear, it is known to results in the sequestration of MF and their eventual destruction by the immune system. (McGarry*et al*., 2005; Taylor *et al*., 2010). These post-treatment responses have been found to be directly proportional to the pre-treatment Mf densities of the affected patients (Kumaraswami *et al.*, 1988).

Extensive studies have attempted to define the specific inflammatory mechanisms involved in these adverse reactions. The main inflammatory mediators evaluated for a potential role in inducing these reactions include histamine, prostaglandins, kinins,

complements and eosinophil granule proteins, but none of these mediators correlated well with the systemic reactions found in treated patients (Ottesen *et al.*, 1997).

2.3.7 Adverse reactions to anti-wolbachial drugs

Implication of *Wolbachia* in the induction of adverse reactions following antifilarial treatment has also been reported, because of sudden release of bacterial mediators such as LPS-like molecule that trigger innate immune system (Taylor, 2000b; Brattig 2000).

A significant association of adverse reactions following macrofilaricidal treatment of *W. bancrofti* infection and elevated *Wolbachia* DNA levels in blood has been reported. (Cross *et al.*, 2001). A similar association was found in onchocerciasis (Keiser *at al.*, 2002). Several factors are thought to contribute to the inflammatory pathogenesis of filariasis which includes the parasite, the immune response and opportunistic infection (Hoerauf *et al.*, 2002b).

All the tetracyclines produce gastrointestinal irritation to varying degrees in some but not in all individuals. Epigastric burning and distress, abdominal discomfort, nausea, and vomiting may occur (Sande and Mandell, 1992).

However, gastric distress can be controlled by administration of the tetracycline with food (not dairy products); nausea and vomiting often subside as medication continues and can often be controlled by temporary reduction in dose or by the use of small amounts at more frequent intervals (Sande and Mandell, 1992).

Tetracyclines are contraindicated in people with hepatic and renal impairment, pregnant and breastfeeding women, people with systemic lupus erythematosus and children under 12 years of age (Sande and Mandell, 1992).

It is important to recall that the slow doxycycline –mediated depletion of *Wolbachia* from the filariae over 4-6 weeks apparently does not liberate enough LPS-like and other bacterial molecules to induce *Wolbachia* depended side effects.

Furthermore doxycycline does not kill MF directly but blocks microfilarae regeneration during embryogenesis thus leading to a reduction of MF according to their half life (Hoerauf, 2003b). Therefore doxycycline may be considered as a first step to safely reduce high MF loads and avoid severe adverse effects of microfilaricidal drugs.

Rifampicin has proved to be a valuable antibiotic with relatively few major adverse effects. The toxicity of rifampicin is predominantly hepatic and immunoallergic in character (Grosset and Leventis, 1983). Hepatic toxicity has been reported in many patients and it has been found to be dose related. The immunoallergic effects are usually associated with intermittent or prolonged therapy. These immunoallergic effects may be minor (a cutaneous, gastrointestinal, or influenza-like syndrome) or major (hemolytic anemia, shock, or acute renal failure). There has also been reported case of an association of rifampin with Fanconi syndrome presenting as hypokalemic paralysis, which is a manifestation of acutetubulo-interstitial nephritis (ATIN). Kidney function and the markers of proximal tubular injury should be carefully monitored in patients receiving rifampin (Min *et al*., 2013). Rifampicin is an anti-wolbachia drug whose action is not directly on the filarial. Therefore the adverse reaction is expected to be minimal due to its anti-wolbachia properties.

2.4 Single Nucleotide Polymorphism (SNP)

A single-nucleotide polymorphism (SNP) is a DNA sequence variation that occurs when a single nucleotide — A, T, C or G — in the genome differs between members of a biological species or paired chromosomes in a human (Yuzhalin *et al*., 2012). They are very common genetic variations that are densely distributed throughout the genome accounting for more than 90% of all differences between unrelated individuals (Richard and Twyman, 2005).

SNPs usually consist of two alleles (the major allele and the minor / rare allele) where the rare allele frequency is >1%). Since there are variations between human populations, a SNP allele that is common in one geographical or ethnic group may be much rarer in another (Kim and Misra, 2007).

SNPs may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions (regions between genes). SNPs within a coding sequence do not necessarily change the amino acid sequence of the protein that is produced. In the coding region there are of two types, synonymous and nonsynonymous SNPs. Synonymous SNPs do not affect the protein sequence while nonsynonymous SNPs change the amino acid sequence of protein. The nonsynonymous SNPs are of two types: missense and nonsense.

These genetic variations between individuals (particularly in non-coding parts of the genome) are exploited in DNA fingerprinting, which is used in forensic science (Kim and Misra, 2007).

2.4.1 Use and importance of SNPs

In humans, variations in the DNA sequences can affect disease development and respond to pathogens, chemicals, drugs, vaccines, and other agents (Taylor MJ, 2002; Zabeleta *et al.*, 2008). SNPs are also critical for personalized medicine (Carlson, Bruce, 2008). However, their greatest importance in biomedical research is for comparing regions of the genome between cohorts (such as with matched cohorts with and without a disease) in genome-wide association studies.

2.4.2 SNP genotyping

SNP genotyping is a high throughput method which is used for the measurement of genetic variation of single nucleotide polymorphisms (SNPs) between members of a species. SNPs are found to be the etiology of many human diseases (International Hapmap Project, 2003; Richard and Twyman, 2005) and are becoming of particular interest in pharmacogenetics.

Technologies for SNP genotyping typically involve the generation of allele-specific products for SNPs of interest and its subsequent detection for genotype determination.

Most of the Current genotyping technologies require the PCR (Polymerase Chain Reaction) amplification step, to introduce specificity and increase the number of molecules for detection (Kim and Misra, 2007).

Products from various allele-discriminating reactions are then analyzed for allelic differences. Major detection methods include mass spectrometry, fluorescence, and chemiluminescence (Kim and Misra, 2007).

Current genomic databases contain information on several million SNPs. More than ten million SNPs have been identified and the information is publicly available through the efforts of the SNP Consortium and other data bases (Hirschhorn and Daly, 2005).

The National Center for Biotechnology Information (NCBI) plays a major role in facilitating the identification and cataloging of SNPs through creation and maintenance of the public SNP database (dbSNP) by the biomedical community worldwide and stimulate many areas of biological research including the identification of the genetic components of diseases (Javed and Mukesh., 2010).

2.5 Cytokines

Cytokines are proteins that are produced by cells. Cytokines interact with cells of the immune system in order to regulate the body's response to disease and infection. Cytokine Cytokines also mediate normal cellular processes in the body (Smith and Humphries, 2008).

Cytokines are diverse, meaning, they are not all alike. The body produces different types of cytokines:

- colony stimulating factors (stimulate production of blood cells)
- growth and differentiation factors (function primarily in development)

- immunoregulatory and proinflammatory cytokines (interferon, interleukins, and TNF-alpha that function in the immune system).

In helminth infections, a number of cytokines that are mainly immunoregulatory and proinflammatory have been implicated.These include cytokines that are known to elicit dominant T-helper-2 (Th-2) responses such as interleukin-5 (IL-5) and interleukin-13(IL-13) [Yazdanbkhsh *et al*., 2001]. A key paradigm in filariasis is that patients with elevated levels of regulatory responses have high parasite numbers and low pathological symptoms whereas patients with few or no parasites and deliberating pathology mount strong filarial-specific responses (Maizel and Lawrence, 1991; Maizel *et al.*, 1999).

Studies in lymphatic filariasis have focused on the immunological differences between patients presenting different degrees of pathology (Nutman and Kumaraswami, 2001). For example, patently-infected individuals with no clinical signs of disease are characterized by down-regulated IL-2 and Interferon gamma responses with a shift towards Th2 (IL-4, IL-5) and T-regulatory cytokine (IL-10 and TGF-ß) responses (Nutman *et al.*, 1987; Dreyer *et al*., 2000; Hoerauf *et al*., 2005; Anuradha *et al*., 2012).

In contrast, patients with chronic pathology display a stronger Th1 immune response (Maizel and Lawrence, 1991; Nutman and Kumaraswami, 2001) or even a Th17 response (Babu *et al*., 2009) which in turn induces the secretion of VEGFs which is associated with the pathology of lymphatic filariasis (Debrah *et al.*, 2006; 2009).

CHAPTER 3

3.0 General Materials and Methods

3.1 Study Communities

The study was undertaken in 43 communities in the Ahanta West and Nzema East Districts in the Western Region of Ghana. These are areas endemic for lymphatic filariasis (Dunyo *et al*., 1996; Debrah *et al*., 2011). The communities were mainly located along the coast and the occupation and lifestyles of the inhabitants expose them to the bites of the vector mosquitoes. For instance the inhabitants sleep along the shores of the coast without bed nets (Plate 4). The inhabitants of the study villages are engaged in either fishing or small scale farming with cassava, maize and vegetables being the main crops. They speak Nzema, Ahanta, and Fanti languages. At the time of the study, most houses in all the 43 communities had mud walls and roofs thatched with dried coconut leaves or bamboo sticks. This made it easier for the vector (*Anopheles* mosquito) to transmit the parasite from an infected individual to an uninfected person. The duration of the study was three years from 2008 to 2011.

- Some of the study communities

Plate 4: Graphical presentation of the study villages**. Source:**

Plate 5: Study participants sleeping at the shore (own souce)

The communities included; Busua, Dixcove, Butre, Asemasa, Asemkow, Ampatano, New Akwadaa, Old Akwadaa, Cape Three Point, Achowa, Kamfakrom, Dzapkasu, Ketakor, Akyenim, Punpunie, Egyam, Enyiwaehu, Funkoe, New Amanful, Aketenchie, Duahorodo, Komanfokrom, Aketechi, Princes Town, Asuboi, Seremou, Mpeasem, Kwasikrom, Ehuntumano, Adjua, Asanta, Sanwoma, Ampain, Miamia, Agyambra, Ainyinasi, Bakanta, Telebokazo, Apataim, Bobrama, Esiama, Azulenuanu.

These communities were selected mainly because,

- The prevalence of the disease in these areas have been determined previously (Dunyo *et al*., 1996; Debrah *et al*., 2006; 2007b; 2011).

- The village health workers were ready to assist in mobilising the participants for drug administration

- The villages were relatively small in size and the populations were relatively stable and hence facilitated drug administration.

3.2 Ethical Clearance

Ethical clearance was obtained from the Nzema East and Ahanta West District Health Administrations by the Department of Clinical Microbiology through the Ethical Committee of the School of Medical Sciences of the Kwame Nkrumah University of Science and Technology, Kumasi, the Ethics Committee of the University of Bonn (Germany) and the University of Liverpool acted as a control body since this work was sponsored by the Bill and Melinda Gates Foundation. The study was registered at current controlled trials (ISRCTN15216778).

Before commencement of data collection, meetings were held in the study communities to explain in detail using the local languages the purpose and procedures of the study. The inconveniences involved, such as repeated night blood sampling was also explained to the participants.

Verbal consent was obtained from community leaders i.e. chiefs and elders of the communities and written informed consent was obtained from all participants. The study was undertaken according to the principles of the Helsinki Declaration of 1964.

3.3 Selection of Study Participants

3.3.1 Selection of study participants for the clinical trial

A cohort of 261 males without any clinical condition requiring long-term medication but suffering from lymphatic filariasis infection were recruited for a clinical trial after screening 1980 male volunteers. Patients who had active infections with living filarial worms detectable by ultrasonography, microscopy or ELISA (enzyme linked immunosorbant assay) were recruited for the clinical trial.

3.3.2 Selection of study participants for the genetics study

In all 1306 volunteers comprising, 266 lymphedema patients and 691 infected patients and 349 endemic normals were recruited for the genetic study. Endemic normals were patients who were not suffering from any of the disease conditions even though they had stayed in the endemic communities for more than ten years. An endemic control was selected when an individual proved negative upon microscopical examination to detect microfilaria, ultrasonographic examination for detection of adult worms in scrotum and spermatic cords and also negative for ELISA for detecting circulating filarial antigen (CFA). Lymphedema patients were selected based on history and different examination procedures (Appendix 1).

3.3.3 Inclusion criteria for the clinical trial;

- Males aged 18-55 years with at least 10MF/ml in the blood (microscopy), or filarial dance sign positive (ultrasonography)
- A minimum body weight of 40 kg
- In good health, and without any chronic clinical condition requiring long term medication

3.3.4 Exclusion criteria

- Patients with other acute or chronic diseases e.g. diabetes, epilepsy, etc.
- Patients with mental disorders
- Patients on regular intake of other drugs
- Patients with hepatic or renal dysfunction

3.3.5 Inclusion criteria for the genetic study

- lymphedema patients of ages 18-70 years
- Volunteers who have stayed in the endemic community for more than 10 years without any disease manifestations (endermic controls)
- Microfilaraemic positive patients of ages 18-55 years

3.4 Field Examinations

3.4.1 Selection of microfilaria positive patients (MF+)

Microfilaria shows a periodical pattern of moving to the peripheral blood vessels at night reaching peak concentrations at midnight that coincide with the local feeding habits of their mosquito vector (Taylor *et al*., 2010) For this reason microfilaria positive patients were identified by microscopic examination of finger prick blood samples taken the night (Dzodzomenyo *et al.,* 1999; Gatika *et al*., 1994; Simonsen *et al*., 1997). About 20µl of blood from the finger was pipetted onto a microscopic slide, covered with a cover slip and microfilarimia was assessed and quantified under 10X objective of the light microscope.

3.4.2 Selection of patients by ultrasonography

Ultrasonography was done in the males to identify patients who had hydrocele as well as patients who had worm nest evident by the filarial dance sign (FDS). For ultrasound examinations a hand carried machine (SonoSite 180 Plus® sonosit inc. Wasghinton USA.) equipped with an L 38 mm, 5-10 MHz linear transducer plus Pulse Wave- and Colour Doppler device was used. The volunteers were examined in a supine position. This position was necessary to avoid interference by movements of the patient themselves. Ultrasound gel was kept at room temperature to reduce artefacts from the cremasteric muscle due to a low temperature stimulus. First the scrotum was scanned in each patient in transverse sections of the right and the left side of the scrotum, followed by longitudinal sections of both sides and a transverse scan of the backside.

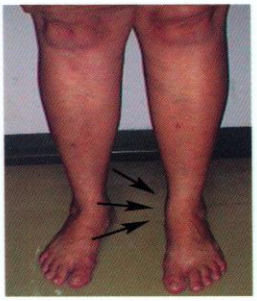
The transducer was positioned in panoramic mode at each section to provide optimal information. Patients were screened for worm nests detectable in lymphatic vessels of the scrotum and spermatic cord.

3.4.3 Selection and staging of lymphedema legs

Patients with edema of the limbs in the filarial endemic communities were subjected to questionnaires which included information on the etiology of the pathology, and the behavoral patterns (Appendix1).

History and physical examination which are acceptable diagnostic testing was undertaken to select patients with lymphedema (National Lymphedema Network, 2011). Patients were then staged from one to seven (Plate 6) as has been described by Mand *et al*., 2008. Patients were then asked to donate 10ml of venous blood which was put into EDTA monovettes and sent to the laboratory for circulating filarial antigen (CFA) test and also for genetic analysis.

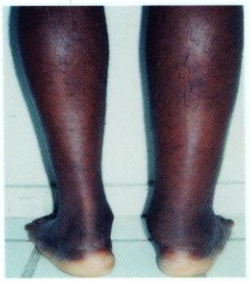
LYMPHOEDEMA STAGING AND MANAGEMENT FOR FILARIASIS–ENDEMIC AREAS



Stage 1

Characteristic feature: Swelling is reversible (goes away) overnight.

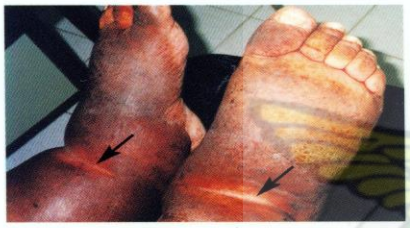
In stage 1 lymphoedema, the swelling increases during the day and goes away overnight when the patient lies flat in bed. To accurately classify lymphoedema in patients with stage 1 disease, it is best to examine the leg in the late afternoon, when the swelling is most visible, and again in early morning, to see that the swelling is gone. If there is swelling in both legs, it may be necessary to rely on the patient's report of normal-size legs in the morning, because comparison with the patient's "normal" leg is not possible.



Stage 2

Characteristic feature: Swelling is not reversible (doesn't go away) overnight.

The main difference between stage 2 lymphoedema and stage 1 is that the swelling does not go away without lymphoedema management. Occasionally, patients with stage 2 lymphoedema will have acute attacks. They also may have entry lesions between the toes, and a mild bad odor.



Stage 3

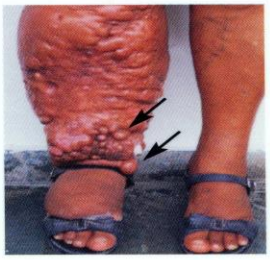
Characteristic feature: Shallow skin folds.

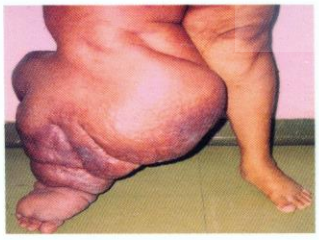
The principal feature of stage 3 lymphoedema is the presence of one or more shallow skin folds. Shallow folds are those in which the base of the fold can be seen when the patient moves the leg or foot so that the fold "opens up". Even very thin lines or creases, which are not seen on normal legs, are considered shallow folds. Early shallow folds are much easier to see when the patient is standing. Thus, it is important to have the patient standing when you are staging the lymphoedema.

Stage 4

Characteristic feature: Knobs.

The main feature of stage 4 lymphoedema is the presence of knobs. Knobs are bumps, lumps, or protrusions of the skin. The importance of knobs comes from the fact that they predispose the leg to further trauma and, therefore, to additional entry lesions, especially if the skin at the site of the knob is less sensitive than the surrounding skin.

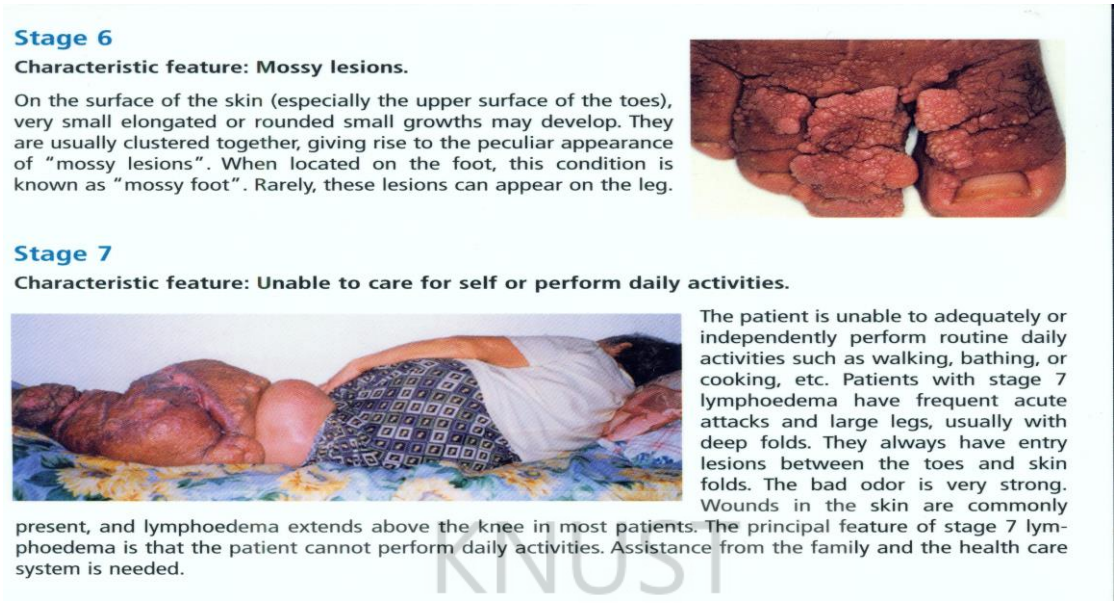




Stage 5

Characteristic feature: Deep skin folds.

The presence of one or more deep skin folds is the main feature of stage 5 lymphoedema. Deep folds are those whose base cannot be seen when the patient moves the leg or foot so that the fold "opens up"; rather, the base of the fold can be seen only when the edges are actively separated by hand.



Stage 6

Characteristic feature: Mossy lesions.

On the surface of the skin (especially the upper surface of the toes), very small elongated or rounded small growths may develop. They are usually clustered together, giving rise to the peculiar appearance of "mossy lesions". When located on the foot, this condition is known as "mossy foot". Rarely, these lesions can appear on the leg.

Stage 7

Characteristic feature: Unable to care for self or perform daily activities.

The patient is unable to adequately or independently perform routine daily activities such as walking, bathing, or cooking, etc. Patients with stage 7 lymphoedema have frequent acute attacks and large legs, usually with deep folds. They always have entry lesions between the toes and skin folds. The bad odor is very strong. Wounds in the skin are commonly present, and lymphoedema extends above the knee in most patients. The principal feature of stage 7 lymphoedema is that the patient cannot perform daily activities. Assistance from the family and the health care system is needed.

Plate 6: Staging of lymphedema patients

Source: Dreyer *et al*., 2002 (Basic Lymphedema Management)

3.5 Laboratory Examinations

3.5.1 Microfilarial count before treatment

Depending on the microfilarial density that was observed in the field (MF >10 =25µl and MF <10= 100µl of blood), 25µl or 100µl of blood was taken into an eppendorf tube and diluted in 900ml of 3% acetic acid to haemolyse the red blood cells. This was then counted in a Sedgewick counting chamber for microfilarial load in each patient (McMahon *et al*., 1979), and expressed as MF/ml of blood.

As confirmation, 100µl or 1000µl (if the sample had very low Sedgewick count) blood was diluted in aqua bidest and filtered using 25µm filters (Whatman's nucleopore, Kent, UK). Filters were then fixed in methanol and dried over night on a glass slide. These filters were then stained with Giemsa in a dilution of 1:20 buffer (2.8g Na_2HP04 and 1.19g K_2HP04) for 30 minutes and the filters were counted for MF load.

3.5.2 Leucocyte counts

Leucocyte counts were performed to assess the level of leucocytes in the study participants since rifampicin is known to cause some side effects such as leucopenia.

A dilution of 1/20 of the blood with 2% acetic acid solution stained with 1ml Methylene blue was prepared and placed in a test tube. The solution was gently mixed and then left for 2 minutes for complete haemolysis. Coverslip was fixed on Neubaeur counting chamber and the blood- diluents suspension was gently mixed for a few seconds.

The counting chamber was then filled using capillary tube and left for some few minutes for the cells to settle. The preparation was then viewed under the microscope at 10x objective. The cells were counted in the 4 corner squares and the calculations were done using the formula below;

$$\text{WBC count (per litre)} = \frac{\text{cells counted x dilution x } 10^6}{\text{area counted x depth of fluid}}$$

Where; Dilution = 20

Area counted = 4 x 1 sqmm

Depth of fluid = 0.1mm (constant)

Factor = 10^6

3.5.3 ELISA (Enzyme linked immunosorbant assay)

This laboratory test was also done as one of the parameters to select endermic controls (Volunteers who have stayed in the endemic community for more than 10years without any disease manifestations).

Circulation filarial antigen (CFA) was measured with the TropBio® ELISA test kit (TropBio, Townsville, Australia). About 50µl of plasma was diluted in 150µl of sample diluents and the mixture was added to a pre-coated plate, and the plates were incubated overnight. The optical density at 414 nm was then measured. Antigen units were determined using a standard curve from standards provided by the manufacturer (Turner *et al.*, 2006).

3.5.4 DNA extraction from tissue (MF)

A volume of blood containing 500 or 1000 MF was diluted in agua bidest, filtered and preserved in liquid nitrogen (-140^{o}C) for PCR analysis to determine the degree of *Wolbachia* depletion in each regimen.

DNA was extracted with commercial kit from Qiagen (Hidden, Germany). 20µl proteinase K (20mg/ml) together with 180µl Lysis buffer (ATL) were added to digest the tissues. The mixture was vortexed and incubated at 56^{o}C overnight in a rocking platform of a thermomixer. A second mixing was done for 15 seconds and 200µl buffer (AL) was added to the sample and then it was mixed by pulse vortexing for 15seconds. The mixture was then incubated at 70^{0}C for 10 minutes. 200µl of ethanol (96-100%) was added to the mixture and it was mixed thoroughly by vortexing to yield a homogenous solution. The mixture was then pipetted into the DNeasy spin column placed in a 2ml collection tube and it was centrifuged for 1minutes at 8000rpm.The flow through was then discarded and the DNeasy spin column containing the DNA was placed on another 2ml collection tube.

It was washed first with the 500µl AW1 wash buffer and centrifuged for 1 minutes at 8000rpm.The flow through was discarded and a second wash with 500µl AW2 buffer was done. Centrifugation at full speed for 3minutes to dry the DNeasy membrane was done to ensure that no residual ethanol was carried over during the following elution. The spin column was carefully removed to prevent contact with flow through and placed in 2ml microcentrufuge tube with 200µl AE buffer directly pipetted onto the Dneasy membrane. It was then incubated at room temperature for 1 minutes and then centrifuged for 1minutes

at 8000rpm to elute. The elution step was repeated once more. During DNA preparations standard precautions such as the use of safety pipette tips and performing DNA and PCR preparations in separate rooms to prevent contamination were applied.

3.5.5 DNA extraction from blood (genetics protocol)

Equal volumes of blood in 8M urea was prepared and in the end a 10ml volume was obtained for the extraction of DNA for genetic analysis. The Chemagen platform (Chemagen Biopolymer-Technologies AG, Baesweiler, Germany) was used for DNA extraction. The chemagic magnetic separation module 1 which is an automated system with two main protocols were used and these are:

A – Lysate mixing for chemagic DNA blood 10k v050624.txt

B – Chemagic DNA blood 10k V050624.txt was used in the extraction process.

In preparing the blood lysate a 50µl protease was added to the 10ml of blood and 8ml lysis buffer 1 was added. The mixer was placed on a racking system and programme A was selected. In this system there was mixing and lysing for 20 minutes. 28ml of binding buffer as well as magnetic beads were added to the lysate .

Tubes prefilled with wash buffers as well as elution buffer were also placed on the racking system and programme B was selected. The DNA was bounded to the beads and the unwanted materials were washed off using the wash buffers. Purified DNA was eluted using the elution buffer.

3.5.6 PCR analysis

PCR analysis was partly done at the Kumasi Centre for Collaborative Research into Tropical Medicine (KCCR) in Kumasi and the other part was done in the Laboratory of the Institute of Medical Microbiology Immunology and Parasitology in Bonn University, Germany, to assess *Wolbachia* depletion in the various treatment regimens, at various time points.

Genotyping was done using the MassARRAY (Sequenom, San Diego, USA) platform for SNP genotyping, from 64 genes, to determine whether there was association of certain SNPs in lymphedema development (See Section 5.3 of Genotyping materials and methods for details).

3.5.6.1 Assessment of *Wolbachia* levels in microfilariae by PCR.

Wolbachia content was quantified by real-time PCR of the *W. bancrofti Wolbachia–ftsZ* gene (NCBI Accession number AF081198) derived from 500-1000 microfilariae using a RotorGene™ 6000 (Corbett Research, Inc., Sydney, Australia) at pre-treatment and 4 months after treatment.

For quantification, primers and a Taqman hybridization probe with the fluorescent dye 6-FAM (6-carboxyfluorescein; Qiagen) were used to amplify a 286- bp fragment of the *W. bancrofti Wolbachia* - ftsZ (accession number AF081198). Primers used were as follows;

FtsZ_Forward: 5′-GCTTGGGCTTGAAGAATTACAAA-3′

FtsZ_Reverse: 5′- GCTATCAGTGCTGCAGAAGC-3′ and

FtsZ_hybridization: 5′-ACGTAGACACGCTTATTGTCATTCCAAACC A-3′.

Real time PCR amplifications were performed using Hotstar *Taq* DNA polymerase, with a half-life of 10 minutes at 97^{o}C according to the manufacturer. With a reaction volume of 10 µl, the PCR mix contained 1µl of 10X PCR buffer (10 mM TRIS-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X–100), 1.2µl of 25mM $MgCl_2$, 0.05µl of 40mM deoxynucleotide triphosphates (dNTPs) mixture, 0.3µl of 10 pmoles of forward primer, 0.9µl of 10 pmoles of reverse primer, 0.1µl of 5µm hybridization probe, 2µl of DNA template solution, 0.05µl of Hotstar *Taq* polymerase, and 4.4µl of double distilled water.

The thermocycling programme used for the *Wolbachia* ftsZ was 10 seconds of denaturation at 95^{o}C, 15 seconds of annealing at 60^{o}C and 20 seconds of extension at 72^{o}C for a total of 45 cycles. A signal was acquired on the FAM channel and the products were quantified by comparing with a standard primers amplified at 256bp fragment of the *Wolbachia-ftsZ* gene and a TaqMan probe (Operon, Cologne, Germany) for the same sequence..

CHAPTER 4

4.0 Clinical Trial

4.1 Introduction

Wuchereria bancrofti, together with *Brugia malayi* and *Brugia timori* are causative agents for lymphatic filariasis. These organisms induce alteration of the lymphatic vessels, both vessel dilatation and obstruction with subsequent development of pathology such as lymphedema and hydrocele. It is a major cause of morbidity and it hinders socio-economic development in parts of Africa, Asia, and the western Pacific (WHO, 1992; Ottesen & Ramachandran, 1995). The main interventional measure recommended for the control of lymphatic filariasis is mass drug administration (MDA) of endemic human populations to interrupt transmission, with either a combination of diethylcabamazine (DEC) and albendazole, or in Africa, ivermectin and albendazole, for a period of four to six years (Ottesen, 2000; Molyneux and Zagania, 2002).

These drugs have been used for the past two decades as major mode of intervention for filarial infection in successful mass drug administration (MDA) programmes (WHO, 2007a; WHO 2007b; WHO, 2010). However, they have been found to be mainly microfilaricidal, thus killing of the baby worm, (ivermectin and albendazole), and the macrofilaricidal activity shown by DEC is only partial. Great concern has also been raised with regard to the development of drug resistance against the existing drugs.

Already, sub-optimal efficacy of ivermectin and/or ivermectin resistance in humans has been reported in onchocerciasis patients in Ghana where despite multiple treatments with ivermectin, microfilaridermia persisted in some patients (Awadzi *et al.,* 2004a; Awadzi *et al.,* 2004b). This was later confirmed by Osei-Atwebeboana *et al.,* (2007).

The pathological developments of LF such as lymphedema and hydrocele are caused by the adult worms (Taylor *et al*., 2010) and as such drugs that are directed towards the elimination of the adult worm would be most preferred. Research is on going in many areas to discover antifilarial drugs with strong macrofilaricidal activity (Hoerauf *et al*., 2011).

The discovery of *Wolbachia* over 30 years ago in most filarial nematodes [Order Rickettsiales] **(**Kozek, 1977) has given a different dimension to the treatment of filarial diseases. These endosymbiotic bacteria are found in the hypodermis of male and female worms, in the oocytes, embryos, and also larval stages (Kozek, 1977). As in many animal filarial species, endobacteria are present in human filariae including *W. bancrofti, Brugia* species, and *O. volvulus* (Hoerauf *et al.,* 2000; Hoerauf *et al.,* 2001; Molyneux *et al.,* 2003), with the exception of a few species such as *Loa loa* (Buettner *et al.,* 2003; McGarry *et al.,* 2003).

The discovery of the essential role of the endosymbiotic *Wolbachia* in worm fertility and survival has resulted in the development of an anti-filarial chemotherapy with doxycycline; an antibiotic which is capable of depleting *Wolbachia* (Taylor and Hoerauf, 1999). The depletion of the *Wolbachia* from the worm results in a long term sterilizing effect, both in animal models (Hoerauf *et al.*, 2000; Taylor *et al.,* 2001; Langworthy *et al.,*

2000; Gilbert *et al.,* 2005) and in humans (Hoerauf *et al.,* 2000; 2001; 2003c; Debrah *et al.,* 2006; 2007a; 2009). The high macrofilaricidal effect which is a better efficacy than the Mass Drug Administration (MDA) combination used in Africa (ivermectin and alberndazole) was also shown with doxycycline when it was used for four, six and eight weeks with 200mg daily dose (Debrah *et al*., 2006; 2007a; Taylor *et al*., 2005). However, these durations were considered too long and that a shorter duration would be advantageous for the treatment of the individual, e.g. in outpatient clinics.

Wolbachia have also been known to be activators of innate immunity that can end up among others in induction of pro-inflammatory cytokines such as Il-6 , INFγ and also members of the vascular endothelial growth factors (VEGF) family (Brattig *et al.,* 2000; Taylor *et al.,* 2000b). In addition to the possible role of *Wolbachia* as a chemotherapy target as enumerated above, evidence suggests also that *Wolbachia* antigens can stimulate host immune responses that may be associated with development and progression of pathogenesis of filarial disease (Saint Andre *et al.,* 2002; Hise *et al.*, 2004; Taylor, 2003) indicating the importance of *Wolbachia* in this pathogenesis.

A three-week course of doxycycline followed by a single dose of ivermectin and albendazole treatment has been shown to reduce *Wolbachia* single gene copies per microfilaria by 86% and also sustained amicrofilaremia for more than one year (Turner *et al.,* 2006). However, this treatment did not show a macrofilaricidal activity. Nevertheless, three weeks doxycycline in combination with a single dose of DEC did show a macrofilaricidal effect (Mand *et al*., 2009), demonstrating that different combinations with

doxycycline could lead to a shorter regimen with macrofilaricidal effect. This then calls for different drugs to assess the macrofilaricidal effect. A pilot work that was recently undertaken by Debrah *et al* (2011) indicated that combination of two weeks doxycycline with rifampicin results in a partial macrofilaricidal effect. This present study therefore sought to confirm the pilot work by Debrah and others and secondly to identify the actual effective regimen using doxycycline and rifampicin to achieve macrofilaricidal activity in the shortest possible time.

4.1.1 Aim of the study

To determine an effective regimen of doxycycline alone, or / and combination of doxycycline and rifampicin for the treatment of lymphatic filariasis

4.1.2 Specific objectives

The specific objectives of the present study were to;

1. Define the minimum and ideal regimen of doxycycline in combination with rifampicin treatment needed to achieve macrofilaricidal effect in lymphatic filariasis.
2. Define the minimum and ideal regimen of doxycycline in combination with rifampicin treatment needed to achieve the depletion of *Wolbachia* and complete sterilization of adult female worms in the shortest possible time in lymphatic filariasis.

3. To assess whether or not 100mg of doxycycline (which can be taken without any liver and kidney function test) would have a treatment option equal or better than the 200mg used in previous studies.

4.2 Materials and Methods

4.2.1 Overall design

The study was a randomized, placebo-controlled and double blind trial (Phase 2a) using doxycycline either 200mg or 100mg alone or in combination with rifampicin (10mg/kg) for treatment. In all, 261 men took part in the double-blinded study. Men only were selected for this study because the primary aim of the study was to assess the macrofilaricidal activity of a particular treatment regimen using USG on the scrotum for detection of filarial dance sign (FDS). The study included 143 patients taking doxycycline and 78 patients taking combination of doxycycline and rifampicin and 40 patients taking placebo. Four months after the start of treatment all the patients were treated with ivermectin and albendazole in accordance with the national mass administration programme which was underway in the study area. Each patient received a single dose of 150 μg/kg of ivermectin and 400mg of albendazole.

The participants were randomly assigned to one of the six different treatment groups, as shown below in section 4.2.6. The grouping of the patients was performed by random allocation of numbers one to six to the study individuals.

The consignments of drugs were sent to Bonn University Clinic at the Institute of Microbiology Immunology and Parasitology (IMMP), where they were coded by a scientist who was not part of the study team. Coding was carried out independently for each group and sealed copies of the codes were kept in Liverpool School of Tropical Medicine until the end of the study when they were decoded.

The study procedure and the symptoms of the potential side effects of doxycycline and rifampicin were explained to the participants. The patients were also asked to report any side effects experienced in the course of the treatment period. Informed consent was also given by each participant who either signed or thumb printed consent forms to take part in the study. All those taking part in the study were informed that they could drop out of the study at any time they wanted.

Treatment was directly observed (DOT). Daily tablet intake was supervised by a trial clinician because of possible side effects.

4.2.2 Clinical chemistry text

Since doxycycline and rifampicin are both eliminated from the body through the kidney and the liver (Sande and Mandel, 1992), clinical chemistry tests were done to assess the state of the patient's kidney and liver, using stick-technology clinical chemistry test by the Reflotron® system (Boehringer Mannheim,Germany, now Roche).

About 1 ml of blood was centrifuged at a speed of 1000rpm for 10min to separate the plasma from the cells. About 200µl of plasma was taken from each patient into a 1.8ml Nunc® cryotube bearing the code number of the patient.

About 30µl plasma was pipetted from the monovette onto the various Reflotron® test strips using a Reflotron® pipette according to the protocol of the manufacturer (Boehringer Mannheim, UK Ltd.).

These tests were done at the Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR) field laboratory at Dixcove. Parameters measured by the stick-technology were glutamat-pyrivil-transaminase (GPT), gamma-glutamyl-transpeptidase (γ-GT) and creatinine. All patients who had results above the recommended levels i.e. GPT>100µl, γ-GT>50µl and creatinine>1.2mg/100µl were excluded from the study.

4.2.3 Sample collection

Blood samples were collected between 9pm and 12 midnight from all patients included in the study. About 10ml of venous night blood was taken from each eligible patient for accurate quantification of MF as described by Hoerauf *et al*, (2003b). The same volume of blood was taken from each patient at 4, 12, 18 and 24 months post treatment.

4.2.4 Power calculation

The group sizes were obtained by power calculations based on the distribution of MF in the pilot study group (Hoerauf *et al*., 2003b) and its reduction by more than 50% after 4 months (t-test of log-values of MF): reduction of MF by 42% -> power 80%; reduction 50% -> power 93%; reduction 75% -> power 99% (all values with alpha = 0.05). Recruitment of individuals to study groups took into account a 15% dropout rate

throughout the trial period. StatView®, Version 5 for Mac OS 9.5 statistical softwares were used for the calcultions.

4.2.5 Drug administration

In all 261 patients were selected for the clinical trial. Six treatment arms were used as follows;

1. Treatment regimen:

   4 weeks doxycycline 200mg plus

   1week placebo matching doxycycline plus

   3 weeks placebo matching rifampicin

2. Treatment regimen:

   5 weeks doxycycline 100 mg plus

   5 weeks placebo matching Doxycycline (one additional capsule/day) plus

   3 weeks Placebo matching Rifampicin

3. Treatment regimen:

   4 weeks doxycycline 100 mg plus 1 week placebo matching doxycycline plus

   5 weeks placebo matching Doxycycline (one additional capsule/day and 2 additional capsule/day the last week of treatment) plus

   3 weeks placebo matching rifampicin

4. Treatment regimen:

   3 weeks doxycycline 200 mg plus

   2 weeks placebo matching Doxycycline plus

   3 weeks rifampicin

5. Treatment regimen:

   2 weeks doxycycline 200 mg plus

   3 weeks placebo matching Doxycycline

   2 weeks rifampicin plus 1 week placebo matching rifampicin

6. Treatment regimen:

   5 weeks placebo matching Doxycycline plus

   3 weeks placebo matching rifampicin

4.2.6 Laboratory examination

4.2.6.1 Microfilarial count before treatment.

For the determination of MF numbers in patients before treatment, please see section 3.5.1 under General Materials and Methods.

4.2.6.2 PCR analysis (refer to General Materials and Methods)

4.2.6.3 Circulating filarial antigen (refer to General Materials and Methods)

4.2.6.4 Microfilarial count after treatment

Night blood samples were again collected from the participants at 4 months, 12 months, 18 months and 24 months after treatment and examined for MF as before.

4.2.6.5 Ultrasonography (USG)

Ultrasound (USG) examination was carried out by Dr. Sabine Mand as detailed in Mand *et al*, (2003). In brief, a portable ultrasound machine, SonoSite 180 Plus (SonoSite, Washington, USA) equipped with an L-38mm 5-10 MHZ (7.5 MHZ for scrotal USG) linear array transducer was used. The men were examined in a supine position in order to reduce interference by movements of the patients themselves. In each patient the scrotum was first scanned in transverse and longitudinal sections of the right and the left side. The transducer was positioned in panorama-mode at each section to provide more information and better localization of the worm nests.

The examiner took print outs and digital video sequences of the worm nests by keeping the transducer in at a 90^{o} angle to the skin surface in transverse and longitudinal sections.

Every worm nest detected by the typical movements of the adult worms (called filarial dance sign: FDS) was recorded as the presence of lymphatic filarial adult worm. Colour Power Doppler (CPD) and Pulse Wave Doppler (PWD) imaging were used as additional tools to differentiate the filarial dance sign of adult worms in lymphatic vessels from blood flow in veins and arteries.

4.2.7 Data analysis

Differences in the adverse effects of the treated group in comparison with the placebo groups were assessed using the Fischer's exact test. Microfilaraemia loads and *Wolbachia* loads were summarized as geometric means (GM). Summary of the presence of the worm nest detectable by the filarial dance sign (FDS) was done using the mean. Differences in median at baseline and four months follow-up were analyzed using Wilcoxon Signed Rank test of raw data in the *Wolbachia* loads assessment. Proportions of MF positive and negative individuals as well as individuals showing filarial dance signs (FDS) before and after treatment were compared using Fisher's exact tests. Two-tailed p-values lower than 0.05 were considered significant.

Independence of data was tested using multivariate analysis. Analyses were done using StatView®, Version 5 for Mac OS 9.5 and IBM SPSS statistics software version 19 for Windows.

4.3 Results

4.3.1 Participation of patients at follow-up examinations

A total of 345 patients were examined for eligibility for the study. Out of this number, 261 were randomized into the placebo controlled double blind randomized study. The drug administration for the volunteers in the study was as follows:

i) 65 patients took 4 weeks of 200mg doxycycline with 3 weeks placebo matching rifampicin

ii) 39 patients took 5 weeks of 100mg doxycycline with 3 weeks placebo matching rifampicin

iii) 39 patients took 4 weeks of 100mg doxycycline with 3 weeks placebo matching rifampicin

iv) 39 patients took 3 weeks of 200mg doxycycline with 3 weeks rifampicin

v) 39 patients took 2 weeks of 200mg doxycycline with 2 weeks rifampicin

vi) 40 patients took 5 weeks of placebo matching doxycycline with 3 weeks placebo matching rifampicin

Of the 261 patients, 25 representing 9.57% could not complete the full course of the treatment due to the nature of their work as farmers and fishermen and they reported that the time of treatment was not favorable for them thus leaving 236 (90.42%) to complete the treatment. The various distributions of those who could not complete the treatment against those who completed the treatment in the various treatment groups have been represented in Table 1.

Table 1: Patients who completed treatment/could not complete treatment

	Completed Treatment	Not completed Treatment	Total
4 weeks 200mg doxycycline	62	3	65
5 weeks 100mg Doxycycline	37	2	39
4 weeks 100mg Doxycycline	34	5	39
3 weeks 200mg Doxycycline and 3 weeks Rifampicin	35	4	39
2 weeks 200mg Doxycycline and 2 weeks Rifampicin	34	5	39
5 weeks placebo	34	6	40
TOTAL	**236**	**25**	**261**

4.3.2 Demographic data of patients

The distribution of age and weight of all the treated patients were comparable in all the various treatment groups (Table 2). The mean age of the patients in the various treatment regimen ranged between 32 and 38 years. The age ranges were however between 18 and 50 years as stipulated in the study protocol. The mean weight of the patients was between 55kg and 62kg as has been represented in Table 2 below.

Table 2: Weight and age distributions of patients

	4wks 200mg doxy	5wks 100mg doxy	4wks 100mg doxy	3wks 200mg doxy plus rifa	2wks 200mg doxy plus rifa	placebo
Mean weight in kg (Range)	61 (49-75)	58 (48-70)	58 (46-69)	62 (54-69)	55 (41-68)	60 (50-70)
Mean age in years (Range)	33 (18-47)	38 (23-49)	36 (22-48)	33 (19-46)	37 (27-48)	32 (18-50)

Wks= weeks

Doxy= Doxycycline

Rifa= rifampicin

4.3.3 Pretreatment parameters of patients

The geometric mean of pre-treatment microfilarial loads of the microfilaremic patients ranged from 135 to 248 MF/ml. The mean pre-treatment MF loads did not differ between the treated groups in the various treatment regimens (P=0.797) as indicated in Table 3. The geometric mean load of *Wolbachia* per MF was between 17 and 68, and there was no significant difference in the mean *Wolbachial* load at pretreatment among all the treatment groups (p=0.253).

The presence of the worm nest among the various treatment groups was compared at pretreatment. Patients who were worm nest positive in the various groups ranged from 34 to 62 with an average worm nest of 1.6. The difference in the number of worm nests in the various group were not significant at pretreatment (p=0.113). The detail is shown in Table 3.

Table 3: Pretreatment patient parameters

	4 weeks 200mg doxy	5 weeks 100mg doxy	4 weeks 100mg doxy	3 weeks doxy 200mg plus rifa	2 weeks 200mg doxy plus rifa	placebo	P-value
GM mean MF/ml	166	248	135	138	140	148	0.797
Patients examined/MF positives	62/37	37/20	34/22	35/21	34/20	34/21	
Percentage	60%	54%	65%	60%	59%	62%	
GM mean *Wolbachia* loads/MF	68	33	28	65	50	17	0.253
Patients examined/*Wolbachia* positives	62/30	37/20	34/16	35/19	34/16	34/18	
Percentage	48%	54%	47%	54%	47%	53%	
Mean FDS	1.5	1.6	1.4	2.0	1.6	1.7	0.113
Patients examined/FDS positives	62/62	37/37	34/34	35/35	34/34	34/34	
Percentage	100%	100%	100%	100%	100%	100%	

Doxy= Doxycycline

Rifa=rifampicin

4.3.4 Adverse reactions to the trial medications

Patients were assessed for adverse effects after treatment and the results are reported in Tables 4 to 8. The most frequent side effects were discoloration of urine and headache. However, this discoloration of urine also occurred among some patients in other treatment groups where there was no rifampicin administration. The incidence of side effects was not dependent on age. In all the treated groups there were no serious side effects observed in any of the patients.

4.3.4.1 Four weeks 200mg doxycycline / Placebo treatment

From Table 4, 53 out of the 62 patients who completed the 4 weeks 200mg doxycycline treatment experienced side effects, with incidence of 85.5% compared to the placebo (47.1%). Headache was the highest among the treated group with 9 patients compared to 3 patients among the placebo group. Even though the difference between patients who experienced side effects were significantly higher in the treated group than the placebo (p=0.0001) group, no serious side effects were observed. Only one person experienced discolouration of urine for about two weeks in the treated group.

Table 4: Side effects experienced during 4 weeks of 200mg doxycycline / Placebo treatment.

	Treated (N=62)		**Placebo (N=34)**		**p-value**
Side effect	**No. of patients**	**Duration (day)**	**No. of patients**	**Duration (day)**	
Discolouration of urine	4	17	-	-	
Headache	9	5	3	1	
Fever	3	2	2	1	
Nausea	1	1	-	-	
Dizziness	3	1	-	-	
Vomiting	3	1	-	-	
Stomach pain	1	1	-	1	
Abdominal pain	4	1	2	1	
Diarrhoea	4	5	3	2	
Scrotal pain	3	1	-	-	
Itching	6	5	2	3	
Rashes	3	3	-	-	
Chest pain	2	1	-	-	
Body pain	1	1	2	1	
Waist pain	4	6	-	-	
Joint pain & muscle	-	-	1	3	
Blurred vision	1	5	-	-	
Common cold	1	1	1	3	
Total no of patients	**53**		**16**		**0.0001***

*Fischer's exact test

4.3.4.2 : Five weeks 100mg doxycycline / Placebo treatment

From Table 5, when the side effects that occurred in the treated group were compared with the placebo group, the difference was not significant (p=0.056). With the exception of discoloration of urine and waist pains which lasted for 16 and 14 days respectively, all the other side effects did not last for more than one week. No serious side effects were observed in all the patients in this treatment group.

Table 5: Side effects experienced during 5 weeks of 100mg doxycycline /Placebo treatment

	Treated (N=37)		**Placebo (N=34)**		**P-value**
Side effect	**No. of patients**	**Duration/day**	**No. of patients**	**Duration/day**	
Discolouration of urine	3	16	-	-	
Headache	1	1	3	1	
Fever	1	1	2	1	
Nausea	2	5	-	-	
Dizziness	1	1	-	-	
Vomiting	-	-	-	-	
Stomach pain	1	3	-	-	
Abdominal pain	2	2	2	1	
Diarrhoea	3	2	3	2	
Scrotal pain	2	1	-	-	
Itching	1	5	2	3	
Rashes	-	-	-	-	
Chest pain	1	1	-	-	
Body pain	-	-	2	1	
Waist pain	2	14	-	-	
Joint pain	5	7	1	3	
Blurred vision	-	-	-	-	
Common cold	1	1	1	3	
Total no of patients	**26**		**16**		***0.056**

*Fischer's exact test

4.3.4.3 : Four weeks 100mg doxycycline / Placebo treatment

Even though the incidence of side effects was high among the treated group (76% of 36 patients), compared to the placebo group (47% of 36 patients), no serious side effects occurred among any of the patients. The longest duration among the treated patients occurred in one person who experienced abdominal pain for 9 days. The remaining side effects did not go beyond one week (Table 6).

Table 6: Side effects experienced during 4 weeks of 100mg doxycycline /Placebo treatment

	Treated (N=34)		**Placebo (N=34)**		**p-value**
Side effect	**No. of patients**	**Duration (days)**	**No. of patients**	**Duration (days)**	
Discolouration of urine	1	1	-	-	
Headache	2	4	3	1	
Fever	0	0	2	1	
Nausea	0	0	-	-	
Dizziness	0	0	-	-	
Vomiting	0	0	-	-	
Stomach pain	1	1	-	-	
Abdominal pain	1	9	2	1	
Diarrhoea	2	1	3	2	
Scrotal pain	2	4	-	-	
Itching	3	7	2	3	
Rashes	1	1	-	-	
Chest pain	2	1	-	-	
Body pain	-	-	2	1	
Waist pain	5	3	-	-	
Joint pain	2	4	1	3	
Blurred vision	3	1	-	-	
Common cold	1	1	1	3	
Total no of patients	**26**		**16**		***0.0023**

*Fischer's exact test

4.3.4.4 : Three weeks 200mg doxycycline plus rifampicin / Placebo treatment

Side effects that occurred in patients treated for two weeks with combination of 200mg doxycycline and rifampicin was not significant when compared with patients who took placebo (p=0.148).The highest duration of a side effect was in urine discoloration among the treated group. Nonetheless there was no occurrence of this side effect among the placebo patients. All the other side effects were the same between the placebo and the treated groups (Table 7).

Table 7: Side effects during 3 weeks of 200mg doxycycline plus rifampicin /Placebo treatment

	Treated (N=35)		**Placebo (N=34)**		**p-value**
Side effect	**No. of patients**	**Duration (days)**	**No. of patients**	**Duration (days)**	
Discoloration of urine	6	14	-	-	
Headache	1	3	3	1	
Fever	-	-	2	1	
Nausea	1	7	-	-	
Dizziness	1	2	-	-	
Vomiting	-	-	-	-	
Stomach pain	1	1	-	-	
Abdominal pain	2	4	2	1	
Diarrhoea	3	1	3	2	
Scrotal pain	1	4	-	-	
Itching	1	5	2	3	
Rashes	1	5	-	-	
Chest pain	-	-	-	-	
Body pain	-	-	2	1	
Waist pain	2	4	-	-	
Joint pain	2	12	1	3	
Blurred vision	-	-	-	-	
Common cold	1	2	1	3	
Total no of patients	**23**		**16**		***0.148**

*Fischer's exact test

4.3.4.5 : Two weeks 200mg doxycycline plus rifampicin / Placebo treatment

From Table 8 below, there was significant difference between patients who experienced adverse effects in the treated group compared with the placebo group (p=0.0112). However the adverse effects were not serious. Apart from urine discoloration and itching which lasted for more than 10 days, the remaining side effects did not go beyond one week in both the treated and the placebo groups.

Table 8: Side effects during 2 weeks of 200mg doxycycline plus rifampicin /Placebo treatment

	Treated (N=34)		Placebo (N=34)		p-value
Side effect	No. of patients	Duration (day)	No. of patients	Duration (day)	
Discoloration of urine	9	16	-	-	
Headache	2	3	3	1	
Fever	1	1	2	1	
Nausea	1	1	-	-	
Dizziness	-	-	-	-	
Vomiting	1	1	-	-	
Stomach pain	3	1	-	-	
Abdominal pain	1	1	2	1	
Diarrhoea	1	3	3	2	
Scrotal pain	-	-	-	-	
Itching	2	12	2	3	
Rashes	1	1	-	-	
Chest pain	1	3	-	-	
Body pain	-	-	2	1	
Waist pain	2	2	-	-	
Joint pain	2	7	1	3	
Blurred vision	-	-	-	-	
Common cold	-	-	1	3	
Total no of patients	**27**		**16**		**0.0112***

*Fischer's exact test

4.3.5 Assessment of anti-*Wolbachia* effect: Quantification of *Wolbachia* load in microfilariae

Quantitative PCR was done before treatment and 4 months after treatment to assess the degree of *Wolbachia* depletion by various treatment regimens. It is the primary parameter to assess the long term sterilizing effect of the filarial worm. *Wolbachia* depletion was measured when anti-filarial therapy (ivermectin and albendazole) had not been administered to the patients (Fig1 and 2).

4.3.5.1 *Wolbachia* depletion across the various treatment groups

At 4 months time point, there was significant depletion of *Wolbachia* in most of the treatment groups notably the standard ($p=0.001$), 5 weeks 100mg doxycycline ($p=0.019$), 4 weeks 100mg doxycycline ($p=0.03$) and 3 weeks combination treatment ($p=0.028$). However there was no significant *Wolbachia* depletion in the 2 weeks combination group as well as the placebo group ($p>0.05$) [Fig. 1].

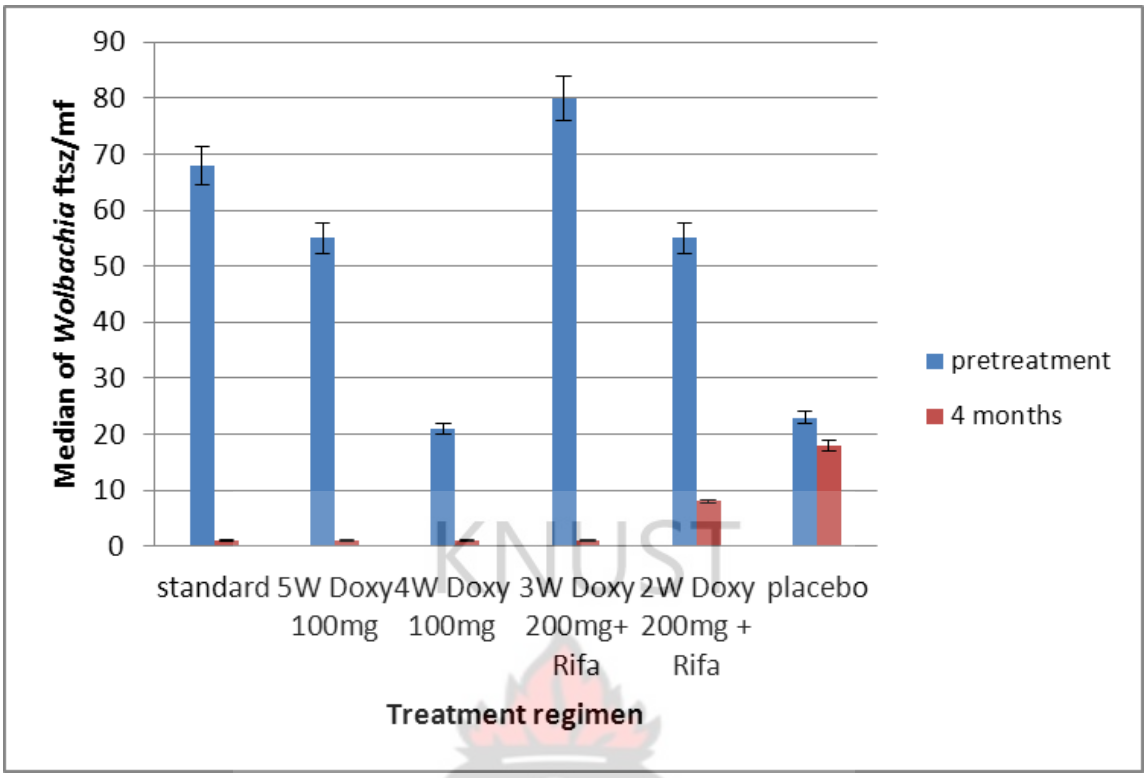


Figure 1: *Wolbachia* load/MF among the various treatment regimens

4.3.4.4 Summary of *Wolbachia* depletion by doxycycline

All the treatment regimens were effective in depleting *Wolbachia* from MF of *W. bancrofti* infected patients to a significant level. However although the 2 weeks group experienced 85% depletion it was not significant (p=0.345) and the placebo group also had only 22% reduction (p=0.859). The percentage *Wolbachia* depletion is shown in Figure 2 below.

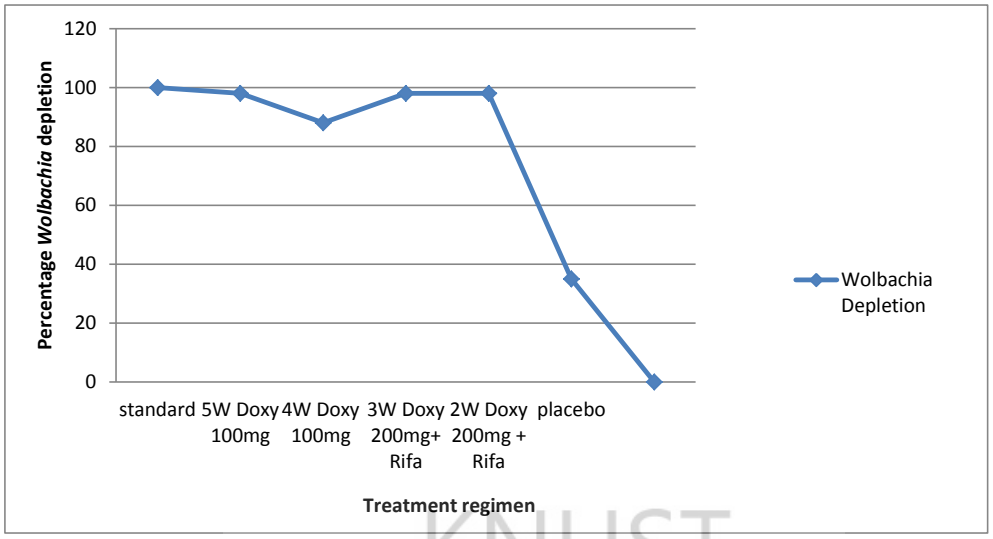


Figure 2: Percentage of *Wolbachia* depletion assessed at 4 months after treatment

4.3.6 Assessment of long term sterilizing effect (absence of microfilaria)

As a secondary parameter to assess the long term sterilizing effect, the absence or presence of microfilaria was compared at pretreatment, 4 months, 12 months, 18 months and 24 months between the treated groups and the placebo group (Tables 9 to 13). All the patients were given ivermectin and albendazole after the 4 months re-examination which is supposed to kill all the microfilariae. Only microfilarial positive patients at pre-treatment were involved in this analysis.

4.3.6.1 Microfilarial depletion in the 4 weeks 200mg doxycycline group (Gold standard)

When the number of microfilarial positive patients were compared between the treated and placebo groups at pretreatment, there was no significant difference. There was also no significant difference at four months follow up between the two groups (P = 0.1282). However, the difference became significant at 12 months (<0.0001) with 100% microfilarial depletion in the treated group but only 32% depletion in the placebo group. At 18 and 24 months follow ups, the depletion of microfilarial load in the treated group reduced to 90% and 80% respectively. But there was still a significant difference when compared with the placebo, which had 32% and 33% microfilarial depletion respectively (Table 9).

Table 9: Comparison of microfilarial depletion between standard and placebo group

Before treatment	MF positive	MF negative	Total	P-value*
4 weeks 200mg doxycycline	37 (100%)	0 (0%)	37	>0.9999
Placebo	21 (100%)	0 (0%)	21	
4 months after treatment	MF positive	MF negative	Total	P-value*
4 weeks 200mg doxycycline	23 (66%)	12 (34%)	35	0.1282
Placebo	18 (86%)	3 (14%)	21	
12 months after treatment	MF positive	MF negative	Total	P-value*
4 weeks 200mg doxycycline	0 (0%)	35 (100%)	35	<0.0001
Placebo	13 (68%)	6 (32%)	19	
18 months after treatment	MF positive	MF negative	Total	P-value*
4 weeks 200mg doxycycline	3 (10%)	27 (90%)	30	<0.0001
Placebo	13 (68%)	6 (32%)	19	
24 months after treatment	MF positive	MF negative	Total	P-value*
4 weeks 200mg doxycycline	5 (20%)	20 (80%)	25	0.0039
Placebo	12 (67%)	6 (33%)	18	

*=Fischer's exact test

4.3.6.2 Microfilarial depletion in the 5 weeks 100mg doxycycline group

As indicated in Table 10, when comparison between 5 weeks 100mg doxycycline treated group and placebo group was done for patients with MF, there was no significant difference at pretreatment ($p>0.9999$) and at four months follow up time points ($p>0.6889$). However, significant difference was observed at 12 months ($p= 0.0057$) with 100% microfilarial depletion in the treated group whereas only 32% microfilarial depletion was achieved in the placebo group at the same time point. At 18 months and 24 months follow up time points, microfilarial depletion of 95% and 94% respectively were achieved in the treated group and 32% and 33% in the placebo group at 18 months and 24 months respectively.

Table 10: Comparison of microfilarial depletion between 5 weeks group and placebo group

Before treatment	MF positive	MF negative	Total	P-value*
5 weeks 100mg doxycycline	20 (100%)	0 (0%)	20	>0.9999
Placebo	21 (100%)	0 (0%)	21	
4 months after treatment	MF positive	MF negative	Total	P-value*
5 weeks 100mg doxycycline	15 (79%)	4 (21%)	19	0.6889
Placebo	18 (86%)	3 (14%)	21	
12 months after treatment	MF positive	MF negative	Total	P-value*
5 weeks 100mg doxycycline	0 (0%)	17 (100%)	17	<0.0001
Placebo	13 (68%)	6 (32%)	19	
18 months after treatment	MF positive	MF negative	Total	P-value*
5 weeks 100mg doxycycline	1 (5%)	18 (95%)	19	0.0001
Placebo	13 (68%)	6 (32%)	16	
24 months after treatment	MF positive	MF negative	Total	P-value*
5 weeks 100mg doxycycline	1 (6%)	16 (94%)	17	0.0003
Placebo	12 (67%)	6 (33%)	18	

*=Fischer's exact test

4.3.6.3 Microfilarial depletion in the 4 weeks 100mg doxycycline group

Comparison of 4 weeks 100mg doxycycline and placebo at pretreatment was not significant (p=>0.9999). At 4 months follow up there was still no significant difference (P>0.05). However, significant difference was observed at 12 months, 18 months and 24 months follow up time points with 100%, 95% and 94% microfilarial depletion in the treated group respectively. In contrast to the placebo group, 32%, 32%, and 33% microfilarial depletion was achieved at 12 months,18months and 24 months follow up respectively (Table 11).

Table 11: Comparison of microfilarial depletion between 4 weeks group and placebo group

	MF positive	MF negative	Total	P-value*
Before treatment				
4 weeks 100mg doxycycline	22 (100%)	0 (0%)	22	>0.9999
Placebo	21 (100%)	0 (0%)	21	
4 months after treatment	MF positive	MF negative	Total	P-value*
4 weeks 100mg doxycycline	16 (80%)	4 (20%)	20	0.6965
Placebo	18 (86%)	3 (14%)	21	
12 months after treatment	MF positive	MF negative	Total	P-value*
4 weeks 100mg doxycycline	1 (6%)	16 (94%)	17	0.0001
Placebo	13 (68%)	6 (32%)	19	
18 months after treatment	MF positive	MF negative	Total	P-value*
4 weeks 100mg doxycycline	3 (16%)	16 (84%)	19	0.0025
Placebo	13 (68%)	6 (32%)	19	
24 months after treatment	MF positive	MF negative	Total	P-value*
4 weeks 100mg doxycycline	4 (25%)	12 (75%)	16	0.0204
Placebo	12 (67%)	6 (33%)	18	

*=Fischer's exact test

4.3.6.4 Microfilarial depletion in the 3 weeks doxycycline plus rifampicin treated group

When the number of microfilarial positive patients was compared between the treated and placebo groups at pretreatment and 4 months follow up, there was no significant difference between the two groups (p=0.9999) [Table 12]. However, the difference became significant at 12 months (p=0.0002) with 90% microfilarial depletion in the treated group but only 32% in the placebo group. Even though the microfilaricidal activity in the treated group reduced to 76% at 18 and 24 months follow up time points, the difference was still significant (Table 12).

Table 12: Comparison of microfilarial depletion in the 3 weeks group against placebo group

	MF positive	MF negative	Total	P-value*
Before treatment				
3 weeks 200mg doxycycline plus rifampicin	21 (100%)	0 (0%)	21	>0.9999
Placebo	21 (100%)	0 (0%)	21	
4 months after treatment	MF positive	MF negative	Total	P-value
3 weeks 200mg doxycycline plus rifampicin	13 (72%)	5 (28%)	18	0.4324
Placebo	18 (86%)	3 (14%)	21	
12 months after treatment	MF positive	MF negative	Total	P-value*
3 weeks 200mg doxycycline plus rifampicin	2 (9%)	19 (90%)	21	0.0002
Placebo	13 (68%)	6 (32%)	19	
18 months after treatment	MF positive	MF negative	Total	P-value*
3 weeks 200mg doxycycline plus rifampicin	4 (24%)	13 (76%)	17	0.0096
Placebo	13 (68%)	6 (32%)	19	
24 months after treatment	MF positive	MF negative	Total	P-value*
3 weeks 200mg doxycycline plus rifampicin	4 (24%)	13(76%)	17	0.0176
Placebo	12 (67%)	6 (33%)	18	

*=Fischer's exact test

4.3.6.5 Microfilarial depletion in the 2 weeks doxycycline plus rifampicin treated group

Twenty patients from the treated group and 21 patients from the placebo group completed the treatment. When the number of microfilarial positive patients was compared between the treated and placebo groups at pretreatment and 4 months follow up, there was no significant difference in the microfilarial depletion between the two groups (Table 13). There was also no significant difference at 12 months, 18 months and 24 months follow up time points with only 47% microfilarial depletion (Table 13).

Table 13: Comparison of microfilarial depletion in the 2 weeks group against placebo group

Before treatment	MF positive	MF negative	Total	P-value*
2 weeks 200mg doxycycline plus rifampicin	21 (100%)	0 (0%)	21	>0.9999
Placebo	20 (100%)	0 (0%)	20	
4 months after treatment	MF positive	MF negative	Total	P-value*
2 weeks 200mg doxycycline plus rifampicin	17 (94%)	1 (6%)	18	0.6094
Placebo	18 (86%)	3 (14%)	21	
12 months after treatment	MF positive	MF negative	Total	P-value*
2 weeks 200mg doxycycline plus rifampicin	8 (53%)	7 (47%)	15	0.4836
Placebo	13 (68%)	6 (32%)	19	
18 months after treatment	MF positive	MF negative	Total	P-value*
2 weeks 200mg doxycycline plus rifampicin	9 (53%)	8 (47%)	17	0.4953
Placebo	13 (68%)	6 (32%)	19	
24 months after treatment	MF positive	MF negative	Total	P-value*
2 weeks 200mg doxycycline plus rifampicin	8(53%)	7(47%)	15	0.4928
Placebo	12 (67%)	6 (33%)	18	

*=Fischer's exact test

4.3.6.6 Summary of microfilarial depletion at 12 months among all the treatment groups

Microfilarial depletion, which is an indication of long term sterilizing effect, was significant at 12 months in all the treatment groups with the exception of the 2 weeks group and the placebo group (Fig. 3 and Tables 9 to 13).

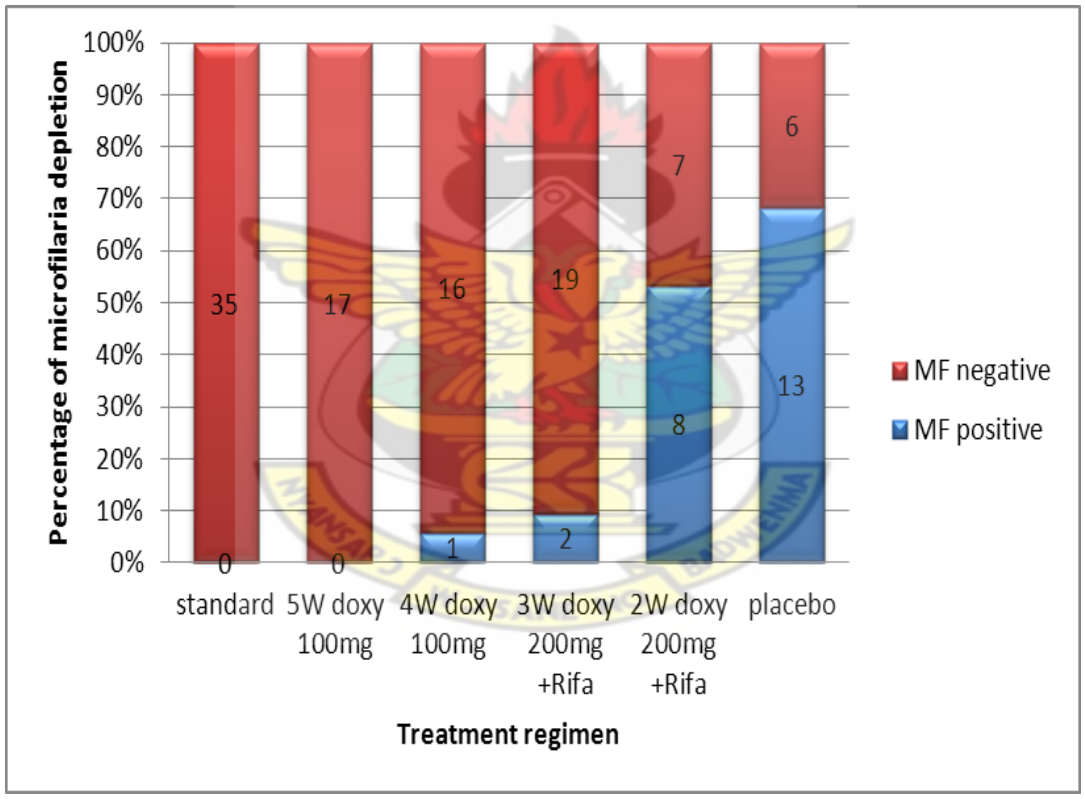


Figure 3: Percentage microfilarial depletion at 12 months time point

4.3.6.7 Summary of microfilarial depletion at 24 months among all the treatment groups

From Figure 4 and Tables 9 to 13 below, even though the percentage of microfilarial depletion decreased from 100% at 12 months to 80% at 24 months in the 4 weeks 200mg doxycycline treated patients, the sterilizing effect was still significant. Significant microfilarial depletion was also shown in the 5 weeks 100mg doxycycline group, 4 weeks 100mg doxycycline group, and 3 weeks combination of 200mg doxycycline and rifampicin group. However, there was no significant microfilarial depletion in the 2 weeks treatment regimen at 24 months follow up time point.

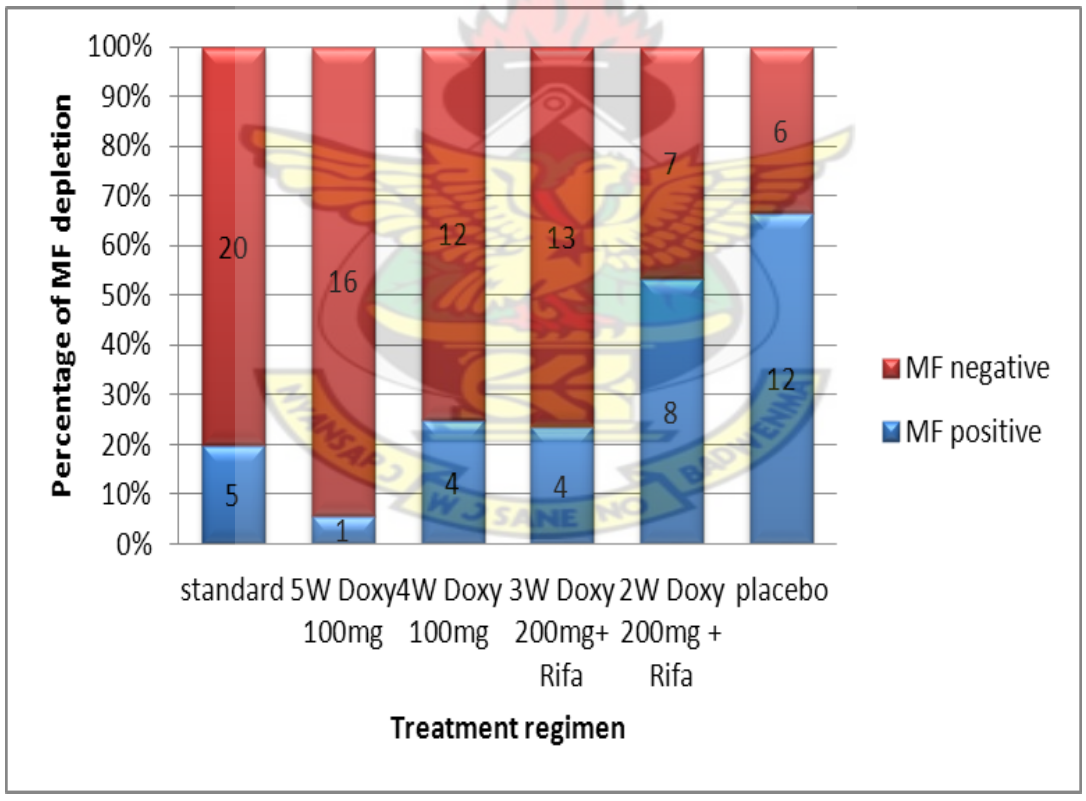


Figure 4: Percentage microfilarial depletion at 24 months time point

4.3.7 Assessment of macrofilaricidal activity using ultrasonography technique.

As the primary aim of this study, ultrasonography (USG) was performed on all the patients for detection of worm nests or filarial dance sign (FDS). The total worm death was assessed in the various groups and the results were compared with the placebo group as well as to the Gold standard (4 weeks, 200mg doxycycline). Patients included in this analysis were those who were present at follow up examinations of the various time points. Assessment of adult worm vitality using ultrasound (USG) technique was done at pre-treatment, 12 months, 18 months and 24 months post therapy. Breakdown of the results are shown in Tables 14 to 18.

4.3.7.1 Assessment of adult worm vitality in the 4 weeks 200mg doxycycline group (Gold Standard)

Sixty two (62) patients from the treated group and 34 from the placebo group completed the treatment. At 12 months time point, 73% of the treated patients lost their worm nest whereas only 37% had their worm nest lost in the placebo group with a significant difference (p=0.0021). The significant difference was sustained at 18 months time point (p=0.0002) and also at 24 months (p=0.005) when comparison with the placebo group was made (Table 14).

Table 14: Comparison of 4 weeks 200mg doxycycline (Gold Standard) and placebo at the various time points for macrofilaricidal activity.

Before treatment	Number of FDS+	Number of FDS-	Total	P-value*
4 weeks 200mg doxycycline	62 (100%)	0 (0%)	62	>0.9999
Placebo	34 (100%)	0 (0%)	34	
12 months after treatment	FDS+	FDS-	Total	P-value*
4 weeks 200mg doxycycline	16 (27%)	43 (73%)	59	0.0021
Placebo	17 (63%)	10 (37%)	27	
18 months after treatment	FDS+	FDS-	Total	P-value*
4 weeks 200mg doxycycline	12 (30%)	40 (70%)	52	0.0002
Placebo	21 (66%)	11 (34%)	32	
24 months after treatment	FDS+	FDS-	Total	P-value*
4 weeks 200mg doxycycline	9 (20%)	36 (80%)	45	0.005
Placebo	16 (53%)	14 (47%)	30	

*=Fisher's exact test

4.3.7.2 Adult worm vitality assessment with 5 weeks 100mg doxycycline

From the 5 weeks 100mg doxycycline treatment regimen, when the placebo group was compared with the treated group at pretreatment there was no significant difference in the adult worm vitality. However, at 12 months follow up time point there was significant difference with 70% adult worm death and this significant difference was sustained at 18 and 24 months (Table 15).

Table 15: Comparison of 5 weeks 100mg doxycycline and placebo at the various time points for macrofilaricidal activity.

Before treatment	Number of FDS+	Number of FDS-	Total	P-value*
5 weeks 100mg doxycycline	37 (100%)	0 (0%)	37	>0.9999
Placebo	34 (100%)	0 (0%)	34	
12 months after treatment	FDS+	FDS-	Total	P-value*
5 weeks 100mg doxycycline	10 (30%)	23(70%)	33	0.0185
Placebo	17(63%)	10 (37%)	27	
18 months after treatment	FDS+	FDS-	Total	P-value*
5 weeks 100mg doxycycline	3 (9%)	32 (91%)	35	<0.0001
Placebo	21(66%)	11 (33%)	32	
24 months after treatment	FDS+	FDS-	Total	P-value*
5 weeks 100mg doxycycline	3 (9%)	29 (91%)	32	0.0002
Placebo	16 (53%)	14 (47%)	30	

*=Fisher's exact test

4.3.7.3 Adult worm vitality assessment with 4 weeks 100mg doxycycline

Thirty four (34) patients completed treatment in both the treated and the placebo groups. Of the 34 patients from the treated group 14 of them representing 54% lost their worm nest at 12 months follow up. In the placebo group 10 patients representing 37% lost their worm nest at 12 months follow up time point and so the difference between the treated and placebo was not significant (P=0.2749). However, the difference became significant at 18 months time point (P=0.0005) and this was sustained at 24 month follow up time point (Table 16).

Table 16: Comparison of 4 weeks 100mg doxycycline and placebo at the various time points for macrofilaricidal activity.

Before treatment	Number of FDS+	Number of FDS-	Total	P-value*
4 weeks 100mg doxycycline	34 (100%)	0 (0%)	34	>0.9999
Placebo	34 (100%)	0 (0%)	34	
12 months after treatment	FDS+	FDS-	Total	P-value*
4 weeks 100mg doxycycline	12 (46%)	14 (54%)	26	0.2749
Placebo	17 (63%)	10 (37%)	27	
18 months after treatment	FDS+	FDS-	Total	P-value*
4 weeks 100mg doxycycline	5 (19%)	22 (81%)	27	0.0005
Placebo	21 (66%)	11 (34%)	32	
24 months after treatment	FDS+	FDS-	Total	P-value*
4 weeks 100mg doxycycline	5 (21%)	19 (79%)	24	0.0241
Placebo	16 (53%)	14 (47%)	30	

*=Fisher's exact test

4.3.7.4 Adult worm vitality assessment with 3 weeks doxycycline plus rifampicin

Thirty five (35) patients from the treated group and 34 patients from the placebo group completed the treatment. There was no significant difference between the two groups (P>0.999) at pretreatment. At 12 months post treatment there was still no significant difference in the number of patients with worm death since only 47% of the patients in the treated group had lost their worm nests compared to 37% of those in the placebo group. However the difference became significant at 18 months post treatment with 63% of the patients in the treated group losing their worm nests whilst only 34% in the placebo group had also lost their worm nests (p=0.0411). At 24 months post treatment, there was no significant difference between the treated group and the placebo group (Table 17).

Table 17: Comparison of 3 weeks combination group and placebo for macrofilaricidal activity.

Before treatment	Number of FDS+	Number of FDS-	Total	P-value*
3 weeks 200mg doxycycline plus rifampicin	35 (100%)	0 (0%)	35	>0.9999
Placebo	34 (100%)	0 (0%)	34	
12 months after treatment	FDS+	FDS-	Total	P-value*
3 weeks 200mg doxycycline plus rifampicin	18 (53%)	16 (47%)	34	0.4498
Placebo	17 (63%)	10 (37%)	27	
18 months after treatment	FDS+	FDS-	Total	P-value*
3 weeks 200mg doxycycline plus rifampicin	11 (37%)	19 (63%)	30	0.0411
Placebo	21 (66%)	11 (34%)	32	
24 months after treatment	FDS+	FDS-	Total	P-value*
3 weeks 200mg doxycycline plus rifampicin	11 (41%)	16 (59%)	27	0.4284
Placebo	16 (53%)	14 (47%)	30	

*=Fisher's exact test

4.3.7.5 Adult worm vitality assessment with 2 weeks 200mg doxycycline plus rifampicin

From Table 18 below, when the number of macrofilarial positive patients were compared between the treated and placebo groups, there was no significant difference at 12, 18 and even 24 months follow up time points (P>0.05) [Table 18].

Table 18: Comparison of 2 weeks combination regimen and placebo for macrofilaricidal activity.

	Number of FDS+	Number of FDS-	Total	P-value*
Before treatment				
2 weeks 200mg doxycycline plus rifampicin	34 (100%)	0 (0%)	34	>0.9999
Placebo	34 (100%)	0 (0%)	34	
12 months after treatment	FDS+	FDS-	Total	P-value*
2 weeks 200mg doxycycline plus rifampicin	18(64%)	10(36%)	28	>0.9999
Placebo	17 (63%)	10(37%)	27	
18 months after treatment	FDS+	FDS-	Total	P-value*
2 weeks 200mg doxycycline plus rifampicin	18(64%)	10 (36%)	28	>0.9999
Placebo	21 (66%)	11 (34%)	32	
24 months after treatment	FDS+	FDS-	Total	P-value*
2 weeks 200mg doxycycline plus rifampicin	16 (59%)	11 (41%)	27	0.7903
Placebo	16 (53%)	14 (47%)	30	

*=Fisher's exact test

4.3.7.6 Adult worm vitality assessment at 12 months time point

Comparison of the various treatment regimens was made against the standard at 12 months. This time point is the primary end point of this study and the results show that, 5 weeks 100mg and 4 weeks 100mg doxycycline are equivalent to the gold standard (4 weeks 200mg doxycycline). The 3 weeks and 2 weeks combination treatment as well as the placebo group showed inferiority to the gold standard at this time point (Fig. 5).

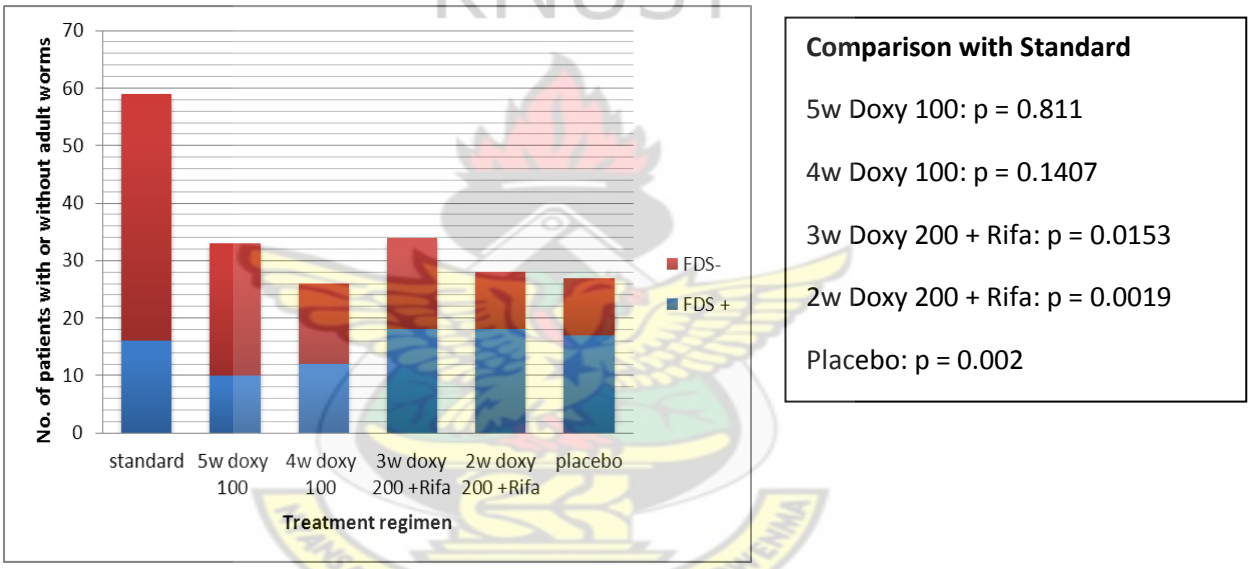


Figure 5: Macrofilarial assesment at 12 months time point (comparison with standard)

4.3.7.7 Adult worm vitality assessment at 18 months time point

When comparison of the various treatment regimens was done against the standard at 18 months time point, there was equivalence of the standard to 5 weeks 100mg doxycycline, 4 weeks 100mg doxcycline and 3 weeks combination of 200mg doxycycline and rifampicin. However there was superiority of the standard to the 2 weeks combination treatment as well as the placebo group (Fig. 6).

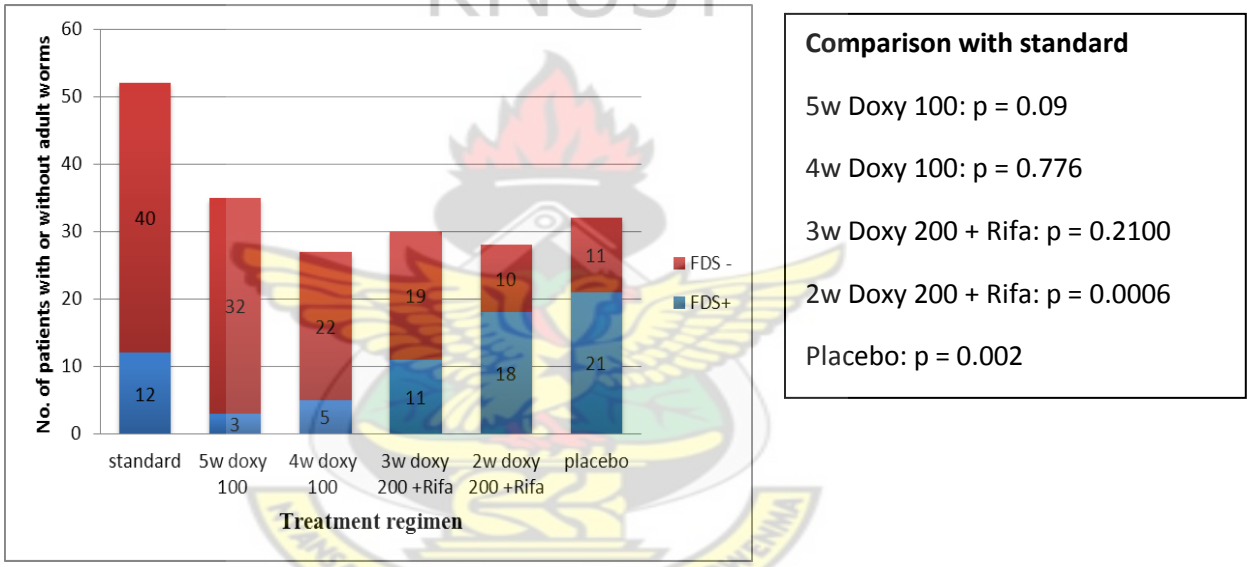


Figure 6: Macrofilaricidal assessment at 18 months time point (comparison with standard)

4.3.7.8 : Adult worm vitality assessment at 24 months time point

The 3 weeks combination treatment was no more equivalent to the standard at 24 months follow up time point in contrast to what was observed at the 18 months time point. The 5 weeks and 4 weeks 100mg doxycycline were still equivalent and effective at 24 months follow up time point. However the superiority of the standard to the 2 weeks combination treatment and the placebo was maintained (Fig. 7).

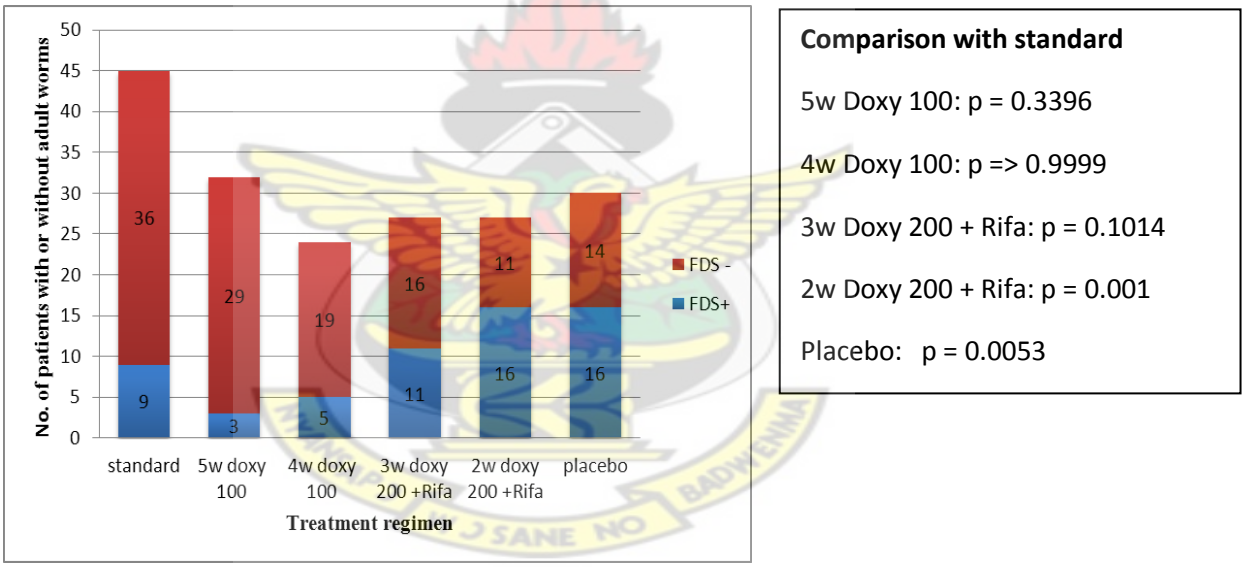


Figure 7: Macrofilaricidal assessment at 24 months time point (comparison with standard)

4.4 Discussion

Infection with the filarial nematode *Wuchereria bancrofti* can lead to lymphedema, hydrocele, and elephantiasis. Since lymphatic filariasis was considered potentially eradicable (Dreyer *et al.*, 1997), a number of antifilarial drugs have been used in the quest to eliminate the disease. Drugs with effective microfilaricidal activity (ivermectin and albendazole in areas where there is coendemicity with onchocerciasis) and those with partial macrofilaricidal activity (diethylcarbamazine (DEC) in areas where there is no coendimicity with onchocerciasis) have been used (Ottesen *et al.*, 1997; Dreyer *et al*., 2006).

Antibiotics such as doxycycline has also been used with the result showing long term sterilizing effect in animal models (Sunish *et al*., 2006) as well as in humans (Hoerauf *et al*., 2003b ; 2003c) in both onchocerciasis and lymphatic filariasis. Macrofilaricidal effect (Debrah *et al*., 2006; 2007a; Taylor *et al*., 2005) has also been achieved with doxycycline and has even resulted in amelioration of lymphatic pathology such as lymphedema and hydrocele (Debrah *et al*., 2006; 2009; Mand *et al*., 2012). Better still, doxycycline has even gone to the extent of being effective in the pathological cases without active infection (Mand *et al*., 2012) and it is also better than the current morbidity management on the basis of hygiene and self care alone (Mand *et al*., 2012). The successes of doxycycline were achieved when it was administered for 4, 6 and 8 weeks, which were considered too long and as such shorter regimen is desirable to complement classical antifilarial drugs (Taylor *et al*., 2010).

When doxycycline alone was used for three weeks, it depleted *Wolbachia* by 86% which was considered not significant although it led to a sustained amicrofilaremia for more than one year and ameliorated adverse reactions to standard anti-filarial drugs used for mass chemotherapy (Turner *et al*., 2006).

Other antibiotics such as azithromycin and rifampicin have been tested in attempts to eliminate lymphatic filariasis and onchocerciasis. Azithromycin was used because it can be given to children thereby closing one of the gaps left by doxycycline. Unfortunately azithromycin did not show any efficacy when used for 5 days (Richards *et al*., 2007) and even when used for 6 weeks (Hoerauf *et al*., 2008). Rifampicin on the other hand has proven to be effective in sterilizing the filarial worm when used within 14 days in animal models (Volkmann *et al*., 2003; Specht *et al*., 2008).

Since combination treatment of 3 weeks doxycycline and DEC resulted in a significant *Wolbachia* reduction as well as macrofilaricidal effect (Mand *et al.,* 2009), it necessitated a trial in humans, using combination of doxycycline and rifampicin in a pilot study. The result from this study showed a partial macrofilaricidal effect (Debrah *et al*., 2011).

Based on the results of the pilot study (Debrah *et al*., 2011), the present double blind placebo-controlled trial was undertaken with different treatment regimens aimed at finding the most effective acceptable regimen that could be used for the treatment of lymphatic filariasis.

4.4.1 Adverse reactions of doxycycline and rifampicin treatment

The different doxycycline treatment regimens as well as the combination treatments were well tolerated in the study population, with relatively mild and few side effects. No life threatening adverse reactions was observed in any of the patients.

Unlike anti-filarial chemotherapy, anti-*Wolbachia* chemotherapy kills worm populations slowly especially the microfilariae (MF). This slow drug action on adult worm populations and MF has a good safety profile because of avoidance of parasite-mediated or *Wolbachia*-mediated inflammatory adverse reactions.

Drugs with a rapid macrofilaricidal activity that directly affect the filarial parasite are associated with potentially severe and debilitating adverse reactions due to both dying adult worms and MF (Taylor *et al*., 2010).

From the present study the most frequent side effects observed were headache and discoloration of urine. But the frequency of the incidence is almost uniform in all the treatment groups as well as the placebo group.

4.4.2 Effect of doxycycline and rifampicin on microfilarial depletion

All patients received 150µg/kg ivermectin and 400mg albendazole four months after the start of treatment.

There was no difference in the number of MFs between the various treatment groups when compared with the placebo group at pretreatment and also at four months follow up. The lack of difference in the MF loads at this time point is not unexpected because it is already

known from previous studies that the sterilizing effect of doxycycline is slow and gradual (Hoerauf *et al.*, 2003b; Taylor *et al.*, 2005; Mand *et al*., 2009). As such four months post therapy is not enough to observe any significant sterilizing effect among the treatment groups.

In a number of trials that have been undertaken with doxycycline, a long term sterilizing effect has been reported, either with doxycycline alone (Taylor *et al*., 2005; Debrah *et al*, 2006; 2007a) or in combination with other antibiotics (Mand *et al*., 2009) at 12 months follow up time point. In much the same way, in this present study, a complete and significant amicrofilaremia (100% MF depletion) was observed in the standard and 5 weeks 100mg groups. The 3 weeks combination treatment group and 4 weeks 100mg doxycycline also showed 90% and 94% microfilarial depletion, respectively. This is an indication of a good sterilizing effect with these treatment groups at 12 months time point. However, the 2 weeks group did not show significant amicrofilaremia in contrast to the observation made in the pilot study (Debrah *et al*., 2011).

The placebo group showed only 32% microfilarial depletion and this is understandable because from previous studies, it is known that MF levels decrease according to its half life even without any intervention (Hoerauf *et al*., 2003b), and in this instance, ivermectin and albendazole were administered.

At 18 months follow up time point the amicrofilaremic effect was sustained within the standard and the 5 weeks group with 90% and 95% respectively.These results were consistent with earlier studies which showed that there is long term sterilization effect

(Debrah *et al*., 2007a; Hoerauf *et al*., 2009) when doxycycline is used for the above durations.

The 4 weeks 100mg doxycycline and 3 weeks combination treatment groups also showed a significant sterilizing effect at 18 months time point with 84% and 76% sterilizing effect respectively. However, the 2 weeks combination treatment did not show any significant sterilizing effect in contrast to what had been observed in the pilot study (Debrah *et al*., 2011). There was also no significant MF reduction in the placebo group with only 34% microfilarial depletion.

The sterilizing effect at 24 months after treatment was significant in the gold standard and the 5 weeks 100mg doxycycline treatment groups. This observation was also made in earlier studies in lymphatic filariasis (Debrah *et al*., 2007a) and onchocerciasis (Hoerauf *et al*., 2009) using similar regimen. The 4 weeks 100mg and 3 weeks combination therapies also gave a significant sterilizing effect at 24 months time point. This is an indication that the sterilizing effect was sustainable up to 24 months even though there was a decrease of 19% and 14% in the 4 weeks and 3 weeks combination groups respectively, from 12 months follow up to 24 months follow up time points. The decrease in microfilarial depletion at 24 months may be due to re-infection at this time point.

New infections with concurrent rise in *Wolbachia* levels following doxycycline has been documented for doxycycline treatment of onchocerciasis, where old, doxycycline-treated and thus *Wolbachia* depleted, female worms were located in onchocercomas next to young, nulliparous worms that were full with *Wolbachia* (Hoerauf *et al.*, 2003c).

However, due to the unavailability of adult worms for histological and PCR analysis in lymphatic filariasis, this new infection could not be formally confirmed for lymphatic filariasis as done for onchocerciasis. Notwithstanding, there have been already reported cases of re-infection in lymphatic filariasis and this is the case because the study participants are still living in the endemic communities and so are still exposed to the infective bite of the mosquitoes (Debrah *et al.,* 2007a*;* 2011).

4.4.3 Effect of doxycycline and rifampicin on *Wolbachia* depletion

Wolbachia depletion was assessed only at 4 months follow up time point. This is because there have been earlier reports of reinfection even at 12months follow ups (Hoerauf *et al.,* 2003b).

The *Wolbachia* depletion saw drastic and significant reduction in most of the groups such as 4 weeks 200mg doxycycline, 5 weeks 100mg doxycycline, 4 weeks 100mg doxycycline and 3 weeks combination therapy of 200mg doxycycline and 10mg/kg rifampicin at four months follow up time point.

For the 3 weeks combination group there was 86% *Wolbachia* reduction in previous studies when doxycycline was used alone (Turner *et al*., 2006) whereas in combination treatment with DEC, a 98% depletion was achieved (Mand *et al*., 2009). The present study with a 3 week combination treatment of doxycycline and rifampicin also saw a 99% *Wolbachia* depletion which was highly significant.

On the other hand even though the 2 weeks combination treatment had 85% *Wolbachia* depletion, it was not significant in contrast to the observation made in the pilot study

(Debrah *et al.*, 2011), where *Wolbachia* loads were significantly reduced by 93%. There was also no significant reduction in the placebo group which had only 22% *Wolbachia* depletion.

The concept of anti-*Wolbachia* chemotherapy of filariasis arose from earlier findings in animal models as well as in humans that depletion of more than ten-fold reduction of the *Wolbachia* endobacteria in adult female worms precedes female worm sterility (Hoerauf *et al*., 2003c) and worm death (Debrah *et al*., 2006, 2007a).

There is also 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 their synthesis (Ghedin *et al*., 2007), while *Wolbachia* have kept all of them (Foster *et al*., 2005, Pfarr and Hoerauf, 2005). As a result, 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 (Hoerauf *et al*., 2003a).

Wolbachia is known to be a stimulus for innate inflammatory reaction (Taylor *et al.*, 2000b; Brattig *et al*., 2004; Hise *et al*., 2004) and it has been shown by investigators that when lymphatic filariasis patients are treated with anti-*Wolbachia* therapy, the depletion of the *Wolbachia* prevented *Wolbachia*–derived mediators of inflammation to the formation of lymphatic dilation, lymphedema and hydrocele development (Debrah *et el*., 2006; 2009).

4.4.4 Effect of doxycycline and rifampicin on adult worms

Adult worm vitality was not assessed at 4 months follow up time point because earlier studies have shown that significant and apparent reductions in worm nests are not seen at this time point since anti-*Wolbachia* chemotherapy kills adult worm populations slowly (Taylor *et al*., 2010).

However at 12 months follow up time point 73% adult worm vitality was observed in the gold standard (four week doxycycline 200mg/d), which was significant and consistent with the results (Hoerauf *et al*., 2003b; 2003c) from previous studies where a four week 200mg/d doxycycline treatment resulted in 71% and 80% macrofilaricidal effect (Debrah *et al*., 2007a; 2011) at 12 months after drug treatment. The five weeks 100mg treatment group was also effective in killing adult worms at this time point which showed 70% macrofilaricidal effect. The two and three weeks combination treatments did not however, show macrofilaricidal activity at this time point. They showed 36% and 47% macrofilaricidal activities respectively. When comparison of the adult worm vitality of the standard was made with the various treatment regimens (Farrington *et al.,* 1990), the 5 weeks 100mg doxycycline and 4 weeks 100mg doxycycline were found to be equivalent to the gold standard. This outcome is a very important milestone in this study in the sense that henceforth, patients can be treated with 100mg doxycycline for 4-5 weeks, which is the normal recommended dosage for patients with other diseases. With 4-5 weeks 100mg doxycycline, there is no need for liver and kidney function teststo be done before treatment which is the case when a patient is treated with 200mg doxycycline.

Adult worm vitality assessed at 18 months follow up showed there was 70% worm death in the gold standard which was statistically significant, signifying and confirming that the gold standard is macrofilaricidal even 18 months post therapy (Hoerauf *et al*., 2003b). With a reduction of dosage from 200mg to 100mg, 91% and 81% macrofilaricidal activity which is also significant was achieved in the 5 weeks 100mg and the 4 weeks 100mg treatment groups respectively. This shows that these treatment regimens are also effective for the treatment of lymphatic filariasis.

With combination treatment of 3 weeks 200mg doxycycline and rifampicin there was also significant worm death at 18 months (p=0.0411). However, the 2 weeks combination treatment group did not show any significant macrofilaricidal effect (36% worm death) when compared with the pilot study that observed 50% macrofilaricidal effect (Debrah *et al*., 2011).

A four-week 200mg/d doxycycline treatment in previous studies resulted in 83% macrofilaricidal effect (Debrah *et al*., 2007a) 24 months after drug treatment. When the four weeks group, which was the gold standard in this study was compared with the previous results, a similar result was achieved with 80% macrofilaricidal effect at 24 months follow up. With a reduction of dosage from 200mg to 100mg, 79% macrofilaricidal activity, which is also significant, was achieved for the 100mg of 4 weeks, indicating that this treatment regimen is also effective in the treatment of lymphatic filariasis.

The five weeks 100mg doxycycline produced a result, which was even better (91% worm death) than the gold standard, and also in onchocerciasis where the same 5 weeks 100mg doxycycline resulted in only 49% female worm death (Hoerauf *et al*., 2009).

With combination treatment of 3 weeks 200mg doxycycline and rifampicin there was no significant worm death at 24 months post therapy, and this could be due to re-infection. The 2 weeks combination treatment group also did not show any significant macrofilaricidal effect when compared with the pilot study that observed 58% macrofilaricidal effect (Debrah *et al*., 2011).

From this study the 2 weeks combination treatment cannot be said to be effective in the treatment of lymphatic filariasis.

4.5 Summary and Conclusion

The current standard treatment of lymphatic filariasis (LF) using antibiotics to target *Wolbachia* endosymbionts is 200mg of doxycycline for 4 weeks. This regimen is known to be effective in depleting *Wolbachia* from lymphatic filarial worms, have high macrofilaricidal activity and a long-term sterilizing effect. However, the 200mg/d dosage is considered high and the 4 weeks is too long for mass application. Therefore, the objective of the present study was to test the efficacy of a reduced dosage of doxycycline from 200mg/d to 100mg/d and to shorten the treatment period using a combination of 200mg doxycycline and rifampicin 10 mg/kg for 2 and 3 weeks.

The 5 weeks 100mg/d doxycycline group showed 70%, 91% and 91% macrofilaricidal activity (absence of FDS) at 12, 18 and 24 months, respectively, compared to 73%, 70% and 80% in the standard and 37%, 34% and 47% in the placebo groups, i.e. showing superiority to both the placebo and standard therapy groups at all time points. Furthermore, 81% and 79% macrofilaricidal activities were observed for the 4 weeks 100mg/d doxycycline at 18 and 24 months respectively.

This is also an indication of effectiveness of this regimen in the treatment of lymphatic filariasis since it is equivalent to the gold standard at 18 months and 24 months and superior to the placebo group. Moreover, 3 weeks combination of doxycycline and rifampicin group produced 63% macrofilaricidal activity at 18 months, which is suitable for treatment of lymphatic filariasis.

The long-term sterilizing effect of the treatment arms was assessed by Mf in the blood as well as *Wolbachia* depletion. Compared to the placebo group, significantly fewer men treated with doxycycline alone for 4-5 weeks or 3 weeks combination of doxycycline and rifampicin were Mf positive at 12 months. The significant sterilizing effect was sustained in the standard, 5 weeks doxycycline 100mg/d group, 4 weeks doxycycline 100mg/d group as well as the 3 weeks combination of doxycycline and rifampicin group. The reduction of the doxycycline from 200mg/d to 100 mg/d (4 or 5 weeks) resulted in >80% macrofilaricidal activity and a long-term sterilizing effect up to two years and therefore should be recommended for treatment of lymphatic filariasis.

This dosage and regimen is now equivalent to that recommended for malaria prophylaxis for travelers and for acne. The anti-*Wolbachia* activity also saw a significant depletion in the standard, 5 weeks 100mg, and 4 weeks 100mg and 3 weeks combination therapies.

4.6 Recommendations

Several compounds assessed for macrofilaricidal activity against filarial worms have failed in the field (Taylor *et al.,* 2000b). Pharmaceutical industries have virtually dropped the idea and pursuit of development of new anti-filarial compounds (Taylor *et al.*, 2000b). This is because the cost of development of a drug is tens of millions of dollars and it takes so long (about 10 years). They are therefore not ready to develop drugs that are expected to be given free of charge like ivermectin (Stevenson, 1999) and albendazole. Research has therefore been on-going to find out whether or not some of the already registered antibiotics have

macrofilaricidal activity as well. Doxycycline 4 weeks 200mg has been the current drug and regimen which has proven to have long term sterilizing and macrofilaricidal effect.

The limitation of this dosage is the long duration and also it is contraindicated in certain groups of people such as children, pregnant women and breast-feeding mothers. Azithromycin was tested because it can be given to children but it was not efficacious. To shorten the duration of treatment, combination treatment was administered and from the present treatment 3 weeks doxycycline plus rifampicin showed a sterilizing effect up to 24 months. Therefore, this regimen can be recommended.

In addition, 4-5 weeks 100mg doxycycline can be recommended because patients need not undergo liver and kidney function tests before treatment, which hitherto is the case.

It is further recommended that other registered drugs such as minocycline and rifapentin which have shown in animal models to be superior to doxycycline and rifampicin in killing filarial worms be tested in humans to assess their efficacy against lymphatic filariasis in future studies.

CHAPTER 5

5.0 Genotyping of Patients

5.1 Introduction

Lymphatic filarial nematodes cause long term chronic infection with few infected individuals developing overt disease. The clinical manifestations such as lymhadenitis and lymphangitis, lymphatic obstruction or dysfunction leading to lymphedema or hydrocele, and tropical pulmonary eosinophilia occur in a subset of infected individuals (Kumaraswami, 2000). These manifestations can be grouped into two major poles (Maizels *et al.*, 1995). One pole being represented by microfilaraemic patients with high parasite numbers and down-regulated cell-mediated responses, and the other by patients with lymphedema and hydrocele, who typically have few or no parasites but exhibit vigorous specific immune reactions (Maizels *et al.*, 1995; Mahanty and Nutman, 1995).

The prevalence of overt clinical manifestations among adult residents of endemic areas is usually less than 10% despite the fact that most individuals are presumably inoculated with infective larvae throughout life (Tisch *et al.*, 2001). In high transmission areas, community-specific infection rates documented by the presence of blood-borne MF and filarial antigenaemia range from 50% to over 80% in persons older than 20 years of age, whereas fewer than 10% have lymphedema of extremities and about 30-40% develop hydrocele (Tisch *et al.*, 2001).

The actual cause(s) of this heterogeneity in infection and disease is not fully known. However, several explanations have been advanced to explain the variability among filarial infected patients. These include:

- differences in immunologic responsiveness caused by human host factors such as cytokines and specific regulatory molecules (Steel and Nutman, 2003; Babu *et al*., 2006; Taylor *et al*., 2000b).
- transmission potential as measured by vector monitoring (King *et al*., 2001).
- in utero exposure to parasite antigens (Steel *et al*., 1994; Malhotra *et al*., 2003).
- secondary bacterial infections superimposed on the lymphatic dysfunction (Mahanty *et al*., 1997).
- modulation of the immune response by parasite factors (Harnett *et al*., 2004)
- the immunogenetics of the host (Hoerauf *et al*., 2002b; Debrah *et al*., 2007b; Cuenco *et al*., 2009).

There has been a growing interest in the immunogenetics of the host contributing to this heterogeneity in filarial disease manifestations. Some studies have suggested that genes associated with Major Histocompactibiliy Complex (MHC) play a role in determining susceptibility to filarial disease and its clinical spectrum (Meyer *et al*., 1994; Yazdanbakhsh *et al*., 1995). A study conducted in India linked the susceptibility of bancroftian filariasis to two candidate genes (CHIT1 and MBL2) (Choi *et al*., 2001).

Though susceptibility to infection, parasite load and pathology has been shown to cluster in families (Cuenco *et al.*, 2004a; Cuenco *et al.*, 2004b; Terhell *et al.*, 2000), the exact genetic factors that lead to families having a greater incidence of filariasis and pathology are just now being elucidated. These include involvement of single nucleotide polymorphisms (SNPs) in the promoter regions of interleukin 10 (IL-10) and vascular endothelial growth factor-A (VEGF-A) associated with microfilaremia (the number of larvae, MF, in the blood) and increased risk of developing hydrocele (Pfarr *et al.*, 2007; Debrah *et al.*, 2007b) respectively. A SNP in the TGF-β1 that changes amino acid from Leucine to Proline has also been found to be associated with the presence of microfilariae and microfilarial load (Debrah *et al.*, 2011) and variants of Endothelin -1 (Ala288Ser) and tumor necrosis factor receptor-II (Met196Arg) are associated with development of either hydrocele or lymphedema (Panda *et al.*, 2011).

Since there have been relatively few or no investigations of host genetic contributions to acquisition of lymphedema, there is the need for studies utilizing a candidate gene approach (SNP genotyping) to understand the different outcome of pathologies such as lymphedema development in lymphatic filariasis. Knowledge of genomic loci linked to pathogenesis would help in understanding pathogy, and could lead to the development of strategies in terms of diagnosis and possible therapy of various forms besides supportive care, to ameliorate lymphedema. In particular, the results may permit better informed genetic counseling. Also, early diagnosis, treatment and the development of more targeted and effective therapeutic regimens for lymphatic filariasis could be a breakthrough for the control of the disease.

5.2 Aim

The aim of the study was to identify single nucleotide polymorphisms (SNPs) that could be associated with the development of lymphedema in lymphatic filariasis.

5.3 Materials and Methods

5.3.1 Study population

The study took place in the Nzema East and Ahanta West Districts in the Western Region of Ghana. In the genetic study, participants numbering up to 1303 comprising 266 lymphedema patients, 691 infected patients as well as 346 endemic control group were involved.

All volunteers included in the study were pricked at night for assessment and quantification of MF in the blood (for detailed methodology, refer to General Materials and Methods in Chapter 3). Lymphedema patients were examined separately by a clinician well conversant with symptomatology of lymphedema (Appendix1).

5.3.2 Genotyping of single nucleotide polymorphisms (SNPs)

The genotyping technique used was the sequenom assay (MassARRAY) comprising a number of steps which included: SNP selection, assay design (specific primers and probes), PCR amplification and sequencing.

5.3.2.1 Selection of single nucleotide polymorphisms (SNPs)

To investigate several candidate genes conferring susceptibility or protection to lymphedema, a number of databases such as National Center for Biotechnology Information (NCBI), and Online Mendelian Inheritance in Man (OMIM) were used. These databases describe the association between polymorphisms and diseases. SNP Annotation and Proxy Search (SNAP) were also used to find proxy SNPs based on linkage disequilibrium, physical distance and/or membership in selected commercial genotyping arrays. Pair-wise linkage disequilibrium was pre-calculated based on phased genotype data from the International HapMap Project (www.hapmap.org).

In all, 64 genes of interest were identified to have a role in inflammation, extravasation of fluid and also in other mechanisms important for tumorigenesis such as cell differentiation or angiogenesis (Appendix 2). A total of 147 SNPs of interest were sequenced by sequenom platform. Selected SNPs for the sequenom have been listed in Appendix 2.

5.3.2.2 Assay design

After the selection of the SNP (mutation) of interest, assay design software which was modified to allow for single base extension (SBE) designs with the modified masses associated with the new termination mix was used to design three sets of primers. The primers included two PCR primers with an amplicon size between 80-120 bp as stipulated in the iplex Gold Application Guide, and a third primer for the extension reaction (massEXTEND).

The software designs the assays, called Plexes, and could theoretically design a Plex where 42 SNPs could be analyzed in one reaction tube. Using the software, four Plexes were designed for 131 SNPs from the list of 147 (Appendix 3 and Table 20). The remaining SNPs were incompatible for genotyping with the other SNPs and were therefore reserved for genotyping by other methods (Table 19). The assay was designed based on a text file containing the SNP identity and the sequence flanking the SNP.

An important (for economical reasons) part of the primer design was to create multiplexed assays (i.e. sets of assays that could be run in the same initial PCR reaction) which, after the extension reaction, were clearly identified in the resulting mass spectrum.

5.3.2.3 MassEXTEND (Sequenom MassARRAY)

This is a process by which a primer extension process is designed using the third primer from the assay design process to detect sequence differences at a single nucleotide level. In this process, one nucleotide was added to the extension primer which then ends in an allele specific difference in mass between the products (Stacey and Liuda, 2009). These differences in mass were automatically translated by the software (MALDI-TOF mass spectrometry) to quickly and cost effectively characterize genotypes with the highest levels of accuracy (Storm *et al*., 2003).

5.3.2.4 PCR amplification

After the genomic DNA was isolated from the whole blood preserved in 8M urea (described in General Materials and Methods in Chapter 2), a DNA stock concentration of 100ng/ml was diluted to 15 ng/µl working concentration with TE buffer. A purity of A260/A280 ratio between 1.7 and 2.0 was measured. The DNA was then divided into aliquots of 2 µl per well into a 384-well plate. PCR reaction mixture of 25µl was performed on a 96 well plate and the mixture was then transferred onto the 384 well plate prior to performing the extension reaction of the SNP of interest. The forward and reverse PCR primers generated during the assay design anneals adjacent to the polymorphic site and are presented together in the multiplexed assay pool. After the PCR product is formed, a SAP (shrimp alkaline phosphate) treatment is performed to dephosphorylate and neutralize the incorporated deoxyribonucleotide triphosphates (dNTPs).

5.3.2.5 Iplex Gold Reaction

Laser beats the analyte molecules out of the matrix and ionises them (desorption/ionisation). In the electric field the analytes then fly differently to the detector because of their different masses. The flight time is proportional to the mass of the molecule. The masses used were between 4.500 - 9.000 Dalton (Fig.8 and Fig 9).

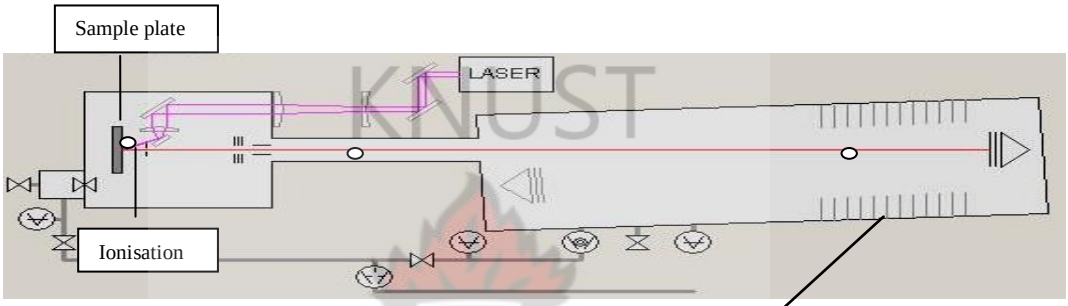


Figure 8: MALDI-TOF-Mass Spectrometer

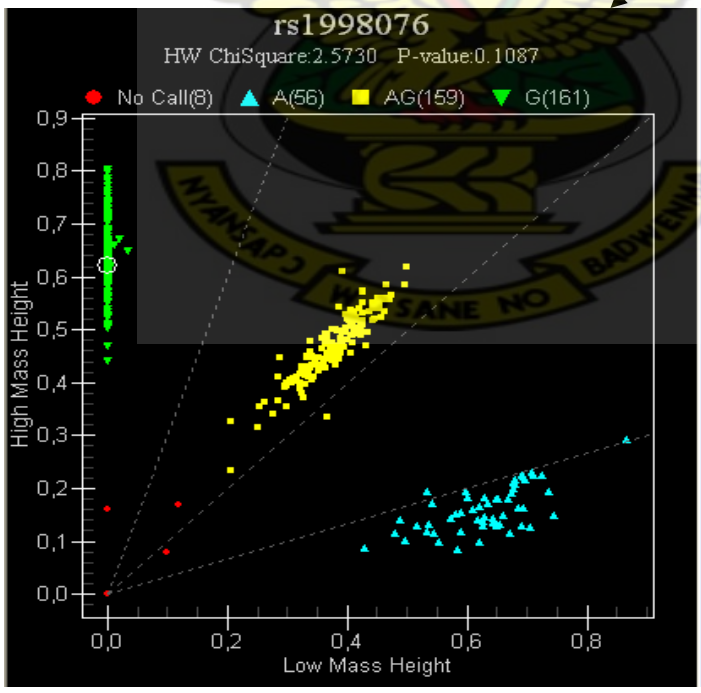


Figure 9: Summary of the MassARRAY® iPLEX® Gold SNP genotyping

5.3.3 Statistical analysis

The famhap19 software was used for analysis of association of the SNPs either in cases (LE) or in controls (CFA+ and endemic normals). Genotype and haplotype frequencies were summarised as percentages (Table 21 and 27). Armitage p-values were calculated separately for each SNP in a single marker analysis. The Armitage test is less influenced by deviation from Hardy-Weinberg Equilibrium (HWE) and the result obtained is valid and acceptable even when a group is not in HWE (Armitage, 1955). Genotype-specific risks were estimated as odds ratios (ORs) with 95% confidence intervals (CIs). Monte-Carlo (MC) simulation-based haplotype tests for case-control data allowing multiple comparison adjustment and correction for multiple testing (Global *P* value) was also done using the famhap19 software.

5.4 Results

5.4.1 Rejected SNPs

In all 147 Single Nucleotide Polymorphisms (SNPs) were selected for genotyping. Out of this number, 16 were rejected during the assay design. This is because the primer sequences produced were likely to form a primer dimerisation or the masses of the sequences were too close to be distinguished in the MALDI-TOF-Mass Spectrometer. The rejected SNPs are presented in Table 19. Nevertheless, with the exception of tumour necrosis factor alpha (TNF-α) and interleukin -4 (IL-4), all the other SNPs had a representative SNP in the sequenom.

Table 19: SNPs rejected by the sequenom

Gene	Reference sequence (rs)
COL1-A2	42524
COL1-A1	2075555
GATA-2	2335052
VEGF-A	1570360
TLR-4	4986790
FOX-P3	17847095
CTLA-4	3087243
TNF-alpha	1800629
TNF-alpha	1799964
TNF-alpha	1799724
MBL-2	7095891
TLR-6	5743810
IL-4	2243250
Decorin	1803344
TGF-beta	1800471
TGF-beta	1800470

5.4.2 Wells from the assay design process

In all 132 SNPs were included from the assay design process.These SNPs fitted into 4 wells and the distribution is shown in Table 20 below. The detailed SNPs distribution is shown in Appendix 3.

Table 20: SNP distribution per well

Well	Number of SNPs
Well 1	38
Well 2	36
Well 3	31
Well 4	27
Total	**132**

5.4.3 Single Nucleotide Polymorphism analysis

Single marker analysis between the lymphedema patients as cases were compared with non-lymphedema patients as controls. SNPs with armitage p-value of $P<0.05$ were considered significant. The genotype distribution and their odds ratios are shown in tables 21 and 27.

5.4.3.1 Analysis between lymphedema patients (Cases) and infected patients (Controls)

Out of the 132 SNPs that were put in the sequenom, seven showed significance when the data was analysed with famhap statistical package. The single marker analysis was between 266 lymphedema patients and 691 infected patients without lymphedema. All the seven SNPs had missing rates of less than 5%. An eighth SNP, Interleukin-17 (IL-17A), however, showed a trend (p=0.058) [Table 21].

Table 21: Single marker analysis of lymphedema and infected patients

Gene Rs number	**Genotypes**	**Cases**	**%**	**Controls**	**%**	**Odds ratio (OR)**	**p-value**
FLT4/VEGFR3 75614493	CC	263	99	667	97	3.2	0.0496
	CT	3	1	24	3	0.3	
	TT	0	0	0	0	0	
MMP-2 1030868	AA	61	23	115	17	1.5	0.0264
	AG	128	48	342	49	0.9	
	GG	77	29	234	34	0.8	
MMP-2 2241145	CC	77	29	161	23	1.3	0.035
	CG	129	48	338	49	1.0	
	GG	60	23	192	28	0.7	
CEACAM-1 8110904	AA	24	9	58	9	1.1	0.0315
	AG	170	67	389	59	1.4	
	GG	61	24	216	32	0.6	
CEACAM-1 8111171	GG	57	22	208	31	0.6	0.025
	GT	134	51	315	46	1.2	
	TT	69	27	159	23	1.2	
IGF-1 7136446	CC	19	7	25	4	2.0	0.0309
	CT	94	35	230	33	1.1	
	TT	153	58	435	63	0.9	

NFKB-inhibitor alpha 696	CC	45	17	61	9	2.1	0.00102
	CT	115	43	301	44	1.0	
	TT	105	40	326	47	0.7	

5.4.3.2 Allelic frequencies of associated SNPs between lymphedema and infected patients

The frequencies of the various alleles from SNPs that had an association when lymphedema was compared with infected patients are presented in Figures 10 to 14. At 95% confidence interval the odds ratio for the alleles of the associated SNPs are also presented in Tables 22 to 26.

5.4.3.2.1 Allelic distribution of VEGFR3

The allelic frequencies of vascular endothelial growth factor receptor 3 (VEGFR3) rs75614493 SNP shows that the allele C which has a frequency of 99% occurs more in the LE patients than the infected (98%). Allele T which is the allele with the lowest allelic frequency even among the Yorubian population is more frequent in the infected group. The odds ratio for allele C is 3.1 times higher in the C than the T allele (Fig. 10 and Table 22).

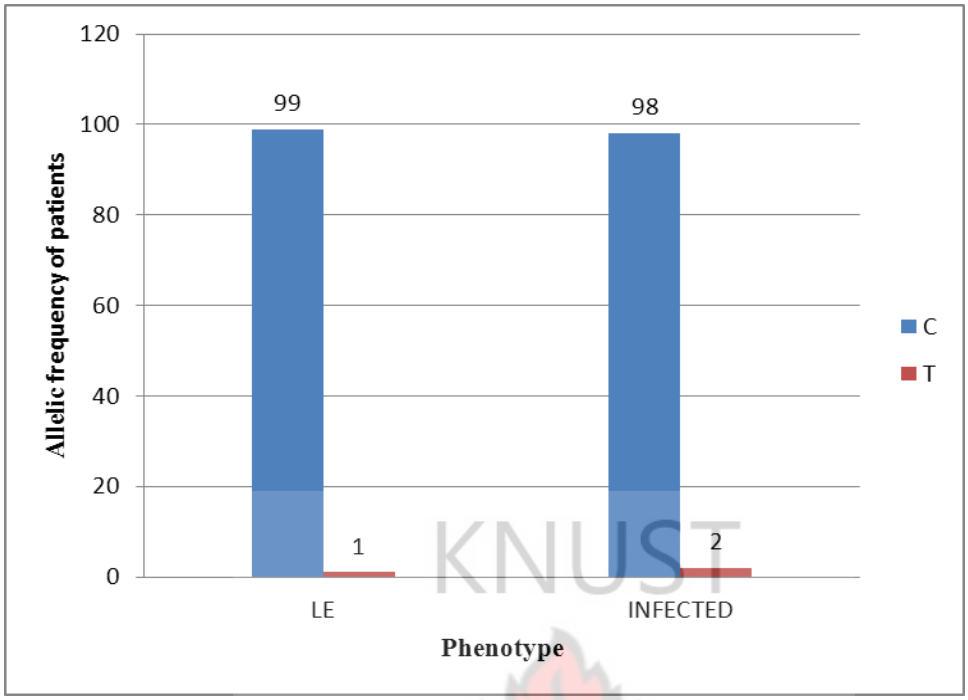


Figure 10: Allelic distribution of VEGFR3 rs75614493

Table 22: Allelic odds ratio of VEGFR3 rs75614493

Alleles	Odds ratio	Range
C	3.1	(0.9-10.4)
T	0.3	(0.1-1.1)

5.4.3.2.2 Allelic distribution of MMP2

The allelic frequencies of MMP-2 rs1030868 SNP shows that 47% of the 266 patients with LE carry the allele A whilst 53% carry allele G. The allelic odds ratio is also higher with allele A than allele G (Fig. 11 and Table 23).

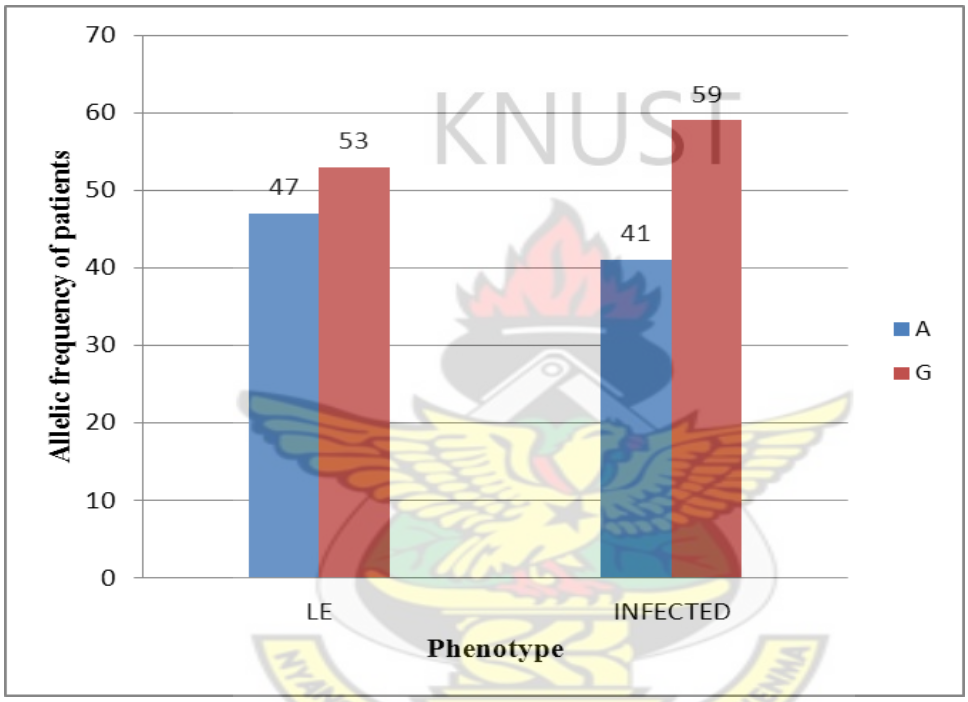


Figure 11: Allelic distribution of MMP2 rs1030868

Table 23: Allelic odds ratio of MMP-2 rs1030868

Alleles	Odds ratio	Range
A	1.3	(1.0-1.5)
G	0.8	(0.7-1.0)

5.4.3.2.3 Allelic distribution of MMP2

The allelic frequencies of MMP-2 rs2241145 SNP shows that the allele G which has a frequency of 53% occurs more in the infected patients than in the LE patients and the complementary allele C which also has a frequency of 53% is more in the LE patients than in the infected patients (48%). The allelic odds ratio is higher with allele C than allele G (Fig. 12 and Table 24).

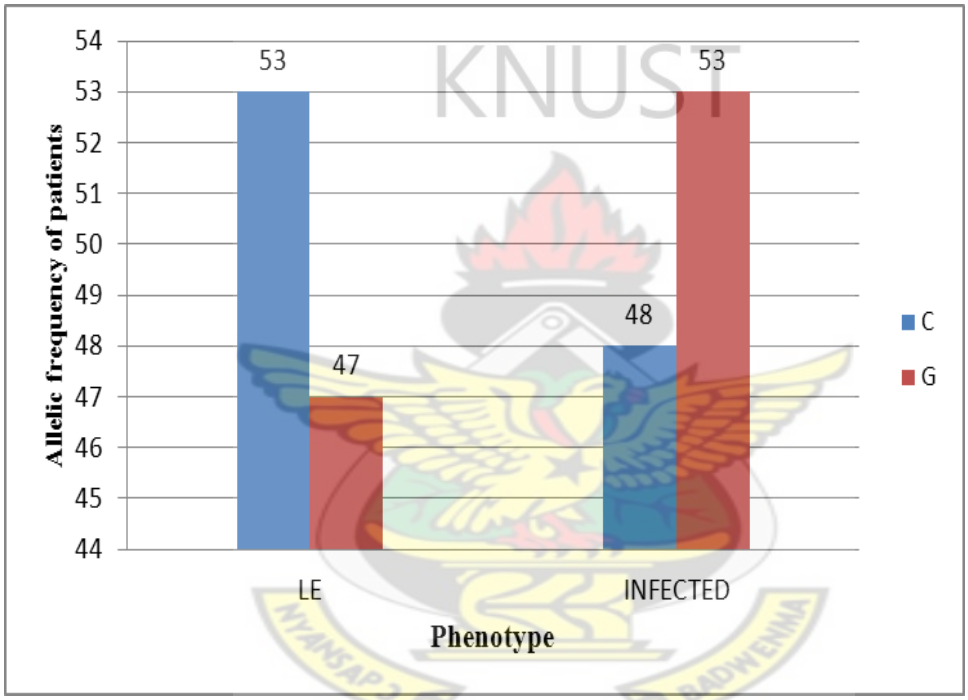


Figure 12: Allelic distribution of MMP2 rs2241145

Table 24: Allelic odds ratio of MMP-2 rs2241145

Alleles	Odds ratio	Range
C	1.2	(1.0-1.5)
G	0.8	(0.7-1.0)

5.4.3.2.4 Allelic distribution of CEACAM 1

CEACAM-1 rs9110904 SNP shows the occurrence of allele G is 5% more in the controls than in the lymphedema patients (LE) whilst allele A is 5% more in the lymphedema patients (LE) than in the controls. The allelic odds ratio is also higher with A than with G (Fig. 13 and Table 25).

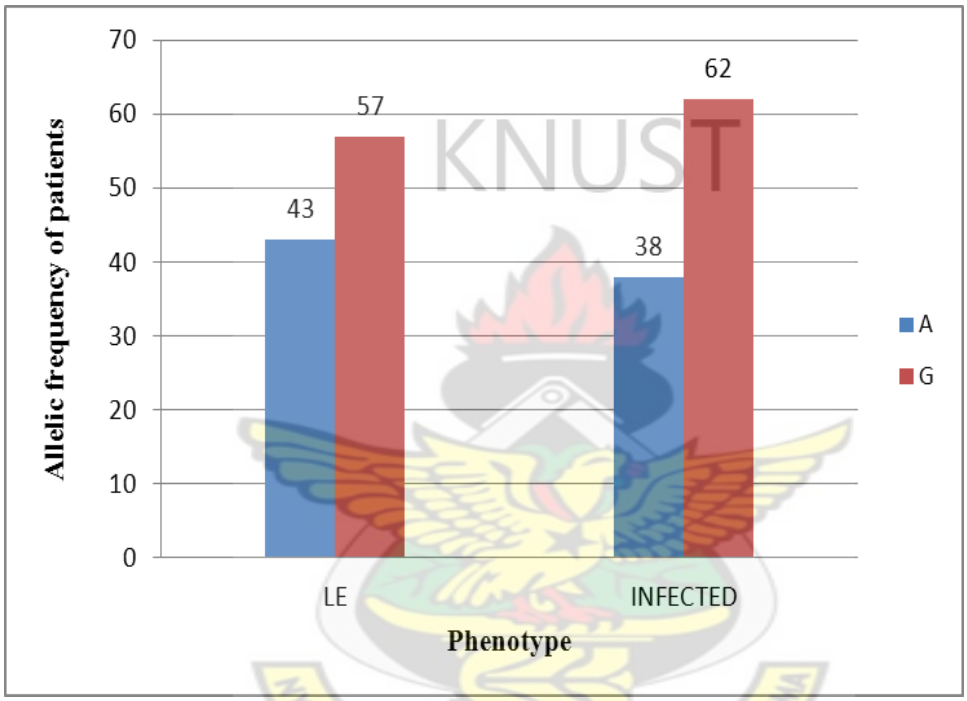


Figure 13: Allelic distribution of CEACAM 1 rs8110904

Table 25: Allelic odds ratio of CEACAM 1 rs8110904

Alleles	Odds ratio	Range
A	1.2	(1.0-1.5)
G	0.8	(0.7-1.0)

5.4.3.2.5 Allelic distribution of CEACAM 1

CEACAM-1 rs8111171 SNP shows the allele T occurs more in the lymphedema patients (LE) compared to the controls whilst allele G is less in the LE than in the controls. The allelic odds ratio is also higher with T than with G (Fig. 14 and Table 26).

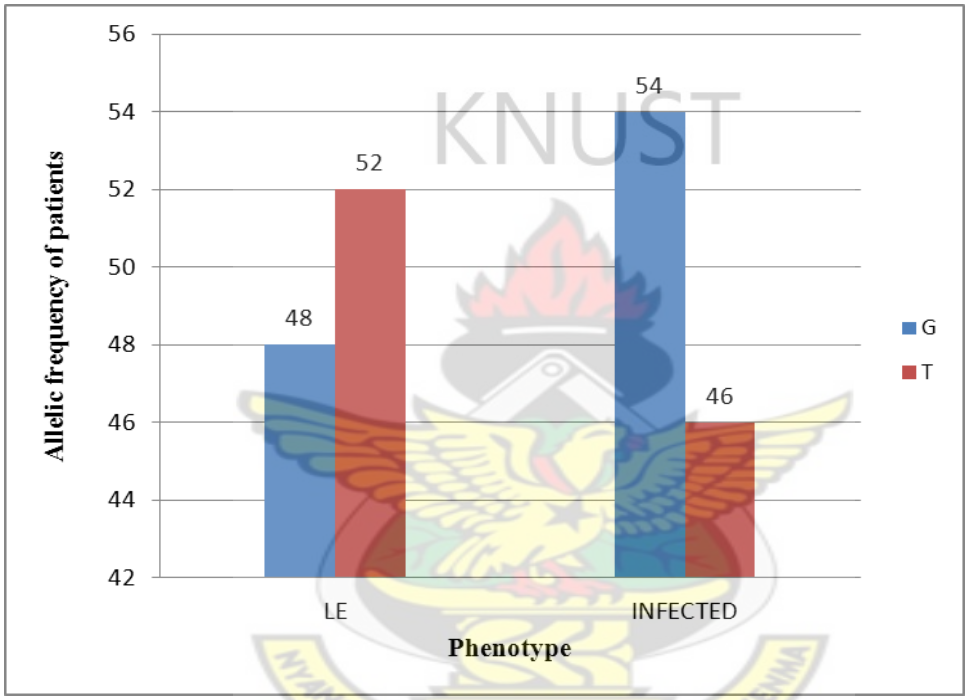


Figure 14: Allelic distribution of CEACAM-1 rs8111171

Table 26: Allelic odds ratio of CEACAM-1 rs8111171

Alleles	Odds ratio	Range
G	0.8	(0.6-1.0)
T	1.3	(1.1-1.6)

5.4.3.3 Analysis between lymphedema patients and endemic controls

From the single marker analysis, 266 lymphedema patients were analysed as cases and 349 endemic normal patients as controls. From the total number of SNPs analysed, four of them showed an association with an armitage P-value of < 0.05. The genotype frequencies and the odds ratio for the SNPs that showed significant association are shown below (Table 27).

Table 27: Single marker analysis of lymphedema and endemic normals

Genes		Cases	%	Controls	%	OR	P-value
IL-10 1800872	GG	78	29	131	38	0.7	0.0122
	GT	129	49	159	46	1.1	
	TT	59	22	56	16	1.5	
IGF1 7136446	CC	19	7	13	4	2.0	0.0438
	CT	94	35	112	32	1.1	
	TT	153	58	221	64	0.8	
NFKB-inhibitor alpha 696	CC	45	17	37	11	1.7	0.00709
	CT	115	43	148	43	1.0	
	TT	105	40	161	46	0.7	
TIMP 2 2277698	CC	225	90	276	81	2.1	0.00407
	CT	24	10	62	18	0.5	
	TT	1	0	2	1	0.7	

5.4.3.4 Allelic frequencies of associated SNPs (lymphedema vs. endemic controls)

The frequencies of the various alleles from SNPs that had an association when lymphedema was compared with endemic controls are presented in Figures 15 and 16. At 95% confidence interval the odds ratio for the alleles of the associated SNPs are also presented in Tables 28to 29.

5.4.3.4.1 Allelic distribution of IL-10

The allelic distribution of IL-10 rs18000872 SNP among lymphedema patients and control shows that the allele G is less frequent in the cases but more frequent in the control group. The reverse is the case with the T allele. The odds ratios of the various alleles show the same trend (Fig. 15 and Table 28).

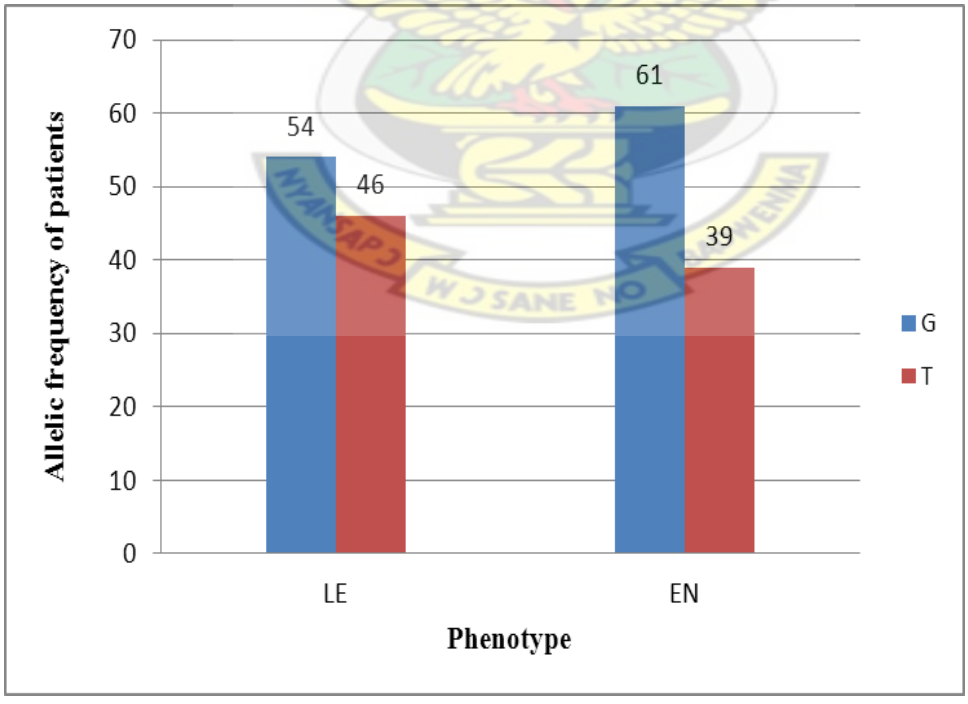


Figure 15: Allelic distribution of IL-10rs1800872

Table 28: Allelic odds ratio of IL-10rs1800872

Alleles	Odds ratio	Range
G	0.7	(0.6-0.9)
T	1.3	(1.0-1.7)

5.4.3.4.2 Allelic distribution of TIMP-2

When comparison of the allelic frequencies were done between the cases and controls for TIMP-2 rs2277698, the allele C in the cases was found to be higher than in the controls and allele T was less in the cases than in the controls. The odds ratios for alleles also follow the same trend with the C being higher than the T (Fig. 16 and Table 29).

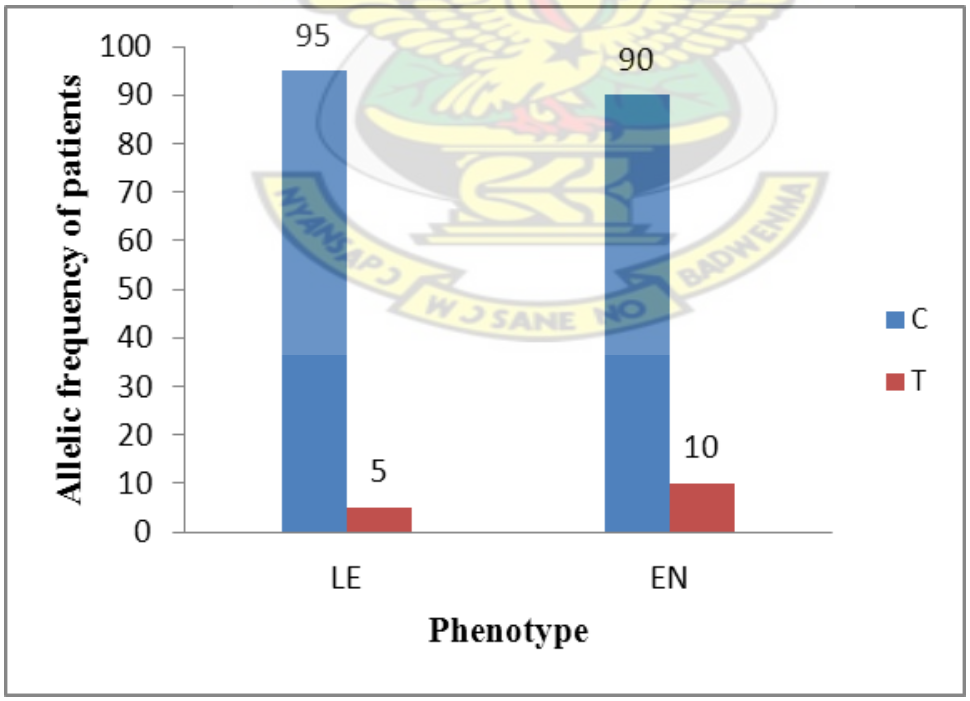


Figure 16: Allelic distribution of TIMP-2 rs2277698

Table 29: Allelic odds ratio of TIMP-2 rs2277698

Alleles	Odds ratio	Range
C	2.0	(1.2-3.1)
T	0.5	(0.3-0.8)

5.4.3.5 Allelic frequencies of SNPs that showed association with both control groups

From the single marker analysis, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (NF-κBIA) and Insulin-like growth factor 1 (IGF-1) showed significant association when lymphedema was compared with infected patients and also when lymphedema was compared with endemic controls. The allelic frequencies and their odds ratios are shown in Figures 17 and 18, and Tables 30 and 31.

The IGF-1 rs7136446 SNP was found to have significant association when lymphedema was compared with infected patients (P=0.0309) and also when lymphedema was compared with endemic controls (P=0.0438). In both instances the T allele was less frequent in the cases than in the controls, which means the T allele gives protection. The odds ratios also show the C allele is higher than the T allele (Fig. 17 and Table 30).

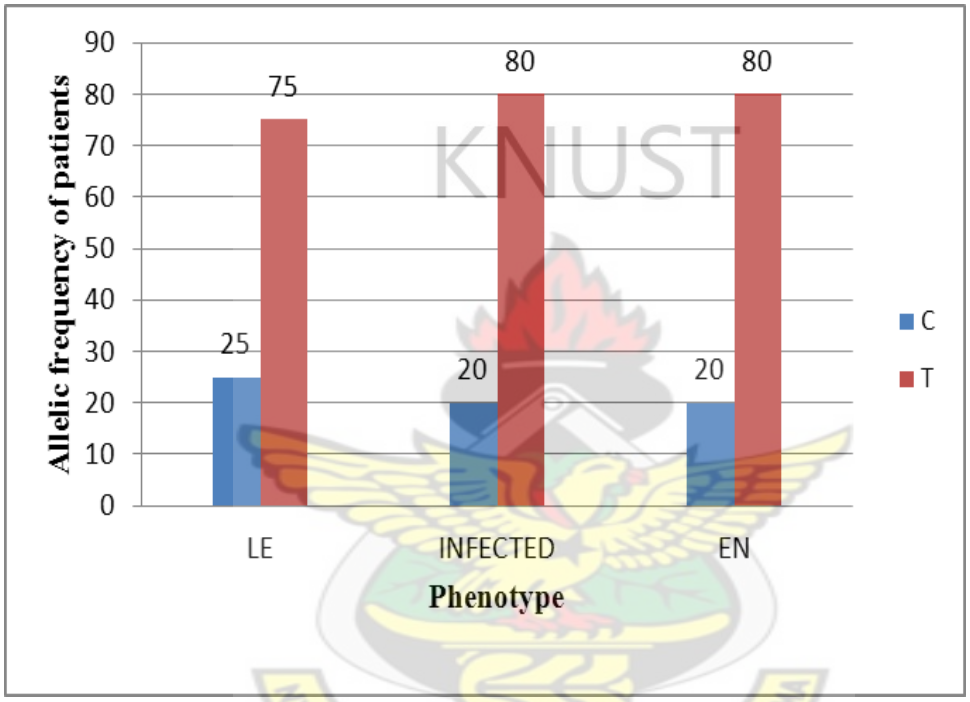


Figure 17: Allelic distribution of IGF-1 rs7136446

Table 30: Allelic odds ratio of IGF-1 rs7136446

CASES vs CONTROLS	Alleles	Odds ratios	Range
LE vs INFECTED	C	1.3	(1.0-1.7)
	T	0.8	(0.6-1.0)
LE vs ENDEMIC NORMALS	C	1.3	(1.0-1.7)
	T	0.8	(0.6-1.0)

From the single marker analysis, the NF-κBIA rs696 SNP was found to have significant association when lymphedema was compared with infected patients (Fig.16 and Table 31 p=0.00102) and also when lymphedema was compared with endemic controls (p=0.0195). In both instances the T allele was less frequent in the cases than in the controls and the C allele was more frequent in the cases than the controls. The odds ratio also shows that the C allele is higher than the T allele (Fig. 18 and Table 31).

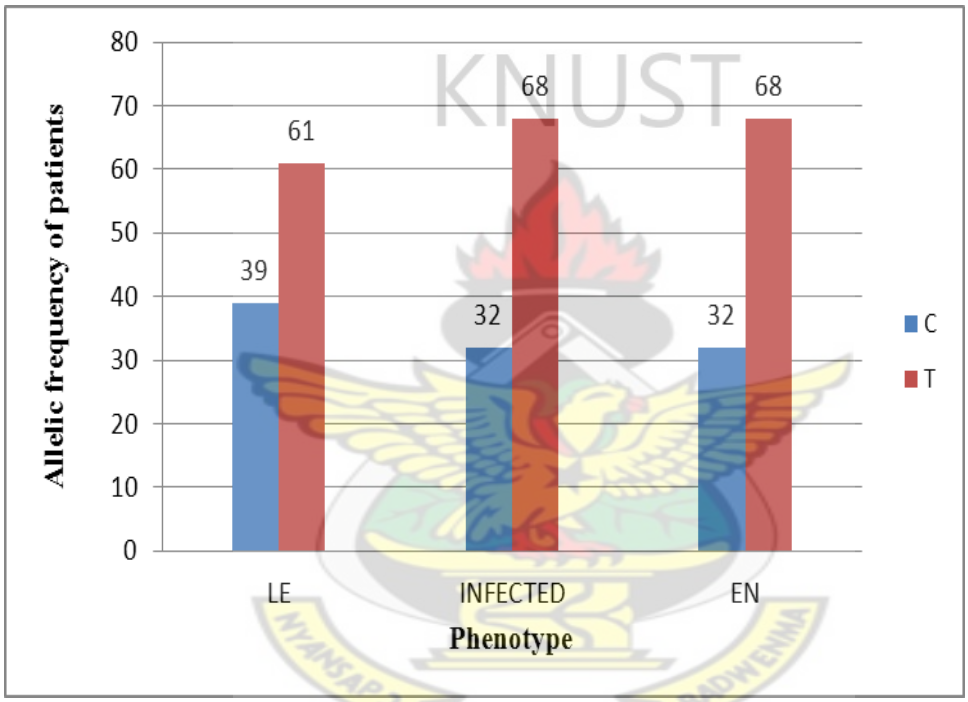


Figure 18: Allelic distribution of NF-κBIA rs696

Table 31: Allelic odds ratio of NF-κBIA rs696

CASES vs CONTROLS	Alleles	Odds ratios	Range
LE vs INFECTED	C	1.4	(1.1-1.7)
	T	0.7	(0.6-0.9)
LE vs ENDEMIC NORMALS	C	1.4	(1.1-1.7)
	T	0.7	(0.6-0.9)

5.4.4 Haplotype Analysis

Since it is known that the genetic variation of a population is intrinsically organized into haplotypes (Victor *et al*., 2006), analysis for possible association was done using the FAMHAP software. A likelihood ratio test with one degree of freedom was used to assess the significance of single haplotype frequencies among SNPs on the same gene. To get a corrected p- value an Omnibus statistics with 200,000 simulations was done (Tables 32 to 38).

5.4.4.1 Haplotype analysis of makers on IGF-1 gene

From the dataset that was generated from the sequenom, haplotype analysis was done for the two SNPs that were found on insulin-like growth factor (IGF-1) gene, IGF-1 rs2946834 and IGF-1 rs7136446. IGF-1 rs7136446 was the only SNP that showed an association when lymphedema (cases) was compared with non-lymphedema group (controls) (p=0.0309) from the single marker analysis. Even though there was no significant difference among the single haplotype frequencies, haplotype GC which was less common in both groups showed a trend. After multiple correction there was still a trend (p=0.058) [Table 32].

Table 32: Haplotype frequency estimation of SNPS on IGF-1 gene

rs2946834	**rs7136446**	**Cases (%)**	**Controls (%)**	P-value (x^2)
A	C	23.5	19.9	NS
A	T	34.6	38.4	NS
G	C	1.4	0.3	0.053
G	T	40.5	41.3	NS

Corrected p-value = 0.0585

5.4.4.2 Haplotype analysis of makers on NF-κBIA gene

Four haplotypes were detected for NF-κBIA variant alleles looking at three SNPs for this gene. No significant difference was detected between lymphedema (cases) and non-lymphedema patients (conrols) for haplotype CAC and TGT even though a trend was observed for haplotype TAC. However, haplotype CGT was significantly higher in the lymphedema patients (Cases) than non-lymphedema patients (Controls) [p=0.003]. This association was found when chi-square was done with one degree of freedom and the significance was sustained after multiple correction (p=0.0142). The results are presented in Table 33.

Table 33: Haplotype frequency estimation of SNPs on NF-κBIA gene

rs696	rs2233406	rs3138053	Cases (%)	Controls (%)	P-value
C	A	C	8.9	8.3	NS
C	G	T	29.4	22.2	**0.003**
T	A	C	13.3	17.3	0.061
T	G	T	47.5	51.7	NS

Corrected p-value: 0.0142

5.4.4.3 Haplotype analysis of makers on MMP-2 gene

Six different haplotypes on the MMP-2 gene were observed from the FAMHAP data analysis software (Table 34). Though haplotype GGGG and haplotype TACG had the highest frequencies in cases and controls, there was no significant association among any of the single haplotypes when lymphedema patients as cases were compared with non-lymphedema patients as controls. These p-values are uncorrected using chi-square test with one degree of freedom that is considered conservative. However, the corrected p-value was also not significant (Table 34).

Table 34: Haplotype frequency estimation of SNPs on MMP-2 gene

rs11643630	rs1030868	rs2241145	rs1992116	Cases (%)	Controls (%)	p-value
G	A	C	G	13.8	12.3	NS
G	G	C	A	3.4	3.8	NS
G	G	G	G	30.0	32.9	NS
T	A	C	G	32.6	28.3	NS
T	G	C	G	3.0	2.0	NS
T	G	G	G	16.2	19.0	NS

Corrected p-value- 0.08587

5.4.4.4 Haplotype analysis of SNPs on CEACAM-1 gene

Three haplotypes were generated from the two SNPs on the CEACAM gene. Haplotype GG was significantly higher in the controls than the cases (p=0.0257). A trend was seen for haplotype AT (P=0.0548) but there was no significant difference in haplotype GT. This significant difference was not sustained after multiple correction (p=0.0968) [Table 35].

Table 35: Haplotype frequency estimation of SNPs on CEACAM-1 gene

rs8110904	**rs8111171**	**Cases (%)**	**Controls (%)**	**p-value**
A	T	43.9	39.0	0.0548
G	G	48.3	54.0	0.0257
G	T	7.9	7.0	NS

Corrected p-value = 0.0968

5.4.4.5 Haplotype analysis of SNPS on IL-10 gene

Three different haplotypes were present from analysis of SNPS on IL-10 gene of lymphedema patients (cases) and non lymphedema patients (controls) (Table 36). There was no significant association on haplotype GT but haplotype TT was significantly more frequent in the cases than in the controls. A trend was, however, observed for haplotype GC. After correction for multiple testing the significant association was strengthened (Table 36).

Table 36: Haplotype frequency estimation of SNPS on IL-10 gene

rs1800872	**rs1800896**	**Cases (%)**	**Controls (%)**	**p-value**
G	C	25.4	30.3	0.055
G	T	28.2	30.5	NS
T	T	46.4	39.2	0.011

Corrected p-value = 0.029575

5.4.4.6 Haplotype analysis of SNPS on VEGFR3 gene

When haplotype analysis was done for the two SNPs on the VEGFR3 gene, three haplotypes were observed. Haplotype CT had the highest frequency in both the cases (85.1%) and the controls (84%). Haplotype CC was equally distributed between the cases and the control; however there was significant association with haplotype TT which was not sustained after multiple testing (Table 37).

Table 37: Haplotype frequency estimation of SNPS on VEGFR3 gene

rs75614493	**rs3587489**	**Cases**	**Controls**	**p-value**
C	C	14.3	14.3	NS
C	T	85.1	84.0	N S
T	T	0.6	1.7	0.0366

Corrected p-value = 0.12673

5.5 Discussion

Pathological effects of lymphatic filariasis such as lymphedema and hydrocele are observed in just a subset of individuals in endemic areas even though all inhabitants in an endemic community have equal chance of being inoculated with the parasite (Kumaraswami and Nutman, 2000; Nutman and Kumaraswami 2001). Clinical manifestations that were associated with lymphatic dysfunction and obstruction such as lymphedema are the most debilitating of the clinical manifestations of lymphatic filariasis (Bennuru and Nutman, 2009).

A number of studies to unravel the genetic bases of this heterogeneity have been undertaken with identification of certain genes such as CHIT1 and MBL2 which have been found to be associated with susceptibility to human filarial infection (Choi *et al*., 2001). Single nucleotide polymorphisms (SNPs) in the promoter region of CTLA4 for instance, have also been linked with susceptibility to lymphatic filariasis; and quite recently lymphangiogenic/angiogenic molecules such as vascular endothelial growth factor A have been found to be associated with development of hydrocele (Debrah *et al*., 2007b).

Some biomarkers for primary lymphedema have been identified (NLM, 2011). However, little has been done regarding secondary lymphedema, thus lymphedema due to filarial infection. Consequently, in this study, 147 SNPs from 64 genes were studied. These chosen genes are known to have functions in inflammation, cell proliferation, angiogenesis, cell adhesion and differentiation (Choi *et al*., 2001; Debrah *et al*., 2007b ; Taylor *et al*., 2000b).

A single marker analysis done with the famhap statistical software in this study showed associations with 11 SNPs in eight genes which include: vascular endothelial growth factor receptor 3 (VEGFR3), insulin like growth factor-1 (IGF-1), matrix metallopeptidase 2 (MMP-2), Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (NFKB- inhibition alpha), carcinoembryonic antigen-related cell adhesion molecule 1(CEACAM-1), Tissue inhibitor of metallopeptidase 2 (TIMP) genes, interleukin 10 and interleukin 17.

5.5.1 Vascular endothelial growth factor receptor 3 (VEGFR3)

In this study, a single marker association was found between a vascular endothelial growth factor receptor 3 (VEGFR3) SNP in the exon region of chromosome 5 and lymphedema development (Table 22, p=0.049). From the 266 patients with lymphedema genotyped, 263 patients representing 99% carried the ancestral CC genotype whilst three patients (1%) were heterozygous (CT). There was no patient with the homozygous TT genotype in the study population even though 1% and 2% of patients carried the T allele in the lymphedema and infected groups respectively. The allelic distribution from this study is consistent with a pilot work done in the Yurobian population from dbSNP (NCBI) with assay ID ss222155021. The allelic frequency of the study was 97.5% for the C allele and 2.5% for the T allele (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=75614493#locus).

From this present study, the genotype CC has an odds ratio of 3.2 (0.9-10.6) [Table 21], which means patients having the CC genotype stand 3.2 times risk of developing lymphedema compared with patients with heterozygous CT which has only 0.3 times the

risk of developing lymphedema. On the other hand the very few people who have the T allele stand 0.3 times the chance of being protected from developing lymphedema (Fig 10).

The haplotype analysis showed that the TT haplotype is significantly lower in the lymphedema patients (cases) than the infected patients (controls). However, the occurance of such an allelic combination may be very rare because from this study and the pilot work done in Nigeria the T allele is really rare and has a very low probability of occurring in the population (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=75614493#locus).

VEGFR-1 and VEGFR-2 and VEGFR-3 are the three main receptors for the VEGFs that have been identified. VEGFR-1 and VEGFR-2 are expressed mainly in the blood vascular endothelium, whereas VEGFR-3 is restricted largely to the lymphatic endothelium (Veikkola *et al*., 2001). VEGFR3 gene encodes a tyrosine kinase receptor for vascular endothelial growth factors C and D.

In animal models, over-expression of VEGF-C in the skin of transgenic mice resulted in lymphatic endothelial proliferation and dilation of lymph vessels (Jeltsch *et al*., 1997) with a resemblance to lymphatics infected with filarial parasites (Taylor *et al*., 2001). These transgenic mice then developed a lymphedema-like phenotype characterized by swelling of feet, edema and dermal fibrosis (Kaipainen *et al*., 1995), similar to what is observed in humans.

Studies on the molecular mechanisms controlling the lymphatic vessels have shown that vascular endothelial growth factors C and D specifically control lymphangiogenesis in

humans (Korpelainen and Alitalo, 1998; Achen *et al*., 1998) by activating the VEGF receptor-3 (VEGFR-3) (Veikkola *et al*., 2001; Jeltsch *et al*., 1997).

VEGFR-3, which is a known marker for lymphatic vessels in human adult tissues (Spiegel *et al*., 2006) has been linked to human hereditary lymphedema (Yu *et al*., 2007). Based on the results from the famhap software analysis from this study and other literature reviewed, it can be hypothesized that patients with the C allele or CC genotype are likely to produce more VEGFR3 which may consequently lead to lymphatic vessel dilation and development of lymphedema.

VEGFs and their receptor 3 (VEGFR3) are needed for the development of lymphatic vessels. However, their over-production leads to lymphatic dilation and lymphedema development (Makinen *et al*., 2001). Several studies by Debrah *et al*. (2006; 2007b; 2009) showed that, plasma levels of vascular endothelial growth factors (VEGFs) and its soluble receptor, sVEGFR3, are significantly elevated in patients infected with filarial worms, and a correlation was found between sVEGFR3, lymphatic dilation and pathology development. Targeting the filarial worms by doxycycline reduced the levels of VEGFs/sVEGFR3, with amelioration of dilated supratesticular lymphatic vessels and improvement in the conditions of lymphedema and hydrocele (Debrah *et al*., 2006; 2007b; 2009). Importantly, the VEGF-C and sVEGFR3 levels decreased well before the supratesticular lymphatic dilation improved. Given that the sVEGFR-3 are secreted into the plasma following over-stimulation of the lymphangiogenesis system (Joukov *et al*., 1996), and more importantly their data (Debrah *et al*., 2006, 2007b, 2009) showing that the stimulation of lymphangiogenesis followed by lymphatic dilation was reduced by doxycycline (Debrah *et*

al., 2006; 2007b; 2009), it is an indication that the VEGFs/VEGFR3 system may constitute a major mediator of pathological lymphatic dilation.

Again, the fact that the VEGF and sVEGFR3 reduction preceded the improvement of pathology indicated a possible causal interaction between lymphangiogenic factors reduced by doxycycline treatment and lymphatic pathology, rather than only a co-incidence or an epiphenomenon. This present study has just gone a further step to confirm the possible involvement of lymphangiogenic/angiogenic factors in the development of pathology of LF.

5.5.2 Insulin-like growth factor 1 (IGF-1)

In this study there was a single marker association between IGF-1 SNP (rs7136446) and lymphedema development (Table 30, Fig. 15). IGF-1 rs7136446 is a SNP in the intron region of chromosome 12. With high number of patients carrying the TT genotype in the study population (58% of cases and 63% of controls), the odds ratio of the genotype TT for protection is 0.9, which means the chance of being protected from developing lymphedema is 0.9 times higher compared with patients with the CC or the CT genotype (Table 21). Conversely, the genotype CC is a susceptible one with an odds ratio of 2.0. Therefore, patients having CC genotype stand 2.0 (1.1-3.8) times higher risk of developing lymphedema than those with TT genotype. This association was observed when lymphedema was compared with infected patients as well as endemic controls (Table 21 and 27).

Haplotype analysis, which is a higher informative value, is used to assess the contribution of genetic polymorphisms in diseases (Gabriel *et al*., 2002).

For this reason haplotype association analysis was done with SNPs on the IGF-1 gene to assess whether they are inherited together and as such contribute to the development of lymphedema. The SNPs that were analysed are rs7136446 and rs2946834.

From the single marker analysis, only IGF-1 rs7136446 showed significant association. The haplotype analysis did not show significant association among the single haplotype combinations, however there was a trend in haplotype GC (p=0.053). There was also a trend when simulation with 200,000 permutations was done (p=0.058). This may be an indication that those SNPs may move together to cause an expression.

IGF-1 is also a lymphangiogenic factor like the VEGFs whose expression in lymphatic vessels leads to simultaneous stimulation of angiogenesis and lymphagiogenesis (Cao *et al*., 2005). In addition to its direct role in stimulation of lymphagiogenesis, the IGF-1 signalling system can also up regulate VEGF expression and thus indirectly induce neovascularization (Bermont *et al*., 2000). These VEGFs have been shown to be associated with pathology development as described above (Debrah *et al*., 2006; 2007b; 2009) and their reductions result in improvement in pathology patients (Debrah *et al*., 2006; 2007b; 2009).

In a study conducted by Verheus *et al*. (2008), IGF-1 rs7136446 SNP showed association with elevated levels of IGF-1 and it was further related to high breast density of women before menopause and after menopause (Verheus *et al*., 2008), which means that this SNP may have a causal effect whenever it is expressed.

Since there was at least a trend in the single haplotype GC and also a trend after multiple testing, there is some indication that though there is not a strong haplotype association, those alleles may function together in the development of lymphedema.

5.5.3 Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NF-κBIA).

Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha **(**NF-κBIA), which is part of the immune pathway is known to regulate many cellular functions such as cell proliferation, apoptosis, angiogenesis, cell adhesion and differentiation (Karin and Greten, 2005). Some studies have documented that the SNP rs696 AG in the 3'-UTR of inhibitor nuclear factor–kapppa B alpha is associated with an increased risk of developing colorectal cancer in Chinese elderly population (Gao *et al*., 2007).

In this study a single marker association was found between rs696 SNP and the development of lymphedema. Patients having rs696 CC genotype stands 2.1(1.4-3.2) times higher risk of developing lymphedema than patients with TT genotype. The C allele was observed to provide susceptibility and so patients with the C allele were shown to stand 1.4 (1.1-1.7) times higher the risk of developing lymphedema than patients with the T allele.

Haplotype analysis was also done with NF-κBIA SNPs. These SNPs were NF-κBIA rs696, NF-κBIA rs2233406, and NF-κBIA rs3138053. The results from the haplotype analysis showed an association with the allele combination CGT (p=0.003).

A trend was also observed for haplotype TAC (p=0.061) and there was no significant association with the other haplotypes. Since these p-values were estimated with chi-square,

which is considered conservative (Herold and Becker, 2009) an omnibus statistics with 200,000 simulations was done to strengthen the chi -square results and the overall difference was also significant (p= 0.00385) after correction for multiple testing, indicating that those haplotyes are inherited together.

The induction of vascular endothelial growth factor A (VEGF- A) which is a known marker for hydrocele development (Debrah *et al.*, 2007b) and angiopoietin 1(Ang-1) production by parasite and parasite antigens has been shown to be dependent on the activation of NF-κB (Babu *et al*., 2012). It is reported that inhibition of NF-κB signaling pathways results in decrease in angiogenic factor production. These factors are controlled by Toll-like receptor ligands which significantly induce the expression/production of VEGF-A and Ang-1 in peripheral blood mononuclear cells of individuals with lymphatic pathology (Babu *et al*., 2012). Again, just like the previous two genes discussed so far, namely VEGFR3 and IGF-1, which were found to influence lymphangiogenic/angiogenic pathways, NF-κB is also known to influence the lymphangiogenic/angiogenic pathway (Babu *et al*., 2012), further buttressing the hypothesis that pathology of LF might be influenced by the lymphangiogenic/angiogenic pathway.

5.5.4 Matrix metalloprotease-2 (MMP2)

In this study, Matrix metalloprotease-2 (MMP-2) SNPs, rs1030868 and rs2241145, were also found to be associated with lymphedema development when a single marker analysis with famhap statistical software was done (p=0.0264 and p=0.035 respectively). The haplotype analysis which was done for all the SNPs on the MMP-2 gene in the sequenom (Table 34) did not show any significant association and as such are not dependent on each other to cause an expression.

MMP-2 is also a known angiogenic factor (Bennuru and Nutman 2009) that has been shown to be involved in tissue remodeling and disease states by degrading basement membranes, regulating vascularization and inflammatory responses as well as tumor growth (Candelario-Jalil *et al*., 2011; Peng *et al*., 2011).

Proteins of the matrix metallo metalloprotease-2 (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling. It is also involved in disease processes, such as arthritis and metastasis. Study conducted by Bedi *et al.* (2009) identified the role of this enzyme in tumor growth vascular aneurysmal disease development and tissue remodeling after surgery including rotator cuff repair (Bedi *et al*., 2009). A study Fatar *et al.* (2008) also showed that MMP-2 protein levels were significantly lower in lacunar stroke patients (174 ± 48 ng/dl) when compared with controls (214 ± 56 ng/dl). The authors therefore concluded an association of the MMP-2 gene with the development of lacunar stroke. In another study, mutations in this gene have been associated with winchester

syndrome and nodulosis-arthropathy-osteolysis (NAO) syndrome, showing involvement of this gene with other diseases.

Two transcript variants in the MMP-2 gene (R101H and Y244X) encoding different isoforms have been found (Martignetti *et al*., 2001).

Regarding lymphedema, swelling is caused when localized plasma protein leaks from arterial blood capillary walls and remains trapped in the body's soft tissues. The resultant distention of tissue due to lymphatic dysfunction and the fibroadipose tissue deposition causes morbidity with subsequent enlargement of the affected area (Couto *et al*., 2011). Variants of the MMP-2 SNP could then be said to be potential markers for lymphedema development (Anuradha *et al*., 2012).

Tetracyclines have been shown to profoundly inhibit mammalian MMPs by a mechanism that is independent of their antimicrobial activity. The mechanism is through their ability to modulate endogenous MMP activity to basal levels, thereby reducing excessive degradation or remodeling at the healing enthesis after rotator cuff repair (Pasternak *et al*., 2006; Lo *et al.*, 2004). It has been shown by Debrah *et al*. (2006, 2007b, 2009) that doxycycline improves the condition of pathology of LF patients, and this was attributed to VEGFs. However, since MMPs are also involved in angiogenesis and are influenced by tetracyclines as well, it is likely that they (MMPs) may also play an important role in lymphedema development, still lending strong support to the fact that pathology of LF may be influenced by lymphangiogenic/angiogenic molecules.

5.5.5 Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM 1)

In this study a single marker association was found between carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM 1) SNPs, rs8110904 and rs8111171, and lymphedema development. Patients who were heterozygous for the SNPs stand a higher chance of developing lymphedema than the homozygous patients (Table 21). The A allele was observed to confer susceptibility and as such patients with the A allele stand 1.2 (1.0-1.5) times higher the risk of developing lymphedema than patients with the G allele in CEACAM 1 rs8110904. With CEACAM 1 rs8111171, the T allele confers susceptibility with 1.3 (1.0-1.5) times the risk of developing lymphedema than the G allele.

Haplotype analysis was done with the two CEACAM SNPs that showed single marker association, namely CEACAM 1 rs8110904 and CEACAM 1 rs8111171. Even though there was a single marker association for both SNPs, haplotype association only showed association in haplotype GG (p=0.0257) and it was not strengthen after multiple testing. However, it can still be said that since there was a single haplotype association there is some indication that haplotype GG from the various SNPs could function together and cause an expression.

CEACAM 1 is a type 1 trans-membrane protein involved in cell-to-cell adhesion (Gu *et al*., 2009). It has been shown to be a potent stimulant for VEGF mediated angiogenesis (Ergun *et al*., 2000; Oliveira-Ferrer *et al*., 2004). CEACAM 1 has also been shown to stimulate microvascular endothelial cells grown in the presence of VEGF (Ergun *et al*., 2000).

In both *in vitro* and *in vivo* models in human mammary gland morphogenesism, CEACAM 1 has been shown to be essential for lumen formation (Huang *et al*., 1999; Chen *et al*., 2007).

The expression of CEACAM affects the expression of other factors known to play a role in vasculogenesis (Gu *et al*., 2009) such as the VEGFs. Therefore variants of CEACAM 1 (rs8110904 and rs8111171) could be said to play a role in lymphedema development.

5.5.6 Interleukin 10

In this study there was a single marker association between interleukin 10 SNP, IL-10 rs1800872 and lymphedema development. Patients having IL-10 rs1800872 TT genotype stand a higher risk of 1.5 times developing lymphedema than those with GG genotype. Much the same way patients with the T allele stand a high chance of developing lymphedema than patients with the G allele. Although the serum levels of IL-10 were not measured in the present study, Wang *et al.* (2011) measured the serum levels in patients with Crohns disease (CD) and observed that IL-10 concentration was significantly higher in CD patients than in the controls and that the T allele of rs1800872 was associated with increased serum IL-10 levels.

Haplotype analysis was done with IL-10 genes from the sequenom. From the two SNPs, three haplotypes were observed. The CT haplotype was not significant but the TT haplotype was significantly higher in the cases than in the controls. With strengthening of the significant single haplotype using 200,000 simulations, it is an indication that those SNPs move together and can cause an expression.

IL-10 cytokine has pleiotropic effects in immunoregulation and inflammation (Wang *et al*., 2011). It down-regulates the expression of Th1 cytokines, MHC Class II antigens, and costimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. This cytokine can block NF-kappa B activity, and it is involved in the regulation of the JAK-STAT signaling pathway. Other studies have provided evidence for the involvement of IL-10 in the pathogenesis of Crohn's disease (CD) (Wang *et al*., 2011).

Regarding filariasis, immunological studies done by other authors have shown that reduced IL-10 secretion leads to more inflammatory cells which kill microfilariae, but could also damage host tissue (Doetze *et al.*, 2000; Specht *et al.*, 2004). Because of this strong association of IL-10 with filarial disease, Pfarr *et al*. (2007) examined three promoter SNPs of IL-10 (-1082, -819, -592) in Indonesian patients with lymphatic filariasis and found that individuals with the variant forms of the -819 and -592 SNPs, which express less IL-10, had lower levels of circulating microfilariae (Pfarr *et al.*, 2007). This fits well with data from other studies that immune cells from patients with the variant SNPs secrete less IL-10 (Timmann *et al.*, 2004; Yilmaz *et al.*, 2005). Thus, patients with these two variant SNPs seem to be predisposed to higher microfilaremia (Pfarr *et al.*, 2007). Since lymphedema is an inflammatory disease, it is possible that SNPs in an inflammatory cytokine IL-10 may be involved in this pathology.

5.5.7 TIMP metalloprotease inhibitor 2

Tissue Inhibitors of Metallo Proteases 2 (TIMP 2) is a natural endogenous inhibitor that can suppress capillary endothelial cell function. It provides a system to modulate and balance the complex interplay between the reparative and degradative processes and maintain homeostasis of the extracellular matrix (Bedi *et al*., 2009). It is also an angiogenic factor (Bennuru and Nutman 2009).

TIMP controls the activity of Matrix metalloprotease (MMP). It can bind to zymogen or inhibit the activated forms of MMP (Nagase *et al.*, 1996; Stevenson *et al*., 2006). The induction of tube formation by filarial antigens is dependent on regulation of TIMP-1 and TIMP-2 which in turn is associated with increased levels of MMP-1 and MMP-2 (Bennuru and Nutman 2009).

TIMP-2 rs2277698 SNP was found to have single marker association with lymphedema development (p=0.035) in this study. Since there is no other SNP on the TIMP gene haplotype analysis was not done.

5.5.8 Summary and Conclusion

A cross-sectional study was designed to genotype 1306 unrelated Ghanaian volunteers comprising 266 lymphedema patients, 691 infected patients but without pathology and 349 endemic controls for single nucleotide polymorphisms (SNPs). In all, 132 SNPs from 64 genes were genotyped and analyzed. A single marker analysis done with the famhap statistical software in this study showed associations with 11 SNPs in eight genes which included: vascular endothelial growth factor receptor-3 (VEGFR3), insulin like growth factor-1 (IGF-1), matrix metallopeptidase-2 (MMP-2), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (NFKB- inhibitior alpha), carcinoembryonic antigen-related cell adhesion molecule -1 (CEACAM-1), interleukin-10, tissue inhibitor of metallopeptidase 2 (TIMP) genes and interleukin-17. Apart from the interleukin-10, all the other mentioned genes were associated with lymphangiogenic/angiogenic modulations, which is a hint that lymphedema development may be associated with lymphangiogenic/angiogenic factors.

One of the major thrusts is the diagnosis of an individual's predisposition to common diseases which have partly genetic origin. This will allow preventive measures to be adopted before the person even develops the symptoms of the disease.

CHAPTER 6

6.0 General Discussion, Summary, Conclusion and Recommendations

6.1 General Discussion

Diethylcarbamazine (DEC), the only antifilarial drug that has partial macrofilaricidal activity cannot be used in Africa because of adverse effects associated with its administration in areas coendemic of Loaisis and onchocerciasis (Molyneux *et al.*, 2003; Dadzie *et al.,* 2003). Targeting *Wolbachia* endosymbionts in the filarial worm by doxycycline showed a macrofilaricidal effect and reduced the plasma levels of lymphangiogenic molecules with amelioration of pathology of lymphatic filariasis (Debrah *et al*., 2006; 2007a; 2009). However, the promising results obtained with doxycycline were achieved when used for 4, 6 and 8 weeks.

With 4 weeks 200mg/d doxycycline set as the gold standard, the present study also found 5 weeks 100mg/d, 4 weeks 100mg/d and 3 weeks combination therapy of 200mg/d doxycycline and 10mg/kg rifampicin as effective in depleting *Wolbachia*. Significant microfilarial depletion was also observed in the 4 weeks and 5 weeks 100mg/d doxycycline, as well as the 3 weeks combination therapy groups up to 24 months follow up time point.

Significant macrofilaricidal effect was also observed in the 5 weeks and 4 weeks 100mg/d doxycycline groups, as well as the 3 weeks combination treatment group.

However, the 2 weeks combination regimen was neither effective in sterilizing the adult worms nor had macrofilaricidal effect. This is in contrast to the pilot work that was done in the same area (Debrah *et al*., 2011).

Few studies have shown associations of SNPs with either susceptibility or protection to the disease or severity of pathology in lymphatic filariasis (Choi *et al*., 2001). Host genetics has been shown to be an important determinant of the intensity of infection and morbidity due to human helminths. Epidemiological studies of a number of parasite species have shown that the intensity of infection (worm burden) is a heritable phenotype; and in lymphatic filariasis, there is some evidence for the genetic control, though little information on the disease manifestations is available (Quinnell, 2003).

In this study, important associations between SNPs and lymphedema were found. The associated SNPs with lymphedema were FLT4/VEGFR3 rs75614493, MMP-2 rs1030868, MMP-2 rs2241145, CEACAM-1 rs8110904, CEACAM-1 rs8111171, IGF-1 rs7136446, NFKB-inhibitor alpha rs696, IL-10 rs1800872, and TIMP-2 rs2277698. Aside the single SNP influence on lymphedema development, haplotype analysis also revealed that there wereinter-SNP associations, and specific allelic combinations may contribute to the development of lymphedema as well. The data gathered from this study indicate that lymphedema is a complex and polygenic disease, and therefore, more cytokine SNPs, especially angiogenic, vasculogenic and inflammatory SNPs might be involved.

One of the challenges facing genetics in SNP investigations is the analysis of complex traits and diseases. Complexity arises because there is no simple correspondence between genotype and phenotype (Lander and Schork, 1997).

One way of zeroing in on the candidate genes is through genetic association studies that compare the prevalence of a marker (or a set of markers) between affected and non-affected individuals (De Angelis, 1999). Genotyping large number of SNPs in linkage and association studies is becoming increasingly popular and important, and will shed new light on the understanding of complex disease traits, including many human diseases and drug responses (Roses, 2000). SNPs genotyping is also easier to automate because SNPs can be screened in a digital format by analyzing the presence or absence of a sequence as done in this study.

In addition, organizations such as the National Human Genome Research Institute (http://www.nhgri.nih.gov/) and the SNPs Consortium (Marshall, 1999) are creating public databases cataloguing several hundreds of thousands of these markers. These databases, coupled with efficient methodologies to detect SNPs and the completion of mapping of the human genome, is facilitating the identification of genetic markers responsible for complex disorders such as diabetes, hypertension (De Angelis, 1999), lymphedema and hydrocele, and also drug response genes in non-familial studies (Shi, 2001).

Identification of such genetic markers in high risk members of filarial pathology (lymphedema) families could facilitate the identification and management of environmental factors that influence the expression and severity of lymphedema.

Such knowledge will also be beneficial in terms of diagnosis and possible therapy of various forms of the pathologies associated with lymphatic filariasis. In particular, the results may permit better informed genetic counseling in affected families, early diagnosis and treatment, and the development of more targeted and effective therapeutic regimens for lymphatic filariasis. Knowing genetic markers for LE and 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 that might be applied to school aged children.

With the completion of the human genome map and the SNP map, genotyping SNPs in large scale complex diseases and pharmacogenetic studies will be an integral part of healthcare system. Advanced technologies to identify genetic polymorphisms rapidly, accurately and economically are becoming a priority in the implementation of pharmacogenetics to drugs development, clinical trials, and clinical monitoring for drug efficacy and toxicity.

6.2 Summary and Conclusion

Treatment of lymphatic filariasis with 200mg/d doxycycline has been shown to be effective in the long-term sterility of adult worms as well as having macrofilaricidal effect. The ideal treatment regimen of lymphatic filariasis was assessed with reduction of dosage from 200mg/d to 100mg/d for 5 weeks and 4weeks, and combination of 200mg/d doxycycline plus 10mg/kg rifampicin for 2 weeks and 3 weeks.

The various regimens proved effective in causing *Wolbachia* and microfilarial depletion, with the exception of the 2 weeks combination group.

Comparison done with the standard as well as the placebo for macrofilaricidal activity also showed the various regimens were macrofilaricidal except the 2 weeks combination group.

6.3 Recommendations

It is now known that 4 weeks 100mg/d doxycycline is effective for the treatment of LF. This regimen is therefore recommended, because at this reduced dosage, one does not need to have the liver and kidney function tests done before treatment. Also 3 weeks combination of 200mg/d doxycycline plus 10mg/kg rifampicin could be used when a shorter duration of treatment is preferred.

Since doxycycline cannot be used for mass treatment due to the long duration of treatment (at least 3 weeks), it can be recommended in certain specific situations such as:

- the use of doxycycline to precede anti-filarial drugs to deplete *Wolbachia* that is known to cause adverse reactions in infected patients.
- when infected people are leaving endemic zones and want to be free of the worm.
- with the report of non-respondance to ivermectin in Ghana (Osei-Atweneboana *et al*., 2007), doxycycline can be recommended for use in case of ivermectin resistance in LF.
- people living outside endemic areas who have been infected.

6.4 Future research recommended

The data gathered from this study indicate that lymphatic filariasis is a complex and polygenic disease, and therefore;

- further SNPs and phenotypes in genes of the angiogenesis pathway should be elucidated.
- SNPs already identified in this study should be functionally characterized.
- This study should be replicated in different geographical populations to ascertain whether these same SNPs are associated with filarial pathology elsewhere.

This study has shown that management of lymphedema could be looked at from the lymphangiogenic/angiogenic point of view. Therefore, any further effort directing at diagnosis and drug development for pathology of LF should take into consideration the lymphangiogenic/angiogenic pathway and associated molecules.

CHAPTER 7

7.0 References

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CHAPTER 8

8.0 Appendices

Appendix 1: Questionnaire for selecting lymphedema patients

Name:

Patient ID:

Sex: male ☐ female ☐

Age:

Duration of stay in village:

When did swelling of feet start?

Did swelling go back in morning when you wake up?

Does swelling go back in the mornings now?

Do you get acute attacks?

How often do you get acute attacks?

How long does the attack last?

What form of treatment do you seek when you get the attacks?

Do you know of any one in your family with this condition?

Relationship to that person?

Appendix 2: List of genes genotyped

	Gene name	Rs number	Amino acid change	Gene region
1	TLR 9	352139	NONE	Promoter
2	TLR 9	5743836	NONE	Promoter
3	TLR 2	3804099	199 N/N	Coding exon
4	TLR 2	3804100	450 S/S	Coding exon
5	TLR 2	893629	NONE	Promoter
6	TLR 2	4585282	NONE	Intron
7	TLR 2	7656411	NONE	Downstream
8	TLR 4	5030725	NONE	Intron
9	IL-5	2069807	NONE	Promotor
10	IL-5	2069812	NONE	Promotor
11	IL-5	2069818	128 T/T	Coding exon
12	IL-23R	7530511	310LEU/PRO	Coding exon
13	IL-23R	1884444	3Q/H	Coding exon
14	NFKB INHIBITOR ALPHA	28933100	SER 32 ILE	Coding exon
15	NFKB INHIBITOR ALPHA	696	NONE	3' utr
16	NFKB INHIBITOR ALPHA	2233406	NONE	Promotor
17	NFKB INHIBITOR ALPHA	3138053	NONE	Promotor
18	MYD88 (ACAA1)	4988453	NONE	Promotor
19	ENDOSTATIN (COL18 A1)	12483377	1257 D/N	Coding exon
20	ENDOSTATIN (COL18 A1)	62000965	259 ARG/LEU	Coding exon
21	ENDOSTATIN (COL18 A1)	76547444	517 GLY/SER	Coding exon
22	ENDOSTATIN (COL18 A1)	62000962	841 VAL/ILE	Coding exon
23	I-CAM	5498	469 LYS/GLU	Coding exon
24	I-CAM	5491	LYS56MET	Coding exon
25	V-CAM	3783615	624I/L	Coding exon
26	V-CAM	1041163	NONE	Promotor
27	V-CAM	3176879	LYS644 LYS	Coding exon
28	VEGF-C	7664413	NONE	Intron boundary
29	VEGF-C	41278571	ARG61GLU	Coding exon
30	VEGF-C	55728985	GLU47 VAL	Coding exon
31	VEGF-C	6838834	NONE	Intron
32	VEGF-D	5980152	NONE	Promoter
33	VEGF-D	4830943	NONE	Promoter
34	ANG-2	2515475	NONE	Intron
35	ANGPT 2	7814961	32LYS/ASN	Coding exon

36	BFGF	1449683	NONE	Coding exon
37	VEGF-R1	78147150	LYS861GLU	Coding exon
38	VEGF-R1	35832528	982E/A	Coding exon
39	VEGF-R3	35874891	ASN527SER	Coding exon
40	VEGF-R3	75614493	GLY1154ARG	Coding exon
41	TIMP-1	61756234	MET65LLE	Coding exon
42	TIMP-2	2277698	SER101SER	Coding exon
43	CEACAM-1	8111171	GLN35GLU	Coding exon
44	CEACAM-1	8110904	GLN83LYS	Coding exon
45	LYVE-1	746164	NONE	Intron
46	LYVE-1	16907980	THR214LLE	Coding exon
47	PROX-1	77561623	SER150ARG	Coding exon
48	PROX-1	78142452	THR479LLE	Coding exon
49	MMP-2	11643630	NONE	Promoter
50	MMP-2	2241145	NONE	Intron
51	MMP-2	1992116	NONE	Intron
52	MMP-2	1030868	NONE	Intron boundary
53	MMP-9	17576	GLN279ARG	Coding exon
54	TBK-1	17853341	LYS570GLN	Coding exon
55	TBK-1	35635889	VAL464ALA	Coding exon
56	GJA4	1764391	PRO319SER	Coding exon
57	CD 36	1334512	NONE	Promoter
58	CD 36	3211938	TYR325 X	Coding exon
59	ANGPTL4	1044250	THR266MET	Coding exon
60	CTGF	6918698	NONE	Promoter
61	OLFML3 (OLFACTOMEDIN-LIKE 3)	35317518	ARG187SER	Coding exon
62	OLFML3 (OLFACTOMEDIN-LIKE 3)	11553080	ASP46GLU	Coding exon
63	NOD2/CARD15	3135500	NONE	3'utr
64	NOD2/CARD15	5743291	VAL955LLE	Coding exon
65	NOD2/CARD15	1000331	NONE	
66	NOD 1	2075820	GLU266GLN	Coding exon
67	NOD 1	736781	NONE	Intron
68	ATG 16 L1	2241880	197T/A	Coding exon
69	IL-23R	7517847	NONE	Intron
70	5-LO	2228065	NONE	Coding exon
71	CAVEOLIN-1	4730751	NONE	Intron
72	CAVEOLIN-1	926198	NONE	Intron

73	CAVEOLIN-1	4730748	NONE	Intron
74	MBL-2	7095891	NONE	Promoter
75	MBL-2	10824792	NONE	3' utr
76	FGF-2	308447	NONE	Promoter
77	FGF-2	303379	NONE	Intron
78	HGF	74657718	NONE	Intron
79	IGF-1	2946834	NONE	Downstream
80	IGF-1	7136446	NONE	Intron
81	DESMOPLAKIN	2076299	1512Y/C	Coding exon
82	DESMOPLAKIN	6929069	1738R/Q	Coding exon
83	BETA-CHEMOKINE RECEPTOR D6	2228468	TYR373SER	Coding exon
84	BETA-CHEMOKINE RECEPTOR D6	6779520	LEU311VAL	Coding exon
85	COL1A1	2075555	NONE	Intron boundary
86	COL1A1	2269336	NONE	Near gene five
87	COL1A2	42524	549PRO/ALA	Coding exon
88	COL1A2	1034620	NONE	Downstream
89	CPLA2 ALPHA	3820185	NONE	Intron
90	CPLA2 ALPHA	12749354	NONE	Intron
91	GATA 2	2335052	164 A/T	Coding exon
92	GATA 2	3803	NONE	3'utr
93	GATA 2	2713604	NONE	Intron
94	GATA 3	570613	NONE	Intron
95	GATA 3	3803604	NONE	Intron
96	IL-10	1800896	NONE	Promoter
97	IL-10	1800871	NONE	Promoter
98	IL-10	1800872	NONE	Promoter
99	IL-6R	2228145	ASP358ALA	Coding exon
100	VEGFR-2	2305948	VAL297ILE	Coding exon
101	VEGFR-2	1870377	HIS472GLN	Coding exon
102	VEGFR-2	1531289		Intron boundary
103	TLR-6	13102250	Trp105leu	Coding exon
104	TLR-6	5743810	Pro249ser	Coding exon
105	TLR-6	5743813	Tyr456his	Coding exon
106	IL-4	2243250	None	Promoter
107	IL-13	20541	Arg110gln	Coding exon
108	IL-13	2069739	None	Promoter
109	IL-13	1800925	None	Promoter

110	IL-13	2069743	None	Promoter
111	ENDOTHELIN	5370	Lys198asn	Coding exon
112	ENDOTHELIN-1	1800541	None	Promoter
113	ENDOTHELIN-1	35104761	-1his82pro	
114	TNF	1800629	None	Near gene 5
115	TNFA	1799964	None	Near gene 5
116	TNFA	1799724	None	Near gene 5
117	VEGF-A	833061	None	Promoter
118	VEGF-A	2010963	None	5'utr
119	VEGF-A	1570360	None	Promoter
120	IL-17F	763780	His161arg	Coding exon
121	IL-17A	2275913	None	Promoter
122	IL-6	1800796	None	Promoter
123	IRF5	4728142	None	Promoter
124	IRF5	3807306	None	Promoter
125	TLR4	4986790	Arg229gly	Coding exon
126	IL-18	187238	None	Promoter
127	IL-18	1946518	None	Promoter
128	IL-18	360718	None	5'utr
129	IL-18	1946519	None	Promoter
130	IFN-G	2069720	None	Intron
131	IFN-G	1861494	None	Intron
132	IFN-G	2069707	None	Promoter
133	DECORIN	17018909	Leu64val	Coding exon
134	DECORIN	3138268	Met268thr	Coding exon
135	DECORIN	1803344	Gln273glu	Coding exon
136	DECORIN	1803342	Pro299leu	Coding exon
137	EOSINOPHIL CATIONIC PROTEIN	2073342	Thr124arg	Coding exon
138	IL-4R	1805010	75i/?	Coding exon
139	IL-4R	1049631	Non e	3'utr
140	IL-4R	1805015	Ser503pro	Coding exon
141	TGF-BETA	1800469	None	Promoter
142	TGF-BETA	1800468	None	Promoter
143	TGF-BETA	1800470	Leu10pro	Coding exon
144	TGF-BETA	1800471	Arg25pro	Coding exon
145	FOXP3	55711326	Ser44thr	Coding exon
146	FOXP3	17847095	Val52gly	Coding exon
147	FOXP3	2232369	Val220a	Coding exon

Appendix 3: Results of the Assay Design

WELL	SNP ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W1	rs6779520	acgttggatggaagcggtgactggagaag acgttggatgtccaggtaacagagagcatc e-tgcttttcccccatc	97	45.8	4429.9
W1	rs2075820	acgttggatgacctgctcttcaagcactac acgttggatgaagcgcagcaggaaggcaa aggcaaacacctcct	90	47.4	4506
W1	rs3138053	acgttggatggtgttcataagtagctattcg acgttggatgcagaagtaggctcacgatcc ttctgcgggagcaca	100	51.1	4593
W1	rs78142452	acgttggatgaatggataggccatcaaggg acgttggatgtctctgccaccacgggctt agcttcaccacgtcca	93	48.9	4786.1
W1	rs35874891	acgttggatgtctcccagactgtgagcaag acgttggatgtgttggagaccacacacttg gtacatggcagacacg	95	47.1	4915.2

1st-PCRP - First PCR primer

2nd-PCRP - Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism

AMP_LEN - Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W1	rs5491	acgttggatgtgacatgcagcacctcctgt acgttggatgggagcaactccttttaggc tctctatgcccaacaac	95	46.3	5074.3
W1	rs1764391	acgttggatggcctctatacatactgcttc acgttggatggacccacccccctcagaatg ctcagaatggccaaaaa	99	45	5196.4
W1	rs35832528	acgttggatgggaagataaaagtctgagtg acgttggatgaatatctcagcgcgtaggac caatacctaccctcctct	98	45.4	5314.5
W1	rs4585282	acgttggatgccaactcagcattcacttac acgttggatgtgtggaagaaaaagccactg aagccactgtacaaagtc	91	47.4	5476.6
W1	rs2713604	acgttggatgctgacccttttaagcagag acgttggatgttgcctctgctcaggaggtg actaggacagggtctcgg	100	50.5	5564.6
W1	rs1034620	acgttggatgtgatccaggcacaaggatag acgttggatggaattgttagctcagcttcc ccctaaccctctgatcaag	93	46.3	5692.7

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W1	rs5030725	acgttggatgagccccaaggttgacagaaa acgttggatgttcatctctgttctccaacc acccaaccatggaagctat	100	46.5	5765.8
W1	rs763780	acgttggatgagtggatatgcacctcttac acgttggatggaaggtgctggtgactgttg cttcacccctgtcatccacc	97	54.2	5908.8
W1	rs3803	acgttggatgctggagttcggctatttcag acgttggatgagatttgtggggacctcagc gtagcctgcaccctcttctc	90	54.4	5979.9
W1	rs7136446	acgttggatgacctgctacattgaaagcac acgttggatgttgaaggttgcaactttgcc ctcattttcacatactacgc	100	45.4	5986.9
W1	rs7814961	acgttggatgcaactttcggaagagcatgg acgttggatggaggaaagtgtagctgcagg acatgctggacctgatattg	99	48.3	6132
W1	rs5980152	acgttggatgtgcctcctggtagattaggg acgttggatgccttttgtctaattgctggg tgattgctgggtctgaactc	100	49.7	6139

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W1	rs11553080	acgttggatgatcccttcaggaacggctgg acgttggatgtcttgttcttgaagtcccgc ctcagcatgccgactactctg	100	53	6342.1
W1	rs746164	acgttggatgactctgtcatattgcaactg acgttggatggcttctttgcttcatgcttg ggtgtagaatcattggccttg	99	47.8	6492.2
W1	rs61756234	acgttggatgaaaatgctgggaaagaggcg acgttggatgcaaccagaccaccttatacc ggagcgttatgagatcaagat	100	46.2	6534.3
W1	rs2076299	acgttggatgctggaagatgaaaacgcgag acgttggatgtattgtctccgtcgcactac cctgtttgctttctgcaggtca	98	54.1	6683.3
W1	rs1030868	acgttggatgtgaaagaaatgcacaccccc acgttggatgctgattgctacagcctgctt ttgctacagcctgctttggtca	99	57.7	6692.3
W1	rs17576	acgttggatgaactggcagggtttcccatc acgttggatgttctcccccttttcccacatc aatcgccccaggactctacaccc	99	60.8	6898.5

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_ LEN	Tm(NN)	UEP_ MASS
W1	rs8110904	acgttggatgtgtctctcgaccgctgtttg acgttggatgtggcaaccgtcaaattgtag caaccgtcaaattgtaggatatg	99	49.9	7071.6
W1	rs2241880	acgttggatgtgtctcttccttcccagtcc acgttggatgagtagctggtaccctcactt ttgtgtaccagaaccaggatgag	99	48.1	7112.6
W1	rs2069720	acgttggatggacctatagttactcatttg acgttggatgcctctctatcatcaatctacc atatcactttatacttcagtcttt	100	45.8	7242.7
W1	rs5498	acgttggatgttgagggcacctacctctgt acgttggatgctcacagagcacattcacgg tacagagcacattcacggtcacct	100	59	7281.8
W1	rs75614493	acgttggatgagacgccgcatcatgctgaa acgttggatgtctccaccagctccgagaat aatgcaggtctcgccttggggtctc	93	63.1	7665
W1	rs1946518	acgttggatgtgctgtatcagatgcaagcc acgttggatgactttctgttgcagaaagtg ggtgcagaaagtgtaaaaattatta	100	45.1	7777.1

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W1	rs5743813	acgttggatgaatgtgcttggtgcatgagg acgttggatggttaaccatttcagatacacc taaccatttcagatacacctttttata	80	48.7	7863.2
W1	rs6907980	acgttggatggtttttatggaaactagcacc acgttggatgcagcttcattcttgaatgc agttttcaacaaatggttcagtttct	99	50.5	7950.2
W1	rs1800925	acgttggatggggtttctggaggacttcta acgttggatgtgcagccatgtcgccttttc aatgtcgccttttcctgctcttccctc	80	61.1	8079.2
W1	rs696	acgttggatgaacaaaatgagggctgatcc acgttggatggtgtacttatatccacactgc ggcttatatccacactgcacactgcct	94	57.2	8155.3
W1	rs4988453	acgttggatgttccacatctcttttctac acgttggatgcttaccaactcagttttccc tccctttcaattgtaaaagggaaatag	100	52.3	8306.4
W1	rs2280883	acgttggatgatgggtgttacaaggaaagg acgttggatgccaacgtaccccagtatgtc tgtgtcaatacacccccaactgggcacc	90	63.3	8478.5

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W1	rs2228145	acgttggatgtctcctcttcctcctctatc acgttggatgcaggaatgtgggcagtggta ggaaggggcagtggtactgaagaagaa	100	54.8	8511.5
W1	rs1449683	acgttggatgtctccccaggcggcgtcc acgttggatgcctggtgcgcggcgagcc agccggcggcccgggacctggggttcac	96	74.8	8632.6
W1	rs1531289	acgttggatgctgaatgctgaaaataagcac acgttggatggtatccttatttagcatctc cttatccttatttagcatctcacctcgtc	97	54.3	8704.7
W2	rs833061	acgttggatgtgggtgagtgagtgtgtgcg acgttggatgttggaatcctggagtgaccc tctccccgctccaac	97	50.9	4408.9
W2	rs3138268	acgttggatgcaggtactctggtaagcttg acgttggatgtgttgacaatggctctctgg gctctctggccaaca	100	48.6	4512.9
W2	rs2228065	acgttggatgtgcaaccctgtgttgatccg acgttggatgaggctgcactctaccatctc tcaccgggagcttct	100	49.6	4544

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W2	rs308379	acgttggatgtaggtagcatggagtgtagg acgttggatggtatctccagcctcatttag cctcatttagtccccc	98	45.4	4728.1
W2	rs2232369	acgttggatgtgcccttctcatccagaaga acgttggatgctgtcgtggtcacctgcat attaggcactgccagg	97	48.3	4906.2
W2	rs1800468	acgttggatgactgtgccatcctcccccat acgttggatgtggagtgctgagggactct aggactctgcctccaac	100	49.2	5115.3
W2	rs7530511	acgttggatgtaagtacactcacctgtttc acgttggatgaacaggcaaaaggtactggc gcagccttggagttcac	95	50.9	5186.4
W2	rs78147150	acgttggatggttgaaagccaaactacagc acgttggatgaagaaatcacctacgtgccg gacggactgtggctgtg	94	49.7	5282.4
W2	rs2277698	acgttggatgtttatctacacggccccctc acgttggatgcctgcaatgagatattccttc ctctcctccaacgtccag	100	47.4	5355.5

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_ LEN	Tm(NN)	UEP_ MASS
W2	rs4728142	acgttggatgacttccaggtcacaccccaa acgttggatgccttcctccccatttcttac ccccatttcttactaacac	80	45.4	5642.7
W2	rs12483377	acgttggatgtaccaggtggggtgcctcag acgttggatgctctgagggtccgctgaag cacacgcatcttctccttt	98	47	5649.7
W2	rs1805015	acgttggatgtctgggacacggtgactgg acgttggatgagagacgcccctcgtcatc ggggcgcagcttcagcaac	99	48.4	5838.8
W2	rs1800541	acgttggatgtcttactgggccactgtg acgttggatgcaggttagacaactgagcac attctgtttgttctccacca	91	45.3	6008.9
W2	rs17853341	acgttggatgctgttaaattgcatgacagag acgttggatggcaaattttacttactacg ttacttactacgttctgctt	93	46.5	6023.9
W2	rs3802604	acgttggatgttctttttgactccaccagc acgttggatgaacccagacctgcatgttc cagtacatgaaagtaggcag	99	48.2	6199.1

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_ LEN	Tm(NN)	UEP_ MASS
W2	rs2069743	acgttggatgcccagaaagacctctgaatc acgttggatgccactgtgagagggattgtc agggattgtcaaagttcaga	100	49.5	6205.1
W2	rs3176879	acgttggatgcatgaaggggtcatatagtc acgttggatgctccagttgaacatatcaagc aagcattagctacacttttga	95	48.3	6404.2
W2	rs3820185	acgttggatggaagcagtgagtctgtgcg acgttggatgactgaaatccatctgaccac caaaaaacactctctcttctca	100	48.8	6582.3
W2	rs352139	acgttggatggtgggagggctgtgtgagt acgttggatggaagatgctagaagatgccc ttacgtggagtgggtggaggt	98	53.9	6613.3
W2	rs12749354	acgttggatgtatggcccattctgaatacc acgttggatgtcaatggactacatttgggg aattgttttaagctcactgtca	95	47.2	6699.4
W2	rs2228468	acgttggatggactggcatgaatgaccttg acgttggatgctcaggctgatttattcccc ggggacatcctccttgttaggg	100	48.5	6782.4

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W2	rs6929069	acgttggatgaagaagaactgcggaacctg acgttggatggcatttttatcactgtccgc agtgatcactgtccgcttcgctt	99	55	6981.5
W2	rs76547444	acgttggatgaacacctggctccccctttc acgttggatgcgagccctgtgttctgttta aacattctgtttattcccagggc	83	50.2	6989.6
W2	rs1800469	acgttggatgagggtgtcagtgggaggag acgttggatggagcaattcttacaggtgtc ggctgcctcctgacccttccatcc	100	59.5	7176.6
W2	rs3804099	acgttggatgtatgctgcttcatatgaagg acgttggatggatctacagagctatgagcc aaacagtttgaagtcaattcagaa	99	45.4	7392.9
W2	rs4830943	acgttggatgcatatatacacttttcagta acgttggatgctgacggaactacagttctc actgaactacagttctccttaattt	100	46	7565.9
W2	rs1000331	acgttggatgctctgtgactcagttgcatc acgttggatgtttcagcacttcacctatac cacatttaggtaatgttatccttct	100	49.1	7597

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W2	rs2515475	acgttggatgtatggcatctcccctctcat acgttggatgcctaaatgctacttcagc gggcaaggatcaagaaatgctagac	99	48.1	7773.1
W2	rs7664413	acgttggatgttcatgccacacccagaacc acgttggatgcatggaatccatctgttgag aaatagtcagagaatctttactatac	100	46.5	7961.2
W2	rs2069812	acgttggatgcttgggcacctttcccattg acgttggatgcctgctgctcatgaacagaa gggtctgctcatgaacagaatacata	100	48.2	8003.2
W2	rs3807306	acgttggatgagcccacagtgagtctggtt acgttggatgcccaggggaaacgaaagtg ggccggggaaacgaaagtggctagac	80	55.8	8119.3
W2	rs7656411	acgttggatggtacatgtgagctaaatag acgttggatggtatcaaactatttttgagtc gggagattttgagtcattatgaggaa	98	46.1	8433.5
W2	rs1041163	acgttggatgaaggacctctgggttacttg acgttggatgcacctaccaaatatctaggg ccccccctaccaaatatctagggatcaga	97	51.2	8486.5

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W2	rs187238	acgttggatgacaggttttggaaggcacag acgttggatgcttcttttaatgtaatatcac taatgtaatatcactattttcatgaaat	100	46.3	8559.6
W2	rs28933100	acgttggatgctgaagaaggagcggctac acgttggatgatctgctcgtactcctcgtc gggatcgtctttcatggagtccaggccg	97	61	8636.6
W3	rs55711326	acgttggatgtcagacctgctgggggcccg acgttggatgaagaggaggcatgggccccg tctcggccctggaag	99	50.5	4569
W3	rs6918698	acgttggatgctgatagaacaatggatccc acgttggatgggagttttatataggcaagg ggcaaggacaaggga	96	48.3	4700.1
W3	rs2305948	acgttggatgagtctgggagtgagatgaag acgttggatggtacaatccttggtcactcc gggtcactccgggtta	97	47	4913.2
W3	rs2241145	acgttggatgcagaatggacagtgcccttg acgttggatgcaacaaaatgattgacacc ccttgacacctggcaca	98	48.1	5115.3

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_ LEN	Tm(NN)	UEP_ MASS
W3	rs1805010	acgttggatgtgtgtctgcagagcccac acgttggatgacgtcatccatgagcaggtg tccgttgttctcaggga	100	50.4	5192.4
W3	rs20541	acgttggatgtgatgctttcgaagtttcag acgttggatgccagtttgtaaaggacctgc acttttcgcgagggac	100	50.2	5201.4
W3	rs62000962	acgttggatgagcaatgccctctctgcca acgttggatgcatgtgacccttcagggag aagttggagcagatgga	99	48.2	5323.5
W3	rs41278571	acgttggatgattctgggtagagtacagtc acgttggatgaagcaaagatctggaggagc atctggaggagcagttac	94	48.4	5563.6
W3	rs1861494	acgttggatggcaagggacaatgagagaac acgttggatggttgacaaatccagtgaagag agtaggtgaggaagaagc	99	48.3	5661.7
W3	rs1049631	acgttggatgaggctcggcttctagttcag acgttggatgcaaggcatgttttgcccacc tcctcagatcatggcccac	100	46.3	5708.7

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W3	rs2233406	acgttggatggtggtggttgtggatacctt acgttggatggtaggtcagatagcataaacg gaacacaatagctactctg	100	45.1	5780.8
W3	rs2073342	acgttggatggacctcataaatccaggtgc acgttggatggcaactacatagaacctcct ttcttggtctgtctgcatac	100	45.2	6064.9
W3	rs926198	acgttggatgaagaaaatcacttcccatcg acgttggatgtcttgtgctgaggatgagtc aatacaagatgggcatgttc	94	48.6	6165
W3	rs5743836	acgttggatgttgggatgtgctgttccctc acgttggatgagcagagacataatggaggc cattagacttgggggagttt	100	47.2	6203
W3	rs570613	acgttggatgcacacgccacaacttcagga acgttggatggacagatagagacatttag agagacatttagtatgagga	98	45.1	6229.1
W3	rs1800796	acgttggatgttctgtgttctggctctccc acgttggatgccttgaagtaactgcacgaa cccatgcagttctacaacagcc	100	48	6624.3

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W3	rs6838834	acgttggatggcaattagcacctaatttg acgttggatgcctcaaaccaatctgtac tcaaaccaatctgtacctaaga	98	49.3	6671.4
W3	rs2069807	acgttggatgctcttggttagatgtgtctg acgttggatgcaaacttctgcagtctcaag caaggaaaacatttctcaagtg	100	48.5	6751.4
W3	rs3804100	acgttggatgatttgaacttatccagcac acgttggatgttccagtgtcttgggaatgc ggaggggaatgcagcctgttac	82	51.1	6840.4
W3	rs1870377	acgttggatgtatgcgctgttatctctttc acgttggatgtccacacttctccattcttc tcttggtttgtcactgagacagc	100	51.1	7045.6
W3	rs2010963	acgttggatgtccggcggtcacccccaaaa acgttggatgagagagagcgcgcgggcgt ggatggcgtgcgagcagcgaaag	99	59.8	7203.7
W3	rs1803342	acgttggatgaggtgggcagaagtcacttg acgttggatgctatcgtttcatgttgtagg cctccatgttgtaggttgtctacc	99	48.1	7285.7

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W3	rs2275913	acgttggatgaggaacatgaatttctgccc acgttggatgaaatttccgcccccaatgag aaatgaggtcatagaagaatctct	98	49.1	7408.9
W3	rs4730748	acgttggatgctagattggctaaaagggtc acgttggatgtggccagacttagatctgac aggcctcagtggtcagttttaaatt	99	46.6	7687
W3	rs5370	acgttggatgcttttcatgatcccaagctg acgttggatgggtctgtcaccaatgtgctc ccctccacataacgctctctggaggg	100	55	7892.1
W3	rs3211938	acgttggatggcatttgctgatgtctag acgttggatggacaactattgtttctgcac attatctcaaaaaattgtacatcata	98	45.9	7920.2
W3	rs13102250	acgttggatggtgttttcaagttcaaccag acgttggatgggatggcaggatatcttttg ggggcaactgattatgagataaatcc	96	47.6	8043.3

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W3	rs4730751	acgttggatgctctatcctggtatcttcttg acgttggatgaaagacttgaaataaaacag tgaaataaaacagatatgttaagtagg	100	48	8403.5
W3	rs55728985	acgttggatgaaaccctaacgcagacctac acgttggatgttcgagtccggactcgacct gggaggacgcggagcccgacgcgggcg	94	72.3	8442.4
W3	rs1044250	acgttggatgtgcacggccaggcggctgtt acgttggatgagttctggctgggtctgga ccctctctggagaaggtgcatagcatca	87	56.3	8573.6
W3	rs35635889	acgttggatgctgatacagaaatccaatgtg acgttggatggattacaatgaaactgttcac catgcaactgttcacaaaaagacagaag	82	50.1	8607.6
W4	rs8111171	acgttggatgcctctgcaacattgaatggc acgttggatgttctaaccttctggaacccg ccgcccaccactgcc	92	56.5	4418.9

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W4	rs2069739	acgttggatgtctgagcgggaatccagcat acgttggatgtgtcctcgatggggctcaac tgcttgtgaccctcc	99	48.2	4494.9
W4	rs893629	acgttggatgattcatgagcctgagttcgg acgttggatgatcacctgaaacacaccgtc tccactcctgatgctt	99	47.3	4783.1
W4	rs77561623	acgttggatgtttgctctcaggtgctcatc acgttggatgtgtctttccccttttggcag gcaggcctactatgag	97	45.5	4906.2
W4	rs1800896	acgttggatggacaacactactaaggcttc acgttggatgctggataggaggtcccttac cctatccctacttcccc	95	47.4	4977.2
W4	rs360718	acgttggatgttcccgaagctgtgtagact acgttggatgcagtgcattttgccctcctg ccctcctggctgccaac	100	56.1	5067.3
W4	rs17018909	acgttggatgccttccgctgtcaatgccat acgttggatgggtaggtaaagagaaactgc catcagaacactggacca	99	47.8	5461.6

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W4	rs1800872	acgttggatgcctggaacacatcctgtgac acgttggatgaagcagcccttccattttac gagactggcttcctacag	94	49	5499.6
W4	rs62000965	acgttggatgttcaaactcctcaggcatcc acgttggatgactcaccgtctcctccctga cccctccctgagaagctcc	100	51.3	5669.7
W4	rs10824792	acgttggatgaatgcaccaccctagttcag acgttggatgcctggtagaatagatatccac tccacttgagacagcactat	100	51.2	6061
W4	rs7517847	acgttggatgtaccatctcactgtctcctc acgttggatgttctagcctacatacaggtg cctacatacaggtgtagctg	100	49.6	6117
W4	rs74657718	acgttggatgctgatatttttttcagggc acgttggatgcttgacatgctattgaaggg ggaaccagaggcattgtttt	99	51.6	6172
W4	rs2069818	acgttggatgagactacctgcaagagtttc acgttggatggctgcaacaaaccagtttag tcaactttctattatccactc	100	45	6266.1

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W4	rs2069707	acgttggatgggtatttgacattcttttg acgttggatgtctggaactccccctgggaa taagccccctgggaatattct	95	47.8	6381.2
W4	rs11643630	acgttggatggtaaagaagctggccaagac acgttggatggagtgtggcagtaaggctga tgaaggctgaccagaggtttt	97	52.2	6501.2
W4	rs308447	acgttggatgctgggaaaatacacagtgtc acgttggatgcttttctcattgacagttaag cattgacagttaagatctcttt	100	46.4	6699.4
W4	rs5743291	acgttggatgaagccttggcactgatgctg acgttggatgagagaatcccccaaactcac aaacagagttcttctagcatga	89	46.4	6742.4
W4	rs1334512	acgttggatgaaatatgcaaccacaccccc acgttggatgactgggatctgacactgtag cgtgctttctcttctctttttt	93	47.9	6894.5

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W4	rs35317518	acgttggatgtttccgggcagccatggcaa acgttggatggttagatgggacacagaatg acatcctttgtcttcccaaggctg	99	53.9	7254.7
W4	rs1992116	acgttggatgtaccctaatgccctagttcc acgttggatgaagaatggaaggacagaccc aattaggtcctagatactgcctgc	97	53.6	7327.8
W4	rs736781	acgttggatgcatcagcatggatgttaagg acgttggatgtcccagacaccacagatttc ggggctttcacccttatgttgtcct	98	49.5	7605.9
W4	rs2269336	acgttggatgacatggaggaagaaaggacg acgttggatggaaggacagcaggctatatc gagaaggctatatctaaggaagatg	85	46.3	7803.1
W4	rs35104761	acgttggatgctctttggataataggcacg acgttggatgggaagtaaattctccaaggc ccccactagggcttccaagtccatac	99	52.5	7836.1
W4	rs1884444	acgttggatgaaagggctattactgcatcc acgttggatgttttccagagggaaacagtc aacggcctgcttccagacatgaatca	100	53	7924.2

1st-PCRP - First PCR primer **2nd-PCRP -** Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism **AMP_LEN -** Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism

Appendix 3 continued

WELL	SNP_ID	1st-PCRP 2nd-PCRP UEP_SEQ	AMP_LEN	Tm(NN)	UEP_ MASS
W4	rs3783615	acgttggatgcctccttaataatacctgcc acgttggatgcaagactatatgacccctc gggtgcttttcttgcaaagtaaatta	98	46.2	8015.2
W4	rs3135500	Acgttggatggatgtgtgaaaactggtta acgttggatgggccatgttgtctataagag ataaacttaagacagtaaaacaaagtga	99	50.1	8654.7

1st-PCRP - First PCR primer

2nd-PCRP - Second PCR primer

UEP_SEQ - Sequence of Unique event polymorphism

AMP_LEN - Amplicon Length

Tm (NN) - Melting temperature

UEP_MASS - Mass of Unique event polymorphism