Reduction in Levels of Plasma Vascular Endothelial Growth Factor-A and Improvement in Hydrocele Patients by Targeting Endosymbiotic Wolbachia sp. in Wuchereria bancrofti with Doxycycline

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Abstract. The treatment for hydrocele is expensive, invasive surgery—hydrocelectomy. A drug that could prevent or improve this condition could replace or supplement hydrocelectomy. In Ghana, 42 hydrocele patients participated in a double-blind, placebo-controlled trial of a six-week regimen of doxycycline, 200 mg/day. Four months after doxycycline treatment, patients received 150 μg/kg of ivermectin and 400 mg of albendazole, which is used for mass chemotherapy in this area. Patients were monitored for levels of Wolbachia sp., microfilaremia, antigenemia, plasma levels of vascular endothelial growth factor-A (VEGF-A) and stage/size of the hydrocele. Wolbachia sp. loads/microfilaria, microfilaremia, and antigenemia were significantly reduced in the doxycycline-treated patients compared with the placebo group. The mean plasma levels of VEGF-A were decreased significantly in the doxycycline-treated patients who had active infection. This finding preceded the reduction of the stage of hydrocele. A six-week regimen of doxycycline treatment against filariasis showed amelioration of pathologic conditions of hydrocele patients with active infection.

INTRODUCTION

Lymphatic filariasis is a devastating and disfiguring disease that is a major public health problem with significant socioeconomic impact in Africa, Asia, the Western Pacific, and the Americas. Approximately 120 million persons are estimated to be infected, and at least 40 million persons have severe physical and psychological conditions caused by this disease.1,2 Approximately 1.3 billion persons are at risk for acquiring this infection in more than 80 countries.3 Ninety percent of these infections are caused by Wuchereria bancrofti and the remainder are caused by Brugia species. Pathologic conditions such as hydrocele, lymphedema, and elephantiasis develop in 10–50% of the infected persons.

Hydrocele is the most common manifestation of lymphatic filariasis,4 and it may be accompanied by thickening of the spermatic cord and changes in the scrotal skin and subcutaneous tissue that include edema, fibrosis, formation of nodules, and oozing of lymph through the skin.5 Scrotal swelling may vary in size and severity.5 Approximately 40% of men actively or previously infected with W. bancrofti exhibit hydrocele, and in Tanzania and coastal areas in Kenya in which this disease is endemic, hydrocelectomy accounts for 15–25% of all surgical cases.5–7 The cause of hydrocele development is still not fully known, in part because the contribution of the W. bancrofti antigens is not well understood and because a proportion of the patients do not have an active infection.10 Nevertheless, this finding has been attributed to factors such as immune responses and immunogenetics of the host.11,12 We have shown that hydrocele may be a genetically predisposed disease13 in which persons who produce high levels of plasma vascular endothelial growth factor-A (VEGF-A) have a greater chance of development of this disease. In that study, an association was observed between plasma levels of VEGF-A and the stage/size of hydrocele,13 which is an indication that VEGF-A may play an important role in development and progression of hydrocele.

Vascular endothelial growth factors are key regulators of endothelial cell function required for vasculogenesis and for physiologic and pathologic angiogenesis.14,15 Vascular endothelial growth factor-A is a major mediator of vascular permeability and angiogenesis, and plays a pivotal role in mediating development and progression of many diseases such as cancer and diabetic retinopathy.16,17 This factor also promotes extravasation of fluid and plasma proteins, including fibrin, from blood vessels into surrounding tissues.18,19 Vascular endothelial growth factor-A may be a major angiogenic factor in development of lymphedema and hydrocele15,20 in lymphatic filariasis. Recent studies have shown that VEGF-C is fundamentally important in lymphatic proliferation, and it is believed to be involved in lymphatic dilation and lymphedema development in patients with lymphatic filariasis.21 In that study, levels of VEGF-C and its soluble receptor (sVEGFR-3) were found to be higher in microfilaremic and lymphedema patients than in healthy patients in the same region, and levels of sVEGFR-3 was significantly higher in lymphedema patients than in microfilaremic patients.

Preceding clinical improvement, mean plasma levels of VEGF-C and sVEGFR-3 in patients treated with doxycycline decreased significantly to a level close to that of healthy patients in the same region and resulted in amelioration of the pathologic conditions of patients with lymphedema.21 This finding was attributed to depletion of Wolbachia sp. in W. bancrofti, which are known to induce pro-inflammatory cytokines22 that are also known to induce VEGFs.23 Similarly, a report24 has also demonstrated that sustained swelling of secondary lymphedema in the mouse tail was as a result of up-regulation of VEGF-C leading to lymphatic hyperplasia; continuous up-regulation of VEGF-C exacerbated the edema because hyperplastic vessels were poorly functional. The inves-
tigators therefore concluded that up-regulation of VEGF-C and lymphatic hyperplasia resulting from dermal lymphatic ligation lead to lymphedema, which is similar to our findings. Another report has shown an association of chyluria (a filarial disease) with increased levels of serum VEGF-A. The current treatment of choice for hydrocele is surgical intervention (hydrocelectomy). However, this procedure is sometimes dangerous and patients refrain from seeking treatment because of the fear of surgery. In addition, this procedure is expensive. Therefore, other options need to be investigated.

Two community studies have suggested that the antifilarial drug diethylcarbamazine (DEC) may have a beneficial effect of reducing the size of smaller hydroceles of filarial origin. However, another study using a randomized, placebo-controlled method reported that DEC was not effective in reducing the size of hydrocele of filarial origin. In addition to these equivocal reports about the effects of DEC on hydrocele, DEC cannot be used in many areas in Africa where onchocerciasis is also endemic. Use of this drug can have severe adverse effects on eyesight when Onchocerca volvulus microfilaria are killed in the eyes. Long-term annual treatment (five years) with ivermectin, the antifilarial drug used against O. volvulus, has also had no effect on the community prevalence of hydrocele in Burkina Faso. Therefore, the search for other interventions to treat patients with hydrocele, either by replacing or supplementing hydrocelectomy, has been recommended. Because hydrocele is believed to be associated with over-production of VEGF-A which has been shown to be reduced by doxycycline treatment prior to amelioration of pathologic conditions of lymphatic filariasis, we investigated the possible use of doxycycline to treat hydrocele patients of filarial origin.

MATERIALS AND METHODS

A placebo-controlled, double-blind study was conducted in the Nzema East District in the western region of Ghana. The study was reviewed and approved by the ethical Committee on Human Research and Ethics of the School of Medical Sciences of Kwame Nkrumah University of Science and Technology, Kumasi, Ghana, and the Research Ethics Committee of the Liverpool School of Tropical Medicine. The study conformed to the principles of the Helsinki Declaration of 1975 (as revised 1983 and 2000). The trial registration number is ISRCTN 14757.

Study population. Persons enrolled from the neighboring villages of Adjan, Domunli, and Akonu in the western region of Ghana took part in the study. No other human filarial species were endemic in these villages. The study site was selected on the basis of an established occurrence of lymphatic filariasis within the surrounding region and clinical observations consistent with symptomatic disease in a proportion of villagers. Written informed consent was obtained from all participants. Persons eligible for participation were male adults in good health, 18–60 years of age, with a minimum body weight of more than 40 kg and without any clinical condition requiring chronic medication. Exclusion criteria included abnormal hepatic and renal enzyme levels (γ-glutamyltransferase > 28 U/L, glutamyl pyruvic transaminase > 30 U/L, creatinine > 1.2 mg/100 mL) assessed by dipstick chemistry, intolerance to doxycycline, alcohol or drug abuse, or antifilarial therapy in the past 10 months.

Randomization of patients and treatment regimens. Patients were randomly assigned to groups using StatView software version 5.0 (Abacus, Grand Rapids, MI) for Macintosh (Apple, Cupertino, CA). Blinding was ensured by the exclusion of persons involved in randomization or tablet packaging in any clinical or laboratory assessment. This study was part of a larger study comprising microfilaricercic and lymphedema patients. Because some hydrocele patients were also microfilaricercic, hydrocele patients were not given a separate randomization block; this accounts for the difference in the number of patients in each block in Figure 1.

A total of 68 hydrocele patients (Figure 1) were recruited and treated with doxycycline in this study. Participants received 2 × 100 mg capsules of doxycycline (Vibramycin®; Pfizer, New York, NY) or matching placebo supplied by the manufacturer daily for 6 weeks. Treatment was done and monitored by a trial clinician in the form of daily observed treatment. Four months after the start of treatment, all participants received a standard oral dose of 400 mg of albendazole (GlaxoSmithKline, Brentford, United Kingdom) and 150 μg/kg of ivermectin (Mectizan®; Merck, Sharp & Dohme, Rahway, NJ). All 68 patients completed the doxycycline treatment without any adverse reactions that warranted stoppage of treatment. However, not all patients were present at all time points. Figure 1 shows the compliance of the patients at various time points.

Measurement and staging of hydrocele by ultrasound. Study participants were examined by a clinician (S.M.) conversant with the symptoms of lymphatic filariasis using physical methods.
and a portable ultrasound machine (180 Plus; SonoSite, Bothell, WA) equipped with a 7.5-MHz linear transducer as described previously. The size (longitudinal and transverse section) of the hydrocele was measured with the ultrasound machine and converted to stage. A four-stage grading scheme as described previously was used as follows: stage 1 = sub-clinical hydrocele; stage 2 = maximal longitudinal and transverse diameters of the hydrocele do not exceed 1.9 and 1.6 cm, respectively (half screen), of the ultrasound machine; stage 3 = maximal longitudinal and transverse diameters of the hydrocele do not exceed 3.8 and 3.2 cm, respectively (full screen); stage 4 = maximal longitudinal and transverse diameters of the hydrocele are greater than 3.8 and 3.2 cm, respectively.

**Determination of microfilarial load.** Microfilarial load was determined by microscopic examination of fingerprick night blood samples as published. Subsequently, eligible patients donated 10 mL of venous blood for accurate quantification using the Whatman (Maidstone, United Kingdom) Nucleopore filter method as described. Blood (7–10 mL) was obtained from each patient 4, 12, and 24 months after the commencement of doxycycline treatment. At each time point, plasma was taken from the remaining sample, aliquoted, and frozen at –80°C until used for analysis of antigenemia and lymphangiogenic factors.

**Determination of Wolbachia sp. levels in microfilaria by polymerase chain reaction.** Wolbachia sp. content was quantified by real-time polymerase chain reaction (PCR) of the W. bancrofti Wolbachia sp. ftsZ gene (AF081198) derived from 500–1,000 microfilariae using a Rotor-Gene™ 3000 (Corbett Research, Inc., Brussels, Belgium) at pre-treatment and 4, 12, and 24 months after doxycycline treatment. Briefly, DNA was extracted using the QIAamp kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. For quantification, primers and a Taqman hybridization probe with the fluorescent dye 6-carboxyfluorescein (Operon, Cologne, Germany) were used to amplify a 286-basepair fragment of the W. bancrofti Wolbachia sp. ftsZ gene. Products were quantified by comparing results with a standard curve of the plasmid containing the W. bancrofti Wolbachia sp. ftsZ gene fragment. The number of Wolbachia sp. per microfilaria was then calculated by dividing the ftsZ gene copy number by the number of microfilaria in the PCR sample.

**Identification of circulating filarial antigen.** For identification of circulating filarial antigen (CFA, filarial adult worm antigen), W. bancrofti antigen was measured with the TropBio enzyme-linked immunosorbent assay (ELISA) kit (TropBio, Townsville, Queensland, Australia). The manufacturer’s protocol was followed except that the samples were diluted 1:20 with the diluent before pipetting into the TropBio ELISA test plates. Pre-treatment, 12-month, and 24-month follow-up samples were tested in duplicate. The optical density at 414 nm was recorded from plasma samples. Antigen units were calculated with a standard curve from standards provided by the manufacturer.

**Determination of plasma levels of VEGF-A.** Plasma concentrations of VEGF-A were measured in hydrocele patients before and 12 months after doxycycline treatment using the VEGF-A Quantikine immunoassay ELISA kit according to the manufacturer’s instructions (R & D Systems, Wiesbaden, Germany). After stopping the reaction, plates were read at 450 nm and 540 nm with a microplate reader (SPECTRAmax® 340PC; Molecular Devices, Sunnyvale, CA).

**Data analysis.** Wolbachia sp. loads in worm tissue and microfilaria were summarized as the geometric mean and median. Differences in median at baseline and subsequent follow-ups were analyzed using the Wilcoxon signed rank test and Mann-Whitney U test. Changes in the degree of antigenemia were calculated as percentages from baseline and analyzed between treatment groups at subsequent follow-up time points by the Wilcoxon signed rank test. Differences in plasma levels of VEGF-A within treatment groups before and 12 months after treatment were assessed using the paired t-test. Comparison of the number of patients with an improved condition in the doxycline and the placebo groups was assessed with the chi-square test. A two-tailed P value < 0.05 was considered significant. All analyses were conducted using tests included in the StatView® software version 5 for Macintosh computers.

### RESULTS

Of the 68 hydrocele patients who received treatment (Figure 1), 42 were present at all time points. Therefore, all analyses were based on these 42 patients. Of the 42 patients, 22 were treated with doxycycline and 20 received placebo (Table 1). Of the 22 doxycycline-treated patients, 12 (54.5%) had CFA, 11 (50%) had microfilaria in the blood, and 10 were CFA negative. Of the 20 placebo patients, 11 (55%) were CFA positive, 5 (25%) had microfilaria in the blood, and 9 (45%) were CFA negative (Table 2). All the microfilaricidal patients were also CFA positive.

Prior to treatment, there was no difference in Wolbachia sp. levels in microfilaria between the doxycycline group and the placebo group (Figure 2). However, four months after doxycycline treatment, Wolbachia sp. levels in microfilaria of the microfilariaemic patients were reduced by 93% (P = 0.0051, by Wilcoxon signed rank test) in the doxycycline-treated group but there was no significant change in the placebo group (P = 0.4652). At 12 and 24 months after treatment, the difference in the Wolbachia sp. load in the doxycycline-treated patients was not significant because only one patient was still microfilaricidal; the rest of the patients were amicrofilaricidal (Table 2). In contrast, placebo-treated patients with microfilaria had high Wolbachia sp. loads per microfilaria at all time points (Figure 2).

Microfilariaemia was not significantly reduced four months after doxycycline treatment compared with pre-treatment levels in the doxycycline and placebo groups (Table 2). Doxycycline and placebo patients were given ivermectin and albendazole after the re-examination at four months to clear microfilariaemia. At 12 and 24 months post-therapy, microfilariaemia was absent in the doxycycline group except for one patient, whereas at the same 12 and 24 month time points, two patients (40%) and three patients (60%), respectively, of placebo patients were again microfilaricidal. Because of the low number of microfilaria-positive patients at all time points, no
significant difference was observed at any of the time points between the doxycycline and placebo groups.

Levels of CFA before and after doxycycline treatment. Table 2 shows the levels of CFA before and after treatment stratified according to antigen status (CFA positive or CFA negative). The TropBio ELISA was used to determine CFA status. According to the manufacturer, antigen units less than 108 (standard 3) are considered negative. Our data for persons from areas in Ghana not endemic for lymphatic filariasis also confirm these values as negative (Debrah AY and others, unpublished data). In CFA-positive patients, CFA levels of doxycycline-treated patients were higher than those of placebo patients prior to treatment but there was no significant difference between the two groups (Table 2) (P = 0.1340, by Mann-Whitney U test). The CFA levels had significantly decreased by 72% from pre-treatment levels at 12 months post-doxycycline treatment (P = 0.0180) and by 96% at 24 months (P = 0.0031, by Wilcoxon rank test) in the doxycycline-treated patients. In contrast, at 24 months post-therapy, CFA levels of placebo patients decreased, but not significantly, by 39%. The treatment regimen used in this study, which resulted in significant reduction of CFA levels in the doxycycline-treated patients, is the same as that used in which macrofilaricidal activity after Wolbachia sp. depletion, confirmed by ultrasound, was observed. 21 This finding indicates that there was also adult worm death in this study.

Plasma levels of VEGF-A before and after doxycycline treatment. To assess the effect of anti-Wolbachia sp. (doxycycline) treatment on plasma levels of VEGF-A, plasma levels of VEGF-A were measured before and 12 months after doxycycline treatment. The mean baseline level of VEGF-A in the doxycycline group was higher, but not significantly different, in the CFA positive subgroup than the CFA negative subgroup (Figure 3A compared to Figure 3B) the levels of doxycycline-treated groups (P = 0.2332, by Student’s unpaired t-test) from the levels in the placebo group (P = 0.1956) (Figure 3A). For the CFA-negative groups, there was no significant change in plasma VEGF-A levels before and after treatment in both the doxycycline-treated and placebo-treated groups (Figure 3B). The level of VEGF-A could not

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of patients</th>
<th>Time point after treatment</th>
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<tr>
<td></td>
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<td>Before treatment</td>
<td>4 months</td>
<td>12 months</td>
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<td>P</td>
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<td>Antigen-positive group</td>
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<tr>
<td>Median of microfilaraemia of doxycycline (GM)</td>
<td>11</td>
<td>550 (632)</td>
<td>442 (407)</td>
<td>0 (0)</td>
<td>1.2 (0)</td>
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<tr>
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<td>5</td>
<td>124 (293)</td>
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<td>0 (0)</td>
<td>7 (4)</td>
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<td>P</td>
<td>0.12*</td>
<td>0.49*</td>
<td>&gt; 0.99*</td>
<td>0.47*</td>
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<tr>
<td>Median CFA of doxycycline (GM; %)</td>
<td>12</td>
<td>8,216 (15,709; 100%)</td>
<td>4,827 (4,352; 28%)</td>
<td>183 (667; 4%)</td>
<td>0.003†</td>
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<td>10–90th percentiles</td>
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<tr>
<td>Median CFA of placebo (GM; %)</td>
<td>11</td>
<td>2,723 (3,225; 100%)</td>
<td>1,814 (2,144; 66%)</td>
<td>1,996 (1,980; 61%)</td>
<td>0.16†</td>
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<td>10–90th percentile</td>
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<tr>
<td>Mean stage of hydrocele of doxycycline (range)</td>
<td>12</td>
<td>2.7 (1–4)</td>
<td>2.7 (2–4)</td>
<td>2.0 (0–4)</td>
<td>0.02‡</td>
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<tr>
<td>Mean stage of hydrocele of placebo (range)</td>
<td>11</td>
<td>2.6 (1–4)</td>
<td>2.6 (1–4)</td>
<td>2.5 (0–4)</td>
<td>0.34‡</td>
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<td>Antigen-negative group</td>
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<tr>
<td>Median CFA of doxycycline</td>
<td>10</td>
<td>34</td>
<td>27</td>
<td>26</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>Median CFA of placebo</td>
<td>9</td>
<td>27</td>
<td>35</td>
<td>24</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>Mean stage of hydrocele of doxycycline (range)</td>
<td>10</td>
<td>3.0 (1–4)</td>
<td>3.2 (2–4)</td>
<td>3.0 (1–4)</td>
<td>&gt; 0.99‡</td>
<td></td>
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<tr>
<td>Mean stage of hydrocele of placebo (range)</td>
<td>9</td>
<td>2.7 (1–4)</td>
<td>3.0 (1–4)</td>
<td>3.3 (1–4)</td>
<td>0.32‡</td>
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</table>

GM = geometric mean; CFA = circulating filarial antigen.

* Differences in median of microfilaria values from doxycycline group were compared with those from placebo and assessed by Mann-Whitney U tests of raw data.
† Differences in CFA before and 24 months after treatment in each group were compared by Wilcoxon rank test of raw data.
‡ Differences in the mean stage of hydrocele before and 24 months after treatment in each group were compared by paired t-test of raw data.

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**Figure 2.** Effect of doxycycline treatment on Wolbachia sp. depletion in Ghana. At every examination, blood was obtained from each patient and microfilariae was determined. A total of 500–1,000 microfilaria were isolated from the blood by filtration and DNA was extracted from purified microfilaria. The copy numbers of the ftsZ gene were measured by real-time polymerase chain reaction (1 ftsZ = 1 Wolbachia sp.) and normalized to the number of microfilaria in each sample. After doxycycline therapy, the number of Wolbachia sp. was significantly reduced at all follow-up examinations. The Wolbachia sp. loads recorded for the doxycycline group at 12 and 24 months post-treatment was obtained from one patient who was microfilaric at both time points.
was significantly decreased \((P = 0.0241)\) at 24 months post-doxycline therapy in doxycline-treated patients who were CFA positive, the stage in CFA-positive placebo patients remained the same \((P = 0.3423)\) (Table 2). The mean stage in doxycline-treated and placebo-treated patients who did not have active infection (CFA-negative patients) remained unchanged at 24 months (Table 2). This finding suggests that doxycycline ameliorates pathologic conditions mainly in those with active infections.

Of the 12 CFA-positive patients who received doxycycline, seven (58.3%) had a reduction in the size of their hydrocele, four (33.3%) remained the same, and in one (8.4%) the stage worsened (Table 3). Conversely, of the 11 CFA-positive patients who received placebo, 18.2% showed improvement, 63.6% remained the same, and in 18.2% the stage worsened (Table 3). When we compared the number of patients with improved conditions (7 of 12) in the doxycycline group versus those (2 of 11) with improved conditions in the placebo group, there was a significant difference between the two groups \((P = 0.0487, \chi^2\)-test\) (Table 3). This finding indicates that doxycycline in combination with ivermectin and albendazole reduces hydrocele pathologic condition of lymphatic filariasis more than ivermectin and albendazole.

**DISCUSSION**

Lymphatic filariasis is ranked as one of the leading causes of permanent disability worldwide by the World Health Organization.\(^{34}\) Infection with *W. bancrofti*, which can lead to development of hydrocele and/or lymphedema, is the cause of debilitating hydrocele disease in 27 million men and lymphedema and elephantiasis in an additional 15 million persons.\(^{35,36}\) The actual cause of hydrocele is not known because a proportion of the patients do not have active infection, although prior infection is thought to induce these cases. However, in a previous study,\(^{13}\) it was demonstrated that hydrocele is associated with a single nucleotide polymorphism in the VEGF-A promoter that leads to the production of more VEGF-A and therefore might predispose persons to this disease phenotype. Nonetheless, the actual stimulus of VEGF-A is not clear.

In this study, we have shown that the *Wolbachia* sp. endosymbionts present in *W. bancrofti* might be one stimulus responsible for over-production of VEGF-A in hydrocele patients. Targeting the *Wolbachia* sp. endosymbionts with doxycycline led to a 93% reduction in *Wolbachia* sp. gene copy numbers, a level of reduction shown to cause macrofilaricidal effects on filarial worms and reduction of plasma levels of VEGF-A, which resulted in amelioration of the size of hydrocele. Importantly, the amelioration was observed in doxycycline-treated patients who were CFA positive and had a significant reduction in plasma levels of VEGF-A, but not in doxycycline-treated patients who were CFA negative or the

**Table 3**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of patients present before and at 24 months</th>
<th>No. (%) of patients with improved condition</th>
<th>No. (%) of patients with same condition</th>
<th>No. (%) of patients with worsened condition</th>
<th>(P^*)</th>
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<tr>
<td>Doxycycline</td>
<td>12</td>
<td>7/12 (58.3)</td>
<td>4/12 (33.3)</td>
<td>1/12 (8.4)</td>
<td>0.04</td>
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<td>Placebo</td>
<td>11</td>
<td>2/11 (18.2)</td>
<td>7/11 (63.6)</td>
<td>2/11 (18.2)</td>
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\(*^*\)Changes in the state of hydrocele patients with and without improvement at 24 months after treatment showed significant difference between doxycycline and placebo patients \((P = 0.04, \chi^2\)-test\).

**Figure 3.** Decrease in plasma levels of vascular endothelial growth factor A (VEGF-A) in patients with active infection (determined by circulating filarial antigen [CFA] status) 12 months after doxycycline treatment in Ghana. A. Plasma concentrations of VEGF-A were measured from plasma of hydrocele patients who had active infection (CFA positive) before and 12 months after doxycycline treatment (12 patients treated with doxycycline, 11 patients treated with placebo (see Table 1). The VEGF-A levels were significantly decreased at 12 months (preceding amelioration of hydrocele, see Table 2) in the doxycycline-treated patients \((P = 0.0466, \text{by paired } t\text{-test})\) but were unchanged in the placebo group. B. Plasma measured from the cohort of hydrocele patients without active infection (CFA negative) before and 12 months after doxycycline treatment (10 patients treated with doxycycline, 9 patients treated with placebo, see Table 1) showed no significant differences in the VEGF-A levels at 12 months in both groups. p.t. = post treatment.

**Stage of hydrocele of patients after doxycycline treatment.**

The effect of doxycycline on the stage (scrotal size) of the hydrocele of the patients is shown in Table 2, stratified according to CFA status. Whereas the mean stage of hydrocele be measured at 24 months because of insufficient blood samples at this time point.

**State of hydrocele patients with active infection before and 24 months after doxycycline treatment in Ghana**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of patients present before and at 24 months</th>
<th>No. (%) of patients with improved condition</th>
<th>No. (%) of patients with same condition</th>
<th>No. (%) of patients with worsened condition</th>
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placebo patients. These data are consistent with the hypothesis that over-expression of vascular and lymphangiogenic factors such as VEGF-A may result in progression of infection to hydrocele. The initial stimulus is presence or death of adult filarial worms and microfilaria in the scrotal region of infected persons, followed by (in the CFA-negative phase of the disease) incoming larval stages of the parasite that release Wolbachia sp. upon being killed, thus exacerbating the condition by stimulating more pro-inflammatory cytokines and VEGF molecules.

Over-expression of VEGF-A molecules in the scrotal region of male patients could be responsible for extravasation and accumulation of fluids, plasma and lymph, from the blood and lymphatic vessels into the scrotal region resulting in formation of hydrocele, chyloucele, and lymphoucele; and formation of nodules in hydrocele patients, which are usually observed just before hydrocele development when DEC is administered. Formation of these nodules might result from the death of microfilaria and adult worms caused by DEC, which would lead to release of VEGF-A into the blood, in part caused by release of Wolbachia sp.

It is not yet clear whether VEGF-A is associated with hydrocele (disease), infection, or both. Our previous data support the hypothesis that VEGF-A may be disease associated because of the strong association between hydrocele patients and polymorphism in the VEGF-A gene ($P = 0.0007$) and the positive correlation between plasma levels of VEGF-A and the size of the hydrocele ($P = 0.026$). This association was not found between the VEGF-A gene and microfilaria-infected patients or non-hydrocele patients. Conversely, VEGF-A could be infection related, which is probably why in our previous work, there was no difference in the plasma levels of VEGF-A between microfilaria-positive patients and hydrocele patients. In addition, in this study, CFA-positive and microfilaria-positive patients had higher levels of VEGF-A than CFA-negative hydrocele patients (Figure 3).

It is likely that VEGF-A is not the only factor responsible for hydrocele development. However, in lymphatic filariasis, infection drives hydrocele development in some persons. For instance, in our previous work, nine microfilaria-infected doxycycline-treated patients and six microfilaria-infected placebo-treated patients had lymphatic dilation and high lymphangiogenic factors before treatment. The doxycycline-treated patients showed a significant reduction in plasma lymphangiogenic factors after treatment with subsequent reduction in lymphatic vessel dilation; this was not observed in the placebo group. Hydrocele developed in five of the six placebo-treated patients within the two years of observation, whereas sub-clinical hydrocele developed in only one of nine doxycycline-treated patients during the same period, which provides evidence that these lymphangiogenic factors may be infection related but can also cause development of pathologic conditions. Thus, VEGF-A and the size of hydrocele decrease more in the CFA-positive persons than in CFA-negative persons after doxycycline therapy.

The same explanation may also apply to VEGF-C/sVEGFR-3 levels and lymphedema development in our previous work. In that study, although we saw a difference in the plasma level of sVEGFR-3 between lymphedema patients and microfilaria-infected patients, which is evidence for development of pathologic conditions, there was no difference in VEGF-C levels between lymphedema and microfilaria-infected patients, which suggests that VEGF-C may be associated with infection. However, as explained above for VEGF-A, although VEGF-C may be associated with infection, it can still result in development of pathologic conditions if its levels are not controlled.

Pathology of lymphatic filariasis, such as lymphedema and hydrocele, is complex, multi-factorial, and involves lymphatic dilation and accumulation of fluid in affected tissues. Therefore, any factors such as VEGF-C/sVEGFR-3, which are responsible for lymphatic dilation, and VEGF-A, which is responsible for extravasation of fluid from the blood and lymphatic vessels into the surrounding tissues, could work synergistically in manifestation of the disease. It is known that VEGF-C is associated with lymphatic dilation, and is a hallmark of lymphedema and hydrocele development. Therefore, microfilaria-positive patients with dilated lymphatic vessels and hydrocele or lymphedema patients, who are characterized by dilated lymphatic vessels, could have equally high levels of lymphangiogenic factors such as VEGF-C. It is also possible that levels of these lymphangiogenic factors could remain high in infected persons before pathologic conditions are manifested, as observed in our previous work regarding hydrocele that devolved in microfilaric patients. Such microfilaric patients in whom pathologic conditions developed could have had high levels of VEGF-C equal to those in patients with pathologic conditions, thus explaining why there was no difference between microfilaric patients and patients with pathologic conditions in that study.

Of great interest are findings in animal studies, which show that VEGF-C gene therapy reduces edema. These findings are contrary to those observed in our human study. To understand this dilemma, one must first know that there are two types of lymphedema, primary and secondary. In both types, the underlying physical cause of lymphedema is that the transport capacity of the lymphatic vessels is usually decreased and protein-rich fluid collects in the tissues, which causes chronic swelling. Therefore, mechanisms that affect free flow of the fluid will cause edema.

In primary lymphedema, subcutaneous lymphatic vessels are usually aplastic or hypoplastic because of production of VEGF-C. In such a situation, VEGF-C gene therapy can induce formation of a lymphatic network to reduce chronic lymphatic insufficiency and thus reduce edema. In animal studies in which VEGF-C gene therapy improved edema, the lymphatic systems of the animals were first removed by surgery, which leads to edema. After surgery, VEGF-C gene therapy was used to reconstruct the lymphatic vessel network severed by the incision wound. In contrast to these findings, in some lymphedemas, such as filarial lymphedema, the lymphatic vessels become dilated, which leads to decreased transport capacity of the vessels. This phenotype could be caused by overproduction of VEGF-C because when VEGF-C is overproduced in endothelial cells of lymphatic vessels, these vessels become dilated, which leads to poor drainage of the lymphatic fluid and edema. In such a situation, gene therapy could worsen the condition. An alternative treatment such as doxycycline therapy that reduces inflammation and therefore VEGF-C levels will lead to a reduction in lymphatic vessel dilation, thus improving the drainage system and reducing edema as we observed in our study. Therefore, findings in the animal studies and our studies are not contradictory, but rather reflect different causal
mechanisms of lymphedema that require different treatments. This finding is supported by animal data from one group, which demonstrated that sustained swelling of a secondary lymphedema in the mouse tail was the result of up-regulation of VEGF-C, which leads to lymphatic hyperplasia; continuous up-regulation of VEGF-C exacerbated the edema because hyperplastic vessels were poorly functional. These investigators also concluded that up-regulation of VEGF-C and lymphatic hyperplasia resulting from dermal lymphatic ligation tors also concluded that up-regulation of VEGF-C and lym-

phatic hyperplasia resulting from dermal lymphatic ligation leads to lymphedema, which is similar to our findings. More research is needed to unravel the actual effect of these lymphangiogenic factors on the development of pathologic conditions of lymphatic filariasis.

The most effective current treatment for hydrocele is hydrocelectomy. However, this option is unaffordable to many affected men and has potential dangers because it involves surgery; it is also alarming and fearful to patients. Therefore, alternative and safer options need to be investigated. One alternative may be treatment of hydrocele patients with doxy-
cycline to halt production of pro-inflammatory cytokines and VEGF-A and then aspirating hydrocele fluid from the scrotum. Reduction of plasma levels of VEGF-A by doxycycline (especially in the early-stage patients who have active infec-
tion) could prevent further inflow of fluid into the scrotal sacs, which could prevent the need for repeated aspiration. This method is likely to be safer than hydrocelectomy. Patients with small (stage 1) hydroceles might not need aspiration because such hydroceles sometimes regress after doxycycline treat-

ment (Mand S, Debrah AY, Hoerauf A and others, unpublished data). However, it must be emphasized that because doxycycline was most effective in patients with active infec-
cion (CFA positive), patients need to be treated early in the infection before the CFA-negative phase develops.

Another option might be aspiration of hydrocele fluid and injection of antibodies to VEGF-A into the tunica vaginalis. These two options could stop progression of hydrocele develop-

ment and need to be investigated in larger trials. If con-
firmed, this option could provide hope and relief to patients with debilitating disease and could improve mobility, sexual function, and working capacity of these patients.

The significant difference observed in reduction of mean plasma levels of VEGF-A in the doxycycline-treated group (Figure 3A) compared with the insignificant difference observed in the placebo group (Figure 3A) at the same time point could be caused by the already lower pretreatment mean level of VEGF-A of the placebo group. However, the fact that the mean level of VEGF-A of the placebo group did not remain the same but increased argues against this possi-
bility. We acknowledge that there may not have been enough statistical power to detect all possible differences within this group. However, this work was a pilot study to provide base-
line data upon which to base more precise sample analyses for a second, larger study. Our current study and previous study, showed that VEGF-A might be responsible for develop-
ment and progression of hydrocele in patients infected with W. bancrofti. This information could be explored in special situa-
tions (e.g., setting up outpatient clinics in disease-endemic areas) where anti-Wolbachia sp. or anti-VEGF-A treatment is administered to patients in whom hydrocele develops to ame-
liorate or halt this condition. This treatment, together with current anti-filarial mass treatment in disease-endemic areas, will strongly reduce transmission and lessen or eliminate mor-
bidity. Such strategies would increase compliance for current drug therapies to interrupt transmission because the inability to treat patients with pathologic conditions is a key factor in non-compliance of treatment programs.

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REFERENCES


