HOOKWORM-RELATED CUTANEOUS LARVA MIGRANS IN PATIENTS LIVING IN AN ENDEMIC COMMUNITY IN BRAZIL: IMMUNOLOGICAL PATTERNS BEFORE AND AFTER IVERMECTIN TREATMENT

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Hookworm-related cutaneous larva migrans (Hr-CLM) is caused by animal hookworm larvae migrating in the human epidermis where they elicit an inflammatory response. This study describes the immunological profile in Hr-CLM patients.

In 77 Hr-CLM patients from Manaus, Brazil, peripheral eosinophils were counted, and serum concentrations of total immunoglobulin E (IgE) and selected cytokines were determined by ELISA before and after treatment with ivermectin. Controls included patients' household members (endemic controls), non-endemic Brazilian and Japanese individuals.

Eosinophil counts and total IgE in Hr-CLM patients were higher than in controls and correlated with disease severity. Concentrations of interleukin (IL)-4, IL-5, IL-6, and IL-10 were higher in Hr-CLM patients than in endemic controls ($p < 0.001$) while no differences were detected for interferon (IFN)- γ , tumor necrosis factor (TNF)- α , IL-1 β , IL-2, or transforming growth factor (TGF)- β . Following ivermectin treatment, numbers of eosinophils and concentrations of IL-4, IL-5, and IL-10 decreased whereas IgE, IFN- γ , and TGF- β concentrations increased. The IL-5/IFN- γ ratio declined from 5.9 (interquartile range [IQR] 0.8–31.6) before to 0.1 (IQR 0.05–0.2; $p = 0.001$) after treatment.

Thus, although an impact of other infections on the immune parameters determined cannot be excluded, Hr-CLM in endemic areas is associated with eosinophilia and elevated cytokine levels, particularly of IL-5 and IL-10, which decrease following ivermectin treatment.

Keywords: cutaneous larva migrans, cytokine, immunological profile, treatment, ivermectin

Introduction

Hookworm-related cutaneous larva migrans (Hr-CLM) is a zoonosis caused by larvae of animal hookworms (e.g., *Ancylostoma braziliense*, *Ancylostoma caninum*, or *Uncinaria stenocephala*), which penetrate the human skin. After penetration, the nematode larvae are captured in a biological impasse since they cannot pass the basal membrane of the epidermis of the human host. Therefore, they remain confined to this skin compartment and migrate up to several months until they eventually die *in situ* [1, 2].

In resource-poor communities in developing countries, Hr-CLM is common and its prevalence may be up to 4% in the general population and up to 15% in children. In

exceptional situations, prevalence may reach 50% [3–5]. In resource-poor communities, Hr-CLM is associated with considerable morbidity, such as intense itching, pain, and itching-associated sleep disturbance [6]. In addition, excoriated tracks may become infected by bacteria [3]. This explains why Hr-CLM significantly impairs the quality of life of affected individuals [6, 7].

Since Hr-CLM is an invasive helminthic disease, it is likely accompanied by the development of antigen-specific immunity. As compared to immune responses against other pathogens, however, those against helminths are complex and often involve various T helper (Th) subsets, e.g., Th2 and regulatory T cells, nonlymphoid leukocytes, such as mast cells, basophils, and eosinophils, and antibodies

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of the immunoglobulin E (IgE) isotype [8, 9]. Immune responses to animal hookworm larvae, however, have never been determined, mainly because Hr-CLM is a neglected parasitic disease, which has not attracted much interest of microbiologists or immunologists [4].

Analyses of antigen-specific immunity in patients living in endemic areas may be confounded by (1) already existing immune responses to the same agent and/or (2) previous or simultaneous coinfections with other parasites. We, therefore, included in our analyses household members of the patients who were presumably infected with a similar spectrum of parasites except Hr-CLM at the time when the study was conducted. The analyses were repeated 2 and 4 weeks following treatment with ivermectin, i.e., after the death of the hookworm larvae. We determined serum IgE concentrations and numbers of peripheral eosinophils. Additionally, we analyzed serum concentrations of pro-inflammatory (interferon (IFN)- γ , interleukin (IL)-1 β , and tumor necrosis factor (TNF)- α), Th2-related (IL-4, IL-5), and immunoregulatory cytokines (IL-10, transforming growth factor [TGF]- β). We also included IL-6 in these studies because it is a key pro-inflammatory cytokine but may also have anti-inflammatory properties depending on the signaling [10]. Since pruritus is the main symptom in Hr-CLM, we also analyzed the production of IL-2, which may be related to pruritus [11]. Furthermore, IL-2 may have a critical role in skin inflammation [12].

Here, we demonstrate a distinct immunological profile of patients suffering from acute Hr-CLM infection and show that this profile changes after the death of the parasites.

Materials and methods

Study area and study design

The study was part of a larger research project on the epidemiology, morbidity, and immunology of Hr-CLM, carried out in three resource-poor communities of Manaus, capital of Amazonas state, North Brazil, from October 2008 to February 2009. The socioeconomic and environmental characteristics of these communities have been described previously [6]. In this setting, the prevalence of cutaneous larva migrans is up to 18% in children during the rainy season, and patients frequently have several larval tracks simultaneously present at multiple body sites [6]. Recruitment of patients, diagnostic procedures, estimation of the severity of Hr-CLM by using a severity score (1–10 points), and assessment of skin-associated life quality impairment using the modified Dermatology Life Quality Index (mDLQI) have been described previously [6].

Seventy-seven patients with a total number of 413 tracks (median, two tracks; maximum 51 tracks) were included in this study. Median age was 9 years (range, 5 to 35 years). 39.8% of the patients were females and 61.2% males.

After diagnosis, all patients were treated with a single dose of oral ivermectin (200 μ g/kg; Revectina Solvay Farma Ltda., São Paulo, Brazil). Two and 4 weeks after treatment, the patients were reexamined.

Control groups

Forty-three household members of the patients were included as endemic controls. These individuals were matched by age and had no Hr-CLM at the time of this study. Assumedly, some of the household members had previously experienced Hr-CLM. Median age was 8 years (range, 5 to 49 years). 58.1% of the endemic controls were females and 41.9% males.

For further comparison, serum samples from 27 healthy Brazilian residents who had lived in Japan for more than 5 years (non-endemic controls) were used. Median age was 12 years (range, 7 to 32 years). 74.1% were males and 25.9% females.

As an internal (laboratory) control, serum samples from 11 healthy Japanese individuals were used. Median age was 39 years (range, 20 to 60 years). 54.5% were males and 45.5% were females.

Laboratory investigations

For hematology, 8 ml ethylenediaminetetraacetic acid (EDTA)-anticoagulated peripheral blood was drawn from each participant (Hr-CLM patients and endemic controls). Eosinophils were counted by flow cytometry. More than 500 eosinophils/ μ l were considered as eosinophilia, and >1000 eosinophils/ μ l as hypereosinophilia. Serum samples were aliquoted, and aliquots were stored at -20°C in Manaus and then at -60°C in Tokyo.

Total serum IgE and cytokines were determined by ELISA (IgE: Bethl Laboratories Inc., Montgomery, USA; cytokines: Ready-Set-Go Human cytokines, eBioscience, San Diego, USA) following the instructions of the manufacturer. The detection limits were as follows: 2 pg/ml for IL-4, IL-6, and IL-10; 4 pg/ml for IL-1 β , IL-2, IL-5, IFN- γ , and TNF- α ; 60 pg/ml for TGF- β 1.

Statistical analysis

Statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University), a graphical user interface for R commander (The R Foundation for Statistical Computing, version 2.13.0). Since data did not follow a normal distribution, median and interquartile range (IQR) were used as indicators of central tendency and dispersion of the data, respectively. The Spearman rank correlation coefficient was calculated for correlations between ordinal variables. Relative frequencies were compared using the χ^2 test. The Wilcoxon matched-pairs signed-rank test was used to analyze pairs of variables, i.e., before and af-

ter treatment with ivermectin. The Mann–Whitney and the Kruskal–Wallis test were applied to compare ordinal variables between the different groups.

Ethical considerations

The study was approved by the Ethics Committee of the Fundação de Medicina Tropical do Amazonas, the public reference institution for tropical diseases of Amazonas State. To each participant or in case of minors to their legal guardian, the study objectives were explained in simple and comprehensible Portuguese. The right to withdraw at any time was clarified. Each participant or their legal guardian signed the written informed consent form. In case of illiteracy, consent was given via fingerprint. The Ethics Committee of the Tokyo Medical and Dental University approved the analysis of the sera of Brazilian residents living in Japan and the laboratory controls.

Fig. 1. Hr-CLM patients have higher numbers of eosinophils in the peripheral blood than endemic controls, and these counts decrease following ivermectin treatment. Numbers of eosinophils were determined at baseline (both groups, A) as well as 2 (T1) and 4 weeks (T2) after ivermectin treatment (CLM+ only, B). Box-and-whisker plots indicate the medians, interquartile ranges, and ranges

Fig. 2. High levels of IgE in Hr-CLM patients and their uninfected household members. Concentrations of total IgE in patients (CLM+), endemic (CLM-), non-endemic (NEC), and laboratory controls (LC) were determined at baseline (all groups, A) as well as 2 (T1) and 4 weeks (T2) after treatment with ivermectin (CLM+ only, B). Box-and-whisker plots indicate the medians, interquartile ranges, and ranges

Results

Eosinophils

Numbers of peripheral eosinophils were determined for Hr-CLM patients and endemic controls. At baseline, 77.9% of the patients had eosinophilia (>500 eosinophils/ μ l), and 41.6% had hypereosinophilia (>1000 eosinophils/ μ l). The median eosinophil count in Hr-CLM patients was 707 cells/ μ l (IQR 505–1234 cells/ μ l), almost twice as high as in household controls (median 432 cells/ μ l; IQR 247–731 cells/ μ l; $p < 0.01$) (Fig. 1A). Four weeks after treatment with ivermectin, the numbers of eosinophils had decreased significantly ($p = 0.01$) (Fig. 1B).

Total IgE

IgE serum levels were determined for Hr-CLM patients and all control groups. At baseline, the IgE concentrations were higher in patients (median, 2727 ng/ml; IQR, 1556–4843 ng/ml) than in endemic controls (median, 2164 ng/ml; IQR, 486–3164 ng/ml; $p < 0.001$; Fig. 2A). IgE serum levels in non-endemic controls were significantly higher than in laboratory controls (median, 1335 versus 179 ng/ml; $p < 0.001$; Fig. 2A).

Two weeks after treatment with ivermectin, the median concentration of IgE in Hr-CLM patients had significantly increased (median, 2727 ng/ml at baseline versus 4033 ng/ml after 2 weeks; $p = 0.005$). Four weeks after treatment, the concentrations still remained very high (median, 3691 ng/ml; $p < 0.01$ as compared to baseline; Fig. 2B).

Cytokines at baseline

Serum cytokine levels were determined for Hr-CLM patients and endemic controls as well as for non-endemic and laboratory controls. At baseline, IL-5 and IL-6 as well as IL-4 and IL-10 were detectable at low levels in serum samples from Hr-CLM patients (Fig. 3). In contrast, most samples from endemic controls were negative for IL-6 and IL-10. IL-4 and IL-5 were detectable, but the concentrations were significantly lower than in samples from patients ($p < 0.0001$). Notably, the concentrations of IL-4 in non-endemic controls varied over a wide range, but the median concentration did not differ from that of Hr-CLM patients (median, 4.9 pg/ml versus 10.2 pg/ml; $p = 0.58$, Fig. 3). IL-5 and IL-10 were not de-

Fig. 3. Hr-CLM patients differ from endemic and non-endemic control individuals regarding their serum cytokine pattern. Concentrations of the cytokines indicated were determined in Hr-CLM patients (before ivermectin treatment, CLM+), endemic (CLM-), non-endemic (NEC), and laboratory controls (LC). Box-and-whisker plots indicate the medians, interquartile ranges, and ranges

tected in samples from non-endemic or laboratory controls (Fig. 3). Patients and endemic controls did not differ regarding serum concentrations of IFN- γ , TNF- α , IL-1 β , IL-2, or TGF- β . In the laboratory controls, the concentrations of IFN- γ were significantly higher as compared to the patients (median 20.1 pg/ml (IQR 0.1–23.3); $p = 0.01$).

Changes in cytokine levels following treatment with ivermectin

Four weeks after treatment with ivermectin, the concentrations of IL-4, IL-5, IL-6, and IL-10 in serum samples from Hr-CLM patients had decreased significantly as compared to baseline levels ($p = 0.001$; $p < 0.001$;

Fig. 4. Ivermectin treatment changes considerably the cytokine pattern in Hr-CLM patients. Concentrations of the cytokines indicated determined in Hr-CLM patients at baseline and 2 (T1) and 4 weeks (T2) after treatment with ivermectin. Box-and-whisker plots indicate the medians, interquartile ranges, and ranges

$p = 0.024$, $p = 0.005$, respectively; Fig. 4). At the same time, increased concentrations of IFN- γ were detected (median, 12.5 pg/ml at baseline, 560.4 pg/ml 2 weeks and 420.8 pg/ml 4 weeks after ivermectin treatment; $p = 0.001$ and $p < 0.001$, compared to baseline values, respectively). Furthermore, the concentration of IL-2 slightly increased after treatment (median 14.4 pg/ml versus 46.2 pg/ml; $p = 0.01$). TGF- β temporarily increased 2 weeks after treatment (median, 0.0 pg/ml versus 31.7 pg/ml; $p < 0.001$). TNF- α decreased 2 weeks after treatment and remained low 4 weeks after treatment (median, 221.3 pg/ml, 28.4 pg/ml, and 37.1 pg/ml, respectively ($p = 0.02$ compared to baseline).

To estimate the relative contribution of different Th subsets to the immunological profiles in Hr-CLM infection before and after ivermectin treatment, we calculated various cytokine ratios. Strikingly, the IL-5/IFN- γ ratio changed from 5.9 (IQR 0.8–31.6) at baseline to 0.1 (IQR 0.05–0.22) 4 weeks after treatment ($p < 0.001$). Although at baseline IL-4 was detectable in patients at very low levels only, the IL-4/IFN- γ ratio showed a similar tendency and declined from 1.0 (IQR 0.04–2.73) before to 0.005 (IQR 0.002–0.012) 4 weeks after treatment ($p < 0.001$). Similarly, the IL-10/IFN- γ ratio decreased from 1.0 (IQR 0.008–50.10) at baseline to 0.015 (IQR 0.001–0.100) 4 weeks after treatment ($p < 0.001$).

Fig. 5. Numbers of eosinophils in Hr-CLM patients are associated with the severity of the disease. Regression analyses between numbers of eosinophils and (A) the number of Hr-CLM lesions ($\rho = 0.41$; $p = 0.0002$), (B) the severity score ($\rho = 0.30$; $p = 0.009$), and (C) the mDLQI ($\rho = 0.32$; $p = 0.005$)

Fig. 6. IgE concentrations of Hr-CLM patients are associated with the numbers of eosinophils and the severity of the disease. Regression analyses between the concentration of IgE and (A) the number of eosinophils ($\rho = 0.44$; $p < 0.001$), (B) the number of lesions ($\rho = 0.42$; $p = 0.0002$), (C) the severity score ($\rho = 0.31$; $p = 0.006$), and (D) the mDLQI ($\rho = 0.23$; $p = 0.04$)

Correlations of immunological parameters with clinical findings

The numbers of peripheral eosinophils at baseline correlated with the number of lesions ($\rho = 0.41$, $p = 0.0002$; Fig. 5A), the severity of the disease ($\rho = 0.30$, $p = 0.009$; Fig. 5B), and the mDLQI ($\rho = 0.32$, $p = 0.005$; Fig. 5C) in Hr-CLM patients. The concentrations of IgE significantly correlated with eosinophil numbers ($\rho = 0.44$;

$p < 0.001$; Fig. 6A). IgE concentrations also correlated with the number of lesions ($\rho = 0.42$, $p = 0.0002$; Fig. 6B), the severity score ($\rho = 0.31$, $p = 0.006$; Fig. 6C), and the mDLQI ($\rho = 0.23$, $p = 0.04$; Fig. 6D). In contrast, there was neither a correlation between cytokine concentrations and the number of lesions or the severity of Hr-CLM, nor between the levels of pro-inflammatory cytokines and numbers of excoriated or bacterially superinfected tracks (data not shown).

Discussion

In the present study, we acquired – for the first time – data on the immune response in Hr-CLM patients. Since the patients lived in a highly endemic area, previous exposure to animal hookworm larvae – at least in a part of the patients – is likely. This is important because immune responses may be different in primary and repeated helminth infection [8, 9]. Thus, our study presumably describes the immunological recall response against Hr-CLM derived antigens that were presented during an acute reinfection.

In comparison to endemic controls, i.e., household members who presumably had a similar medical history (including previous Hr-CLM infestations) and who might have suffered at the time when the study was conducted from the same spectrum of other parasitic diseases (except Hr-CLM) as the patients, the latter differed significantly regarding the numbers of peripheral eosinophils, the IgE concentration, a substantial production of IL-5, IL-6, and IL-10, and also the secretion of IL-4 (although the serum levels of this cytokine were very low). Although most IgE produced in response to systemic helminth infections is polyclonal and believed to contribute to protection to a limited extent only [13], it is possible that at least parts of the elevated IgE concentrations were attributable to the acute Hr-CLM infection. This is corroborated by the finding that IgE concentrations in our patients correlated with the number of lesions and the severity of Hr-CLM. Likewise, the temporary IgE increase following ivermectin treatment might be due to a rapid disintegration of killed hookworm larvae (and possibly intestinal nematodes) and a boost of the humoral response by the released antigens.

In healthy individuals, eosinophils account for less than 7% (<350 cells/ μ l) of peripheral white blood cells. In experimental human helminth infection, however, numbers of eosinophils in the blood increased to >1000 cells/ μ l in most individuals infected with *Necator americanus* larvae or *Brugia* spp. and reached values of >10,000 cells/ μ l in some individuals with lymphatic filarial infection [14]. Our patients showed eosinophil counts twice as high as their household members who lacked Hr-CLM at the time of the study. The assumption that the eosinophilia was a direct response to Hr-CLM is corroborated by the observation that the number of eosinophils in the blood correlated with the number of tracks and the degree of morbidity associated with Hr-CLM. Moreover, histological sections of lesions of patients with Hr-CLM revealed eosinophils infiltrating the skin close to the larval tracks (Schuster A et al., manuscript in preparation). Furthermore, some travelers infected with Hr-CLM (and presumably not with other helminths) also developed peripheral eosinophilia [15].

In systemic helminth infections, eosinophils may act as effector cells through killing of invading/migrating larvae *in situ* but also have an immunomodulatory role [16]. These distinct functions are mediated by various proteins that can be released from the granules, by receptors for, e.g., immunoglobulins and complement fragments, by the

expression of various adhesion molecules, and by a range of cytokines and chemokines that can be secreted [17]. Killing of schistosomula, *Trichinella spiralis* newborn larvae, and *Brugia* spp. microfilaria by purified eosinophil granule proteins has been demonstrated *in vitro* [18–20]. *In vivo*, IL-5 transgenic mice, i.e., animals with a constitutive eosinophilia, are more resistant to primary infection with the nematode *Nippostrongylus brasiliensis* than wild-type mice [21], which could be attributed to trapping and immobilization of the larvae accompanied by a strong infiltrate and degranulation of eosinophils at the injection site [22].

IL-5 produced by Th2 cells is the key cytokine for the development of a peripheral eosinophilia [23]. IL-5 serum concentrations were significantly higher in Hr-CLM patients than in endemic controls. A role of IL-5-mediated protection against nematode larvae is supported by data obtained in the above-mentioned murine *N. brasiliensis* model where IL-5 knock-out mice with defective eosinophilopoiesis showed an impaired resistance in early secondary infection to this parasite, i.e., significantly more larvae reached the lungs than in wild-type animals [24]. Likewise, IL-5 knock out mice are more susceptible to secondary *T. spiralis* infection [25].

Eosinophils may also possess an immunoregulatory role in helminth infection [16]. Notably, eosinophils are able to present *Strongyloides stercoralis*-derived antigens *in vitro* and in mice *in vivo* [26, 27], and to secrete Th2-related cytokines, e.g., IL-4 and IL-13, in murine *Schistosoma mansoni* infection [28, 29]. Moreover, eosinophils cultured in the presence of TNF- α plus either IL-4 or IFN- γ *in vitro* produce Th1 or Th2 chemokines, respectively [30]. If this also occurs *in vivo*, this mechanism could account for an increased infiltration of the tissue by the respective Th cell subset.

Although IL-5 was the dominant Th cytokine detectable in Hr-CLM patients, the cytokine profile indicated the expression of a mixed type of immune response rather than a genuine Th2 response, i.e., in addition to IL-5, we detected substantial concentrations of the immunomodulatory cytokine IL-10 while Th1 cytokine concentrations were low. Similar data have recently been reported for children chronically infected with *Ascaris lumbricoides* or *Trichuris trichiura* [31], and for experimental infection with human hookworm [32]. Likewise, mice responded to repeated infection with bird schistosome cercariae by a local production of IL-4 and IL-10, and skin-draining lymph nodes contained Th2 polarized lymphocytes [33]. Possible sources of the IL-10 in Hr-CLM patients are Th2, but also Th1 or regulatory T cells [8]; all of which could contribute to an active immunomodulation to avoid further tissue damage caused by the immune response against migrating larvae. Consequently, 2 weeks after treatment with ivermectin and the subsequent resolution of tracks, IL-10 concentrations decreased to the limit of detectability in the majority of the patients. The rise in IFN- γ at that time point might underline the previous immunosuppression mediated by IL-10.

We detected IL-4 at low levels in Hr-CLM patients whereas most serum samples from endemic controls were negative for this cytokine. Like IL-5, IL-4 is a Th2 cytokine and binds to the same receptor as IL-13, i.e., IL-4R α . Both IL-4 and IL-13 are key cytokines in the protection against helminths [34]. While, in many murine infection models, both cytokines promote immunity, resistance of mice to a secondary infection with *Heligmosomoides polygyrus* critically depends on IL-4; neutralizing IL-4 in previously infected animals results in a considerable loss of protection [35]. Notably, IL-4R α signaling also is essential for the development of lymphocyte hypo-responsiveness caused in mice by multiple invasions of *S. mansoni* cercariae in the skin [36].

IL-6 serum levels also were higher in Hr-CLM patients than in endemic controls. Elevated levels of serum IL-6 (together with IL-10, IL-13, IFN- γ , and a moderate eosinophilia) have also been reported for children with chronic cough associated with toxocariasis [37]. The rapid normalization of the IL-6 concentration after the ivermectin-induced resolution of tracks suggests that the increased IL-6 levels at baseline were the consequence of the inflammatory response against migrating larvae and the concomitant tissue damage.

One limitation of our study is that the presence of other helminths could not be excluded in patients and endemic controls. However, the selection of endemic control individuals living in the same households as the Hr-CLM patients may support our overall conclusions. Future studies should focus on local immune response *in situ* where cytokines may be relevant that were not detectable in serum, e.g., TGF- β could be detected around subcutaneous *Dirofilaria repens* [38]. Additional studies are warranted regarding the specificity of immune parameters correlating with Hr-CLM, e.g., assays with PBMCs restimulated with hookworm-specific antigens could be performed. A proper antigen preparation, however, would be required for such study since larval immunodominant antigens might differ from those derived from adult worms. It also would be interesting to analyze in this context whether animal hookworm larvae may secrete proteins that interfere with the immune response and thereby promote survival as it has been shown for various other helminths [39, 40].

In conclusion, our data indicate that acute (re-) infection with Hr-CLM is reflected by IL-5 secretion recruiting eosinophils from the bone marrow to the peripheral blood from where IL-4 and other cytokines/chemokines may attract the cells to larval tracks in the epidermis. Therefore, eosinophils might contribute to the killing of hookworm larvae but also release Th2 chemokines and thereby influence the cellular infiltrate *in situ*. Tissue damage is reflected by the production of nonspecific pro-inflammatory cytokines, such as IL-6, while the extent of inflammation is controlled by a concomitant down-regulation of these responses through IL-10. Upon treatment with ivermectin, larvae die and inflammation resolves, which is accompanied by a normalization of these immune parameters.

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Disclosure of authors' contributions

Study concept and design were performed by Feldmeier and Ignatius. Examination of patients and hematological data were performed by Schuster and Lesshaft. Acquisition of immunological data was performed by Hata and Shimogawara. Organization, logistics, and quality control were performed by Guedes de Oliveira and Feldmeier. Supervision of fieldwork was performed by Feldmeier. Supervision of laboratory work was performed by Akao. Statistical analysis was performed by Shimogawara, Akao, and Feldmeier. Interpretation of data was performed by Shimogawara, Feldmeier, Ignatius, Akao, and Ohta. Drafting of the manuscript was performed by Feldmeier, Shimogawara, and Ignatius.

Critical revision of the manuscript was performed by all authors.

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