Helminth infections on Flores Island, Indonesia:
Associations with communicable and non-communicable diseases

Aprilianto Eddy Wiria

Helminth infections on Flores Island, Indonesia:
Associations with communicable and non-communicable diseases

Aprilianto E. Wiria

Leiden University Medical Center, 13 June 2013

ISBN: 978-94-6182-276-5

Cover image: Sunset view on the road along the beach from Ende city to Nangapanda.

Lay out and printing by: Offpage, Amsterdam

Financial support for the publication of this thesis was provided by Esaote Benelux, Faculty of Medicine, University of Indonesia, and Indonesia Malaria CARE Foundation.

The research presented in this thesis was performed at the Department of Parasitology, Leiden University Medical Center, Leiden, The Netherlands, the Department of Parasitology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia and the Nangapanda Research Center, Ende, Flores, Indonesia.

Helminth infections on Flores Island, Indonesia:
Associations with communicable and non-communicable diseases

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 13 juni 2013
klokke 10.00 uur

door

Aprilianto Eddy Wiria
geboren te Jakarta (Indonesië)
in 1980

Promotiecommissie:

Promotor:
Prof. dr. M. Yazdanbakhsh

Copromotores:
Dr. T. Supali, University of Indonesia
Dr. E. Sartono

Overige leden:
Prof. dr. P. Kremsner, University of Tübingen
Dr. A.J.F. Luty, L'Institut de recherche pour le développement (IRD)
Dr. P. Soewondo, University of Indonesia
Prof. dr. R. van Ree, Amsterdam Medical Center
Prof. dr. B.J.C. Middelkoop
Prof. dr. J.W.A Smit

Contents

* These authors contributed equally.

chapter 1

Helminth infection in populations undergoing epidemiological transition: a friend or foe?

Helminth infection in populations undergoing epidemiological transition: a friend or foe?

Aprilianto Eddy Wiria · Yenny Djuardi · Taniawati Supali · Erliyani Sartono · Maria Yazdanbakhsh

Received: 28 September 2012 / Accepted: 21 October 2012 / Published online: 6 November 2012

Abstract Helminth infections are highly prevalent in developing countries, especially in rural areas. With gradual development, there is a transition from living conditions that are dominated by infection, poor sanitation, manual labor, and traditional diet to a situation where burden of infections is reduced, infrastructure is improved, sedentary lifestyle dominates, and processed food forms a large proportion of the calorie intake. The combinations of some of the changes in lifestyle and environment are expected to result in alteration of the landscape of diseases, which will become dominated by non-communicable disorders. Here we review how the major helminth infections affect a large proportion of the population in the developing world and discuss their impact on the immune system and the consequences of this for other infections which are co-endemic in the same areas. Furthermore, we address the issue of decreasing helminth infections in many parts of the world within the context of increasing inflammatory, metabolic, and cardiovascular diseases.

Keywords Helminths · Co-infection · Allergy · Metabolic syndrome · Cardiovascular diseases · Epidemiological transition · Immune responses

This article is a contribution to the special issue on Immunoparasitology - Guest Editor: Miguel Stadecker

A. E. Wiria (✉) · Y. Djuardi · T. Supali
Department of Parasitology, Faculty of Medicine,
University of Indonesia,
Jakarta, Indonesia
e-mail: aprilianto.eddy@gmail.com

A. E. Wiria
e-mail: a.e.wiria@lumc.nl

A. E. Wiria · Y. Djuardi · E. Sartono · M. Yazdanbakhsh
Department of Parasitology, Leiden University Medical Center,
Leiden, The Netherlands

The burden of helminth infections

A diverse range of helminth parasites, differing in terms of their size, life cycle, and clinical impact, can lead to chronic infections in humans. Soil-transmitted helminth (STH) infections, together with schistosomiasis and filariasis, form a major proportion of a group of neglected tropical diseases and together affect about one third of the world population [1]. Helminth infections, the ancient companions of poverty, together with the major infectious diseases are the targets of millennium development goals to improve global public health outcomes.

There are more than 20 helminth species infecting humans, of which the majority of infections are light, asymptomatic, and rarely cause attendance to health centers [1, 2]. However, children with heavy and chronic STH (*Ascaris lumbricoides*, hookworms [*Necator americanus*, *Ancylostoma duodenale*], and *Trichuris trichiura*) infections can suffer from malnutrition, growth stunting, intellectual retardation, as well as cognitive and educational deficits [2]. In addition, it is known that a proportion of individuals with intestinal schistosomiasis (caused by *Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma mekongi*, and *Schistosoma intercalatum*) can present with intestinal and hepatosplenic symptoms, while those with *Schistosoma haematobium* infection can suffer from urogenital symptoms [3]. A more serious condition has been seen when eggs get dislodged in the spinal cord or the brain, leading to neurological damage, such as myelopathy (acute transverse myelitis and sub-acute myeloradiculopathy) in *S. mansoni*- or in *S. haematobium*-infected subjects and acute encephalitis of the cortex, sub-cortical white matter, or basal ganglia in *S. japonicum*-infected subjects [4]. In schistosome-naïve travelers, acute infection can result in systemic hyper-reactive symptoms, commonly referred to as Katayama fever [5]. The range of pathologies seen with a third group of major helminth infections, the filarial

nematodes, are lymphatic oedemas caused by *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*, blindness caused by *Onchocerca volvulus*, and calabar swellings caused by *Loa loa* [6, 7]. In lymphatic filariasis, the lymphatic vessels at the site where the adult worms nest are dilated (lymphangiectasia), most commonly seen in the extremities and male genitalia [6]. Individuals with chronic onchocerciasis can develop depigmentation and loss of skin structure and elasticity that can lead to premature skin aging, while ocular lesions resulting from the migration of microfilariae to the eye can lead to severe visual impairment and blindness [6]. The calabar swelling in loaiasis is the typical clinical sign of this infection which is presented as a regional fugitive/episodical angioedema around the migrating adult worms, mostly in the arms and the legs [6, 7]. Although these outcomes of helminth infections are not the norm, their severity can form a strong basis for the urgent calls to control helminth infections worldwide.

It remains interesting that the majority of helminth infections have no outward clinical signs. It is thought that these infections often co-exist in harmony with their human host as a result of long evolutionary co-adaptation. To this end, helminths are able to influence their host to ensure their long-term survival, while this same adaptation might be beneficial to the host if it restricts damage to tissues and organs. The current view is that, although in the majority of infected populations these parasites can manipulate the immune system, in a small proportion of subjects, this manipulation seems to fail and leads to pathologies as described earlier.

The immunological consequences of helminth infections

Helminths are known to skew the immune system towards type-2 immunity characterized by T helper (Th) 2 cells and their cytokines (interleukin (IL)-4, IL-5, IL-9, IL-13), high level of tissue eosinophilia, mucosal mastocytosis, and production of immunoglobulin (Ig) E. Another hallmark of helminth infections is their ability to induce regulatory responses via regulatory T cells (Treg) that express molecules involved in the inhibition of immune responses such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or glucocorticoid-induced TNF receptor [8] and secrete suppressory cytokines (IL-10 and transforming growth factor (TGF) ß) [9]. Helminth infections have also been associated with regulatory B cells (Breg), which can release IL-10 and restrain hyper-inflammatory responses [10]. These parasites seem to also affect the expansion of innate immune cells with the ability to dampen immune response. Among these are the suppressor macrophages [11] as well as the induction of regulatory dendritic cells (DCreg) which are characterized by the expression of IL-10, TGF-β, indoleamine 2,3-dioxygenase, and cyclooxygenase-2 [12, 13]. Moreover, helminth infections result in the skewing of macrophage development away from classically activated macrophages to alternatively activated macrophages (AAM) (which can be induced by IL-4, IL-13, and IL-10) [14]. This army of regulatory cells can modulate immune responses in antigen (Ag)-specific and non-specific manner and can lead to restriction of inflammation. In terms of pro-inflammatory responses, several studies in humans as well as in animal models have indicated that Th1 and Th17 cells can be part of the immune response induced by helminth infections and might play an important role in pathological outcomes.

In schistosomiasis, immunoepidemiological studies have indicated that Th2 responses are associated with resistance to reinfection [15, 16]. However, in animal models, Th1 cells have been shown to also contribute to immunity against these parasites [17, 18]. With respect to pathology, pro-inflammatory responses, represented by high TNF and by the type-1 associated cytokine, interferon (IFN)-γ, were shown to be elevated in the circulation of Kenyan patients with hepatosplenomegaly [19], yet in a study in Brazil schistosome-related pathology was reported to be associated with higher IL-13 production [20]. Notwithstanding the experimental details, these apparently conflicting outcomes might reflect the fact that pathology is a complex process and immunological responses would be expected to be different during the different phases of the development of pathology. With respect to other cytokines, recent studies that have focused on examining the role of Th17 cells have shown these cells to be associated with schistosome-induced pathology in animal models [21, 22] and in humans [23]. In terms of control of the immune responses in schistosomiasis, several mechanisms seem to be involved, most prominent being the down-modulation of effector responses by IL-10 and TGF-ß, which prevent schistosome-induced pathology [24–26]. The exact mechanisms are not fully understood but could involve, in addition to Treg, IL-10 production B cells which have been shown to be present in murine and in human chronic schistosomiasis [27]. Moreover, in a recent review, the role of AAM as key players in controlling the development of immune pathology in schistosomiasis was highlighted [28]. To what extent Treg, B cells, or AAM play a role in the control of immune responses and pathology in human schistosomiasis needs to be determined in well-designed studies.

In STH infections which are mainly restricted to the gastrointestinal tract, acute or early infections, as shown in animal models with *Trichuris muris*, are often thought to be associated with pro-inflammatory cytokines, such as TNF [29, 30]. Th2 responses have been shown to be needed to expel these helminths from the gut [31, 32]. One of the mechanisms involved in the Th2-induced expulsion is the stimulation of higher intestinal epithelial cell turnover [33] and, as recently shown, the alteration of the composition of

the mucus layer [34]. Whereas work in experimental models has elegantly shown how different arms of the type-2 immune system are involved in intestinal worm expulsion [35] and how gut helminths try to avoid expulsion by up-regulation of IFN-γ to combat Th2 responses [36], relatively little is known about the situation in humans. One of the first human studies examining immune response to STH showed that stronger Th2 cytokines were associated with lower infection burden, suggesting that in humans type-2 responses may also be associated with protection from STH [31]. A few studies have reported the up-regulation of suppressory cytokines such as IL-10 and/or TGF-ß [37]. In Cameroon, children living in areas hyperendemic for helminths produce more IL-10 and TGF-ß than children living in mesoendemic areas [38], whereas in a study in Brazil, increased spontaneous IL-10 production was seen in a larger proportion of children infected with STH [37]. The higher production of these cytokines was related to hyporesponsiveness to helminth or bystander antigens [39] and presumably allows worm survival. A more comprehensive analysis of the response to STH infection was undertaken in chronically infected children whose peripheral blood gene expression patterns were studied extensively by using microarrays. The results indicated that chronic STH infection was associated with altered gene expression profiles that suggest these infections to be associated with modified Th2 responses (i.e., up-regulation of IL-5 and IL-10), with down-regulation of neutrophil activation and function and up-regulation of mediators associated with eosinophils [40]. Interestingly, microRNA (miRNA) was also studied using a specific array [40], and the results showed that a single miRNA, hsa-let-7d, was down-regulated during chronic STH infection. This miRNA, already reported to affect iron metabolism [41], is part of the let-7 miRNA family, found to be abundant in the alveolae of normal lungs but almost absent in the lungs of mice with idiopathic pulmonary fibrosis [42], suggesting its possible role in preventing lung fibrosis. Although these studies need to be linked to extensive target identification and functional studies, they indicate the potential for gaining a full picture of how these parasites might affect not only the immune system but also target organs.

In lymphatic filariasis, inflammatory responses occur when the adult worms die, either drug-induced or spontaneously. Granulomas arise around the worms, characterized by macrophages which develop into giant cells as well as plasma cells, eosinophils, and neutrophils [43]. Peripheral Th1 and Th17 cells as well as antibody responses are reported to be up-regulated in patients with chronic pathology [44]. The immune regulatory responses that are up-regulated during microfilaremic state without any signs of pathology are characterized by production of IL-10 and TGF-β [45] as well as with increased Treg. Metenou et al. [46] have shown that individuals infected with *W. bancrofti* and/or *Mansonella perstans* have increased frequency of natural Treg as well as IL-10 producing effector cells. A study in a *B. timori* endemic area, conducted by Wammes et al. [47], showed that the Treg of microfilaremic subjects has stronger suppressory activity than the Treg of endemic amicrofilaremic controls or subjects with chronic pathology. In onchocerciasis, hyper-reactivity has been shown to be correlated with pathology of the skin and lymph nodes [48]. A possible role for IL-13 in the pathogenesis of onchocerciasis was found in an immunogenetic study, which showed that Th2-dominated sowda form of hyper-reactive onchocerciasis was associated with a mutation in the IL-13 gene, a gene known to be linked to allergic hyper-reactivity [49]. As in lymphatic filariasis, Treg has been described in subjects with onchocerciasis [50, 51] and thought to keep the development of pathology at bay.

Taken together, it seems that the pro-inflammatory responses typified by Th1 and Th17 and also by hyperactive Th2 responses might underlie the immunopathology in helminth infections. However, most helminth infections appear to be associated with regulatory mechanisms [52] that try to keep pathological responses under control. These regulatory responses can be helminth Ag-specific or exert non-specific spill over suppressory effects on unrelated Ags.

Helminths and their spillover effects on health outcomes

Various helminth infections seem to have profound effects on the immune reactions against other infections and bystander Ags, be it vaccines, environmental allergens, or self-Ags. However, this is an area full of controversy and clearly needs more research.

Co-infection

It is common that helminth infections are co-endemic with malaria, tuberculosis (TB), and human immunodeficiency virus (HIV) [1]. This situation raises the question of what impact helminth infections may have on the co-endemic infections as they are believed to be able to suppress effector T cell responses. In filaria-infected individuals, the magnitude of inflammatory cytokines, IFN-γ and IFN-γ inducible protein 10, in response to malaria Ag was shown to be affected by filaria-induced IL-10 [53]. Moreover, in malaria-infected individuals, concomitant filarial infection has been shown to decrease the frequencies of malaria-specific Th1 and Th17 cells [54]. The same seems to be the case for mycobacteria Ag-specific responses, which appear to be down-regulated in filaria-infected subjects via increased expression of the negative co-stimulatory molecules CTLA-4 and inhibitory receptor programmed death 1 [55]. Interestingly, although chronic mycobacteria [56] and plasmodia infections [57, 58] on their

own are associated with increased Treg, a study on Flores island, Indonesia, using in vitro Treg depletion has shown that Treg from STH-infected subjects have stronger functional activity than Treg from those free of STH in immune responses to bystander mycobacteria and plasmodia Ag [59].

Although at the immunological level helminths seem to be able to influence responses to co-infections, it is far from clear whether this is translated into clinical and epidemiological outcomes. Epidemiological studies on the relationship between helminths and malaria, TB, or HIV have shown conflicting results [60, 61] as have studies in animal models [62–67].

In some studies, a positive association has been reported between helminths and malarial parasitemia, while in others this has been refuted or even a negative association has been found with clinical outcomes [68–70]. For example, there is evidence that helminth co-infection with malaria is associated with increased risk of anemia [71], but there is also evidence for a protective role against developing anemia [72] or no association [73]. A recent review by Nacher [60] suggests that there are different malaria outcomes with different species of helminths. This is supported by a recent case–control study in Colombia that showed an example of how different helminth species vary in their association with *Plasmodium falciparum* [74]. It showed that whereas *A. lumbricoides* infection had a protective effect, hookworm infection seemed to be a risk factor. In line with this unclear situation on the effect that helminths have on malaria parasites or outcomes are the inconsistent results of the deworming trials [75–77], which might be due to the differences in the characteristics of the populations studied, species of helminths prevalent, and the study designs.

Regarding the relationship between helminth infections and mycobacteria, there is still limited evidence for any significant interaction. In helminth-infected individuals, a higher risk to develop pulmonary TB has been reported [78], as well as IFN-γ production in response to purified protein derivative upon Bacillus Calmette Guerin vaccination [79]. However, in an area highly endemic for helminths in the Amazon, where it was expected that high helminth infection pressure would attenuate the tuberculin skin test (TST), there was no significant suppressive effect of helminth infection on the TST size [80].

The same inconclusiveness applies to the relationship between helminths and HIV. Concerning HIV acquisition and progression, Webb and colleagues [81] have reviewed this subject and indicate that there are inconsistent findings with regard to whether helminth–HIV co-infection can have a more detrimental effect on the host compared to a single HIV infection or the beneficial effect of anthelminthic treatment on HIV. A Cochrane review in 2009 demonstrated a beneficial effect of deworming on both plasma HIV-1 RNA and CD4 counts [82], but a recent report on anthelminthic treatment with albendazole and praziquantel during pregnancy in Uganda indicated that there was no effect of treatment on vertical HIV transmission [75]. However, in this study, the authors acknowledge that the data were generated on a small number of infants born to HIV-infected mothers. For the mothers, anthelminthic treatments resulted in a modest reduction in HIV virus load in the albendazole (to treat hookworm and *T. trichiura*)-treated mothers, but no difference was seen in praziquantel (to treat *S. mansoni*)-treated subjects compared to placebo at 6 weeks after administration of the drug [83]. These results might again be in line with what has been discussed in a meta-analysis of the effect of anthelminthic treatments on HIV that suggests that different helminth species might have different outcomes [84].

It has to be concluded that in order to be able to draw firm conclusions regarding the effect of helminths on malaria, TB, or HIV, well-powered placebo-controlled anthelminthic trials are needed with a streamlined design that will also take care of the issue that different helminth species might have a distinct modulatory role.

Non-communicable diseases

Allergy and asthma

Allergic diseases (allergic asthma, eczema, and rhinitis) are dominated by type-2 responses [85]. The prevalence of these diseases are thought to be relatively low in developing countries especially in rural areas [86–88], which has often been put into the context of the hygiene hypothesis and the high prevalence of helminth infections (Fig. 1). It has been argued that in rural areas of low-to-middle-income countries (LMIC), the exposure to microorganisms and parasites is high and therefore the immune system is educated in a way that it no longer reacts to innocuous substances such as environmental allergens. Although helminth infections skew immune responses toward Th2, they have been reported to be inversely associated with allergic disorders [89]. This has been argued to be due to the ability of chronic helminth infections to induce regulatory responses [90]. This means that helminth infections which have been associated with increased IL-10 or TGF-ß production by several regulatory cells might suppress the effector mechanisms that lead to the development of allergy. In a study in human schistosomiasis in Gabonese children more than a decade ago, it was shown that the anti-inflammatory cytokine, IL-10, in response to the helminth Ags, was associated inversely with skin prick test (SPT) reactivity to house dust mite allergen [89]. However, in a more recent study of Ecuadorian children, *A. lumbricoides* infection, although inversely associated with SPT reactivity to allergen [87], did not involve IL-10 production in response to *A. lumbricoides* Ag [91]. In contrast to the study in Ecuadorian children but in line with the Gabon study, in another study where hookworm-infected

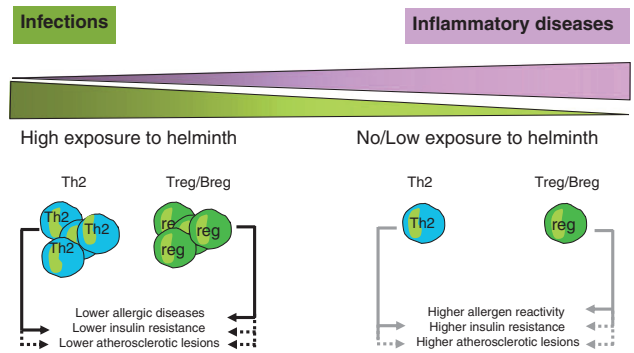


Fig. 1 A schematic representation of epidemiological transition and disease outcomes. Epidemiological transition is associated with changes in disease burden. With decreasing burden of infectious diseases, populations are facing increasing inflammatory noncommunicable disorders. One hypothetical framework for these observed changes is that infections such as helminths which are associated with expansion of Th2 and regulatory T and B cells are disappearing and therefore leading to altered immune profiles. There is evidence, as discussed in this review, that Th2 responses are associated with lower insulin resistance while enhanced Treg and Breg can lead to suppression of allergic diseases in animal models. Therefore, when Th2 and regulatory cells decline, it follows that insulin resistance and allergic diseases become rampant. The *black lines* represent strong modulatory pathways in areas where infections are highly prevalent, while *gray lines* represent weak modulatory pathways in areas where infections are largely controlled. The *solid lines* represent associations based on data available, while *dotted lines* represent theoretical associations that are yet to be tested

schoolchildren were studied in Vietnam, IL-10 production in response to *N. americanus* excretory–secretory Ag was inversely associated with skin sensitization [92]. The discrepancy between these studies might be caused by infection intensity as shown in an animal model [93] or differences in helminth species. Another important aspect of the relationship between helminths and allergies is the issue of IgE cross-reactivity. There is evidence for IgE cross-reactivity between helminth Ags and allergens such as with cockroach [94] or mite [95]. It has been suggested that this cross-reactivity might influence allergy outcomes. Whereas in Europe, high total and specific IgE are associated with increased risk of SPT positivity [96] and allergic disease, this association is less clear in areas endemic for helminth infections [97, 98]. The reason may be that IgE cross-reactive between helminths and allergens has low biologic activity [90]. Thus, in areas where helminths are endemic, early sensitization to helminth Ag might lead to weak IgE binding to cross-reactive allergens, preventing strong allergic responses, and might explain in part the mechanisms whereby helminth infections lead to fewer allergic disorders.

With respect to deworming trials, 1 year of anthelminthic treatment in studies in Ecuador and in Vietnam showed inconsistent results, the former showing no effect, but the latter led to increased SPT reactivity to allergen [92, 99]. In addition, a study in Denmark analyzing records of children born between 1995 and 2008 showed that treatment with mebendazole of children infected with *Enterobius vermicularis* had a marginal effect on the incidence of asthma [100]. Interestingly, in a study of anthelminthic treatment of pregnant women in Uganda, an increased risk of infantile eczema was seen in mothers who received anthelminthics compared to those that received placebo [101]. This raises the issue regarding the timing of deworming; maybe exposure to worms in early life imprints on the immune system, affecting allergic outcomes more profoundly than when helminths are removed later in life [102]. Moreover, there is increasing evidence for the difference in risk factors associated with allergic and nonallergic asthma [103] or maybe even eczema.

The converse experiments have been attempted where patients were infected with helminths to treat allergic disorders. The outcomes of these trials did not show any beneficial effects [104–106]. Randomized placebo-controlled trial of treatment with hookworm in adult patients with allergic asthma [106] showed improvement of bronchial responsiveness in infected group, but this was not significantly different compared to the placebo-treated group. It might indicate that higher doses of hookworm infection are needed. It must be pointed immediately that not all helminth infections can be therapeutically equal; for example, in mouse models for experimental allergic inflammation, previous infections with *Strongyloides stercoralis* [107] or *Nippostrongylus brasiliensis* [108] resulted in suppression of allergic response to ova (as an allergen) challenge, while infection with *Toxocara canis* resulted in exacerbation of the allergic response [109]. Altogether the hypothesis that helminth infection might be beneficial against allergic disease or deworming might increase risk for the disease is still controversial and needs more convincing evidence [110–112]. There has been one very-long-term study where 15–17 years of ivermectin treatment to control

onchocerciasis in Ecuador has resulted in increased SPT reactivity to allergens [113]. Understandably, such studies are difficult to perform and the question remains whether these can be repeated. A recent Cochrane review [104] also acknowledged insufficient evidence on the efficacy, tolerability, and likely costs of using helminth infection as therapy to support their use in the routine management of allergic rhinitis.

Autoimmune diseases and inflammatory bowel disease

There are very few epidemiological studies of autoimmune diseases in LMIC; however, geographical differences have been reported. The conclusion of ecological studies is that the situation of autoimmune diseases is mirroring that seen in the field of allergy. It is suspected that there might be an inverse association between the prevalence of autoimmune diseases and the prevalence of infections including helminths [114]. One of the first studies showing the association between helminth and autoimmune diseases was in an animal model of multiple sclerosis (MS), where injection of mice with *S. mansoni* eggs led to reduced severity of the disease and delayed development of clinical symptoms [115]. Recently, several studies have demonstrated that the course of autoimmunity is determined by a mixture of pathological Th1 and Th17 responses [116, 117], and in the light of immune regulation, the potential role of helminths to reduce the severity of disease in MS patients [118–120] and inflammatory bowel disease (IBD) [121–123] has been shown in a number of trials. A clinical trial using repeated *Trichuris suis* ova treatment resulted in reduced relapses in MS patients [123]. In another study of MS patients, B cells isolated from patients who were infected with helminths produced higher levels of IL-10 than B cells from uninfected patients [120]. These B cells could also produce high levels of brain-derived neurotrophic factor and nerve growth factor which are thought to have a neuroprotective effect. The most detailed cellular and molecular information on changes that take place in the intestinal mucosa has been gathered on one ulcerative colitis patient who self-treated himself with eggs of *T. trichiura* [124]. Ingestion of two doses of helminth eggs (500 and 1,000 eggs) led to nearly 3 years of complete disease remission [124]. As the symptoms began to deteriorate paralleling a decline in egg counts, a third dose (2,000 eggs) was taken and resulted in both symptom and histopathological improvement, the latter seen in biopsies following colonoscopy [124]. Detailed immunological studies showed that active colitis was associated with cytokine-producing CD4+ IL-17+ Th cells, whereas helminth colonization and disease remission were characterized by the presence of IL-22+ Th cells in the colonic mucosa. Moreover, the helminth-colonized ascending colon had higher expression of IL-4, IL-25, and RELMβ [124], while the rectum with active symptoms had elevated IL-17 and TNF as well as reduced expression of RELMβ [124]. With respect to IL-22, gene delivery of this cytokine to a local inflamed colon in a colitis model led to attenuation of colitis, which was shown to be due to the enhancement of intestinal mucus production by goblet cells [125]. The role of other cells such as macrophages is not as clear. The macrophages of the gastrointestinal tract, which are distinct from blood macrophages [14], seem to be essential for keeping the balance of commensal microbiota such that pathology is avoided [126]. There is however still controversy about whether AAM are beneficial or detrimental in this aspect. In a murine model of bacterial colitis, infection with *Heligmosomoides polygyrus*, which was associated with increased AAM, seemed to lead to severer colitis [127]. Yet in another chemically induced colitis model, *Hymenolepis diminuta* infection induced an increase of FIZZ1/RELMα and argininase-1 expression as markers of AAM and attenuated colitis [128]. The discrepancy on the role of macrophages could be the result of the use of different species of helminths in the two studies or the type of colitis model used. Nevertheless, Wolff and colleagues recently summarized that helminthic therapy for autoimmune diseases such as IBD is relatively safe [129] and might provide new therapeutic possibilities for inflammatory diseases such as MS and IBD. It is again important to note that large-scale trials are essential before any firm conclusion can be drawn.

Cardiovascular disease

Cardiovascular disease (CVD) involves the heart and the blood vessels. Atherosclerosis is an important risk factor for developing CVD [130] and is now believed to involve inflammation. Part of the inflammatory process is thought to be mediated by the infiltration and retention of low-density lipoprotein (LDL) in the arterial intima. The transformation of these lipids into oxidized lipids can initiate an inflammatory response that can accelerate CVD progression [131]. The oxidized lipids can stimulate innate immune responses and lead to the recruitment of inflammatory cells, such as monocytes, into the vessel wall that when activated can attract other cells and aggravate the growing plaque (reviewed by Hansson [132]). However, currently available therapeutics against CVD are largely restricted to alleviating hypertension and hyperlipidemia [133, 134], while drugs targeting inflammatory mediators are not yet available. Interestingly, the lipid-lowering drug statin, one of the most frequently used medication, has been shown to have anti-inflammatory effects [135, 136]. Moreover, studies on the role of anti-inflammatory mechanism in atherogenesis have suggested that Treg [137] might be involved to limit inflammation and attenuate atherogenesis. In an experimental model of hypertensive mice, Kassan et al. [138] showed that IL-10 released by Treg could improve microvascular endothelial function by reducing the nicotinamide adenine dinucleotide phosphate oxidase activity and increasing endothelial NOS activity. These data suggest

that indeed the balance between pro- and anti-inflammatory cytokines might be an important element in the regulation of vascular endothelial function.

CVD prevalence is increasing in Asian countries [139]. In this region, rapid socioeconomic development has led to a shift in infrastructure, technology, and food supply that promotes over-nutrition and sedentary lifestyles [140]. Also, in Asia, despite large geographical differences, infectious disease control such as deworming program is underway [141]. Hypothetically, helminths might protect against CVD by reducing risk factors such as nutritional status. Helminths are known to reduce energy intake and to be associated with poor nutritional status, which in turn is associated with beneficial effects on traditional CVD risk factors such as reduced serum lipid levels [142]. Using apolipoprotein deficient (apoE−/−) mice, a transgenic mice that has impaired plasma lipoprotein clearance and develops atherosclerosis in a short time [143], the development of atherosclerotic lesions was reduced by approximately 50 % in mice infected with *S. mansoni* [144]. The same group showed that the cholesterol-lowering effects were mediated by soluble factors released from *S. mansoni* live eggs. A similar experiment, but using frozen eggs, showed a reduction in total cholesterol and LDL in mice injected with schistosome egg compared to those not injected with eggs. However, in the latter study, no difference in atherosclerotic lesion formation was observed [145]. Taken together, these data suggest that helminth infections or their products might be able to act as lipid-lowering agents.

Recently, a number of studies have shown a higher risk of death from CVD among patients with rheumatoid arthritis (RA) [146–148], which would be in line with the notion that increased inflammation such as higher TNF and IL17 in RA would lead to a higher risk of CVD [149]. A meta-analysis by Micha et al. [150] summarized that methotrexate, an anti-RA drug which is also used for the treatment of chronic inflammatory disorders, has a beneficial effect on CVD. Interestingly, the reported immune-modulatory capacity of statins is based on their ability to induce Treg recruitment [151] and to inhibit the pro-inflammatory action of IL-17 and TNF on human endothelial cells [152]. A similar immune suppression by Treg, decreasing pro-inflammatory cytokines such TNF and IL-17, has been shown in helminth-infected subjects [47, 54]. Moreover, a family of antioxidant proteins, the peroxiredoxins (PRDX), essential for the enzymatic scavenging of hydrogen peroxide [153], has been shown to play a role as immune modulators [154]. There is evidence that PRDX-1 and -2 are able to interfere with endothelial activation and to regulate pro-inflammatory responses in apoE−/− mice, respectively [155, 156]. Another PRDX family, PRDX-4 seems to protect against the formation of unstable atherosclerotic plaques [157]. Interestingly, PRDX is also one of the molecules secreted by several helminth parasites and is thought to help the parasites to get through the defense barriers and avoid attack mounted by the host [158–160]. Therefore, an interesting area of research would be to test the role of helminth-secreted PRDX and possibly other helminth-derived products on CVD as potential anti-atherogenic therapy (Fig. 1).

Diabetes

Obesity, DM2, and CVD share a metabolic profile characterized by insulin resistance (IR) and chronic sub-acute inflammation [161]. It has been shown that the pro-inflammatory cytokine TNF is able to induce IR [162, 163]. This was shown in murine models where a lack of TNF function results in improved insulin sensitivity and glucose homeostasis, while administration of TNF leads to IR [164]. As already discussed in earlier sections, helminth infections seem to affect inflammation. In this regard, cross-sectional studies in southern India showed an inverse association between DM1 [165] and DM2 [166] with lymphatic filariasis. In our study in an area endemic for STH on Flores Island, Indonesia, individuals infected with STH showed lower IR (Wiria et al., unpublished data). The influence of helminth infections has been shown in animal models of DM1 [167] and DM2 [168]. Inhibition of DM1 development by helminth infections appeared to be due to the ability of helminths and their products to induce IL-10 production by DC, B cells, AAM, and Treg. Likewise in an experimental model of DM2, Ricardo-Gonzales et al. [169] demonstrated an improvement of glucose metabolism mediated by the IL-4/STAT6 immune axis, a key pathway in helminth immunity and Th2 response. They showed that IL-4 administration improved insulin action, lowered levels of insulin, total cholesterol, and triglyceride, and protected the mice from diet-induced obesity. Another study showed that helminth-induced eosinophilia forestalls obesity and IR [168]. Wu et al. [168] showed that in mice kept on high-fat diet, infection with *N. brasiliensis* induced sustained eosinophilia. Eoshinophils migrated into adipose tissue, secreted IL-4, and then induced AAM in the tissue; these cells are able to sustain insulin sensitivity [168]. Despite the extensive population studies on the role of helminths in allergy or autoimmune diseases, epidemiological or mechanistic data on the relationship between helminths and DM2 are still lacking, and even less is known about the effect of deworming programs on IR and emergence of DM2 (Fig. 1).

Helminth-derived molecules

Recent years have seen a surge in efforts to identify helminth-derived molecules with modulatory activity [170]. The characterization of such molecules would be beneficial on two fronts: (1) they can serve as vaccines if

used by parasites to enhance their survival—their neutralization would be expected to enhance worm expulsion [35] and (2) they have the potential to be used to tame hyper-inflammatory disorders. Early studies have shown that schistosomes carry lyso-phosphatidylserine that are able to modulate DC and induce Treg [171]. In addition, the ability of schistosomes to stimulate Th2 responses [172] was shown to be mediated via immune-modulatory molecules present in the extracts of eggs or in products secreted by eggs (omega-1, IPSE, PRDX). These molecules can work via modulating DC [173] and basophils [174] and via machrophages [158]. Other human helminth parasites are also known to release modulatory molecules, such as AvCystatine, which is derived from *O. volvulus* worms and stimulates the preferential production of IL-10 by macrophages [175]. Much work is underway to characterize molecules from worms that are used as models of human infection. For example, from *Fasciola hepatica*, a molecule which is called *F. hepatica* helminth defense molecule-1 [176] can prevent LPS-induced activation of innate immune responses in macrophages, and from *H. polygyrus* the excretory–secretory antigen (HES) is able to induce Foxp3+ Treg via a TGF-ß-like activity [177]. Interestingly, vaccination by HES resulted in antibody production and protection against infection with *H. polygyrus* [178]. Thus, the study of immune modulatory molecules may provide new candidates for treatment of inflammatory diseases on the one hand and vaccines against parasites on the other.

Conclusions

Studies of helminth infections which seem to be able to modulate the immune system have provided detailed information on cellular mechanisms involved in pathology, immunity, and tolerance. Of particular interest has been the ability of these parasites to exert anti-inflammatory responses. On the one hand, this could potentially have detrimental consequences for co-infections or vaccination programs, while on the other hand it could exert beneficial effects on diseases that stem from strong inflammatory reactions. The identification of helminth-specific molecules with immune modulatory activities holds great potential as new therapeutics. However, the clinical implications of these parasites and their immune modulatory activities have yet to be determined in well-designed population studies.

Acknowledgments This work was supported by the Royal Netherlands Academy of Arts and Sciences (KNAW) in the form of funding for the ImmunoSPIN project (KNAW-05-PP-35).

Conflict of interest All authors declare that they have no conflict of interest.

References

1. Hotez PJ, Mistry N, Rubinstein J, Sachs JD (2011) Integrating neglected tropical diseases into AIDS, tuberculosis, and malaria control. N Engl J Med 364:2086–2089
2. Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D, Hotez PJ (2006) Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet 367:1521–1532
3. Gryseels B (2012) Schistosomiasis. Infect Dis Clin North Am 26:383–397
4. Ross AG, McManus DP, Farrar J, Hunstman RJ, Gray DJ, Li YS (2012) Neuroschistosomiasis. J Neurol 259:22–32
5. Ross AG, Vickers D, Olds GR, Shah SM, McManus DP (2007) Katayama syndrome. Lancet Infect Dis 7:218–224
6. Taylor MJ, Hoerauf A, Bockarie M (2010) Lymphatic filariasis and onchocerciasis. Lancet 376:1175–1185
7. Hoerauf A, Pfarr K, Mand S, Debrah AY, Specht S (2011) Filariasis in Africa—treatment challenges and prospects. Clin Microbiol Infect 17:977–985
8. Sakaguchi S, Yamaguchi T, Nomura T, Ono M (2008) Regulatory T cells and immune tolerance. Cell 133:775–787
9. Maizels RM, Yazdanbakhsh M (2008) T-cell regulation in helminth parasite infections: implications for inflammatory diseases. Chem Immunol Allergy 94:112–123
10. Hussaarts L, van der Vlugt LE, Yazdanbakhsh M, Smits HH (2011) Regulatory B-cell induction by helminths: implications for allergic disease. J Allergy Clin Immunol 128:733–739
11. Atochina O, Daly-Engel T, Piskorska D, McGuire E, Harn DA (2001) A schistosome-expressed immunomodulatory glycoconjugate expands peritoneal Gr1(+) macrophages that suppress naive CD4(+) T cell proliferation via an IFN-gamma and nitric oxide-dependent mechanism. J Immunol 167:4293–4302
12. Everts B, Smits HH, Hokke CH, Yazdanbakhsh M (2010) Helminths and dendritic cells: sensing and regulating via pattern recognition receptors, Th2 and Treg responses. Eur J Immunol 40:1525–1537
13. Broere F, du Pre MF, van Berkel LA, Garssen J, Schmidt-Weber CB, Lambrecht BN, Hendriks RW, Nieuwenhuis EE, Kraal G, Samsom JN (2009) Cyclooxygenase-2 in mucosal DC mediates induction of regulatory T cells in the intestine through suppression of IL-4. Mucosal Immunol 2:254–264
14. Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8:958–969
15. Dunne DW, Butterworth AE, Fulford AJ, Kariuki HC, Langley JG, Ouma JH, Capron A, Pierce RJ, Sturrock RF (1992) Immunity after treatment of human schistosomiasis: association between IgE antibodies to adult worm antigens and resistance to reinfection. Eur J Immunol 22:1483–1494
16. Roberts M, Butterworth AE, Kimani G, Kamau T, Fulford AJ, Dunne DW, Ouma JH, Sturrock RF (1993) Immunity after treatment of human schistosomiasis: association between cellular responses and resistance to reinfection. Infect Immun 61:4984–4993
17. Betts CJ, Wilson RA (1998) Th1 cytokine mRNA expression dominates in the skin-draining lymph nodes of C57BL/6 mice following vaccination with irradiated *Schistosoma mansoni* cercariae, but is down-regulated upon challenge infection. Immunology 93:49–54
18. Hoffmann KF, James SL, Cheever AW, Wynn TA (1999) Studies with double cytokine-deficient mice reveal that highly polarized Th1- and Th2-type cytokine and antibody responses contribute

equally to vaccine-induced immunity to *Schistosoma mansoni*. J Immunol 163:927–938
19. Mwatha JK, Kimani G, Kamau T, Mbugua GG, Ouma JH, Mumo J, Fulford AJ, Jones FM, Butterworth AE, Roberts MB, Dunne DW (1998) High levels of TNF, soluble TNF receptors, soluble ICAM-1, and IFN-gamma, but low levels of IL-5, are associated with hepatosplenic disease in human schistosomiasis mansoni. J Immunol 160:1992–1999
20. Alves Oliveira LF, Moreno EC, Gazzinelli G, Martins-Filho OA, Silveira AM, Gazzinelli A, Malaquias LC, LoVerde P, Leite PM, Correa-Oliveira R (2006) Cytokine production associated with periportal fibrosis during chronic schistosomiasis mansoni in humans. Infect Immun 74:1215–1221
21. Rutitzky LI, Bazzone L, Shainheit MG, Joyce-Shaikh B, Cua DJ, Stadecker MJ (2008) IL-23 is required for the development of severe egg-induced immunopathology in schistosomiasis and for lesional expression of IL-17. J Immunol 180:2486–2495
22. Rutitzky LI, Stadecker MJ (2011) Exacerbated egg-induced immunopathology in murine *Schistosoma mansoni* infection is primarily mediated by IL-17 and restrained by IFN-gamma. Eur J Immunol 41:2677–2687
23. Mbow M, Larkin BM, Meurs L, Wammes LJ, de Jong SE, Labuda L, Camara M, Smits HH, Polman K, Dieye TN, Mboup S, Stadecker MJ, Yazdanbakhsh M (2012) Th17 cells are associated with pathology in human schistosomiasis. J Infect Dis (in press)
24. Baumgart M, Tompkins F, Leng J, Hesse M (2006) Naturally occurring CD4+ Foxp3+ regulatory T cells are an essential, IL-10-independent part of the immunoregulatory network in *Schistosoma mansoni* egg-induced inflammation. J Immunol 176:5374–5387
25. Herbert DR, Holscher C, Mohrs M, Arendse B, Schwegmann A, Radwanska M, Leeto M, Kirsch R, Hall P, Mossmann H, Claussen B, Forster I, Brombacher F (2004) Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. Immunity 20:623–635
26. Turner JD, Jenkins GR, Hogg KG, Aynsley SA, Paveley RA, Cook PC, Coles MC, Mountford AP (2011) CD4+ CD25+ regulatory cells contribute to the regulation of colonic Th2 granulomatous pathology caused by schistosome infection. PLoS Negl Trop Dis 5:e1269
27. van der Vlugt LE, Labuda LA, Ozir-Fazalalikhan A, Lievers E, Gloudemans AK, Liu KY, Barr TA, Sparwasser T, Boon L, Ngoa UA, Feugap EN, Adegnika AA, Kremsner PG, Gray D, Yazdanbakhsh M, Smits HH (2012) Schistosomes induce regulatory features in human and mouse CD1d(hi) B cells: inhibition of allergic inflammation by IL-10 and regulatory T cells. PLoS One 7:e30883
28. Barron L, Wynn TA (2011) Macrophage activation governs schistosomiasis-induced inflammation and fibrosis. Eur J Immunol 41:2509–2514
29. Hayes KS, Bancroft AJ, Grencis RK (2007) The role of TNF-alpha in *Trichuris muris* infection II: global enhancement of ongoing Th1 or Th2 responses. Parasite Immunol 29:583–594
30. Artis D, Humphreys NE, Bancroft AJ, Rothwell NJ, Potten CS, Grencis RK (1999) Tumor necrosis factor alpha is a critical component of interleukin 13-mediated protective T helper cell type 2 responses during helminth infection. J Exp Med 190:953–962
31. Jackson JA, Turner JD, Rentoul L, Faulkner H, Behnke JM, Hoyle M, Grencis RK, Else KJ, Kamgno J, Boussinesq M, Bradley JE (2004) T helper cell type 2 responsiveness predicts future susceptibility to gastrointestinal nematodes in humans. J Infect Dis 190:1804–1811
32. Else KJ, Grencis RK (1991) Cellular immune responses to the murine nematode parasite *Trichuris muris*. I. Differential cytokine production during acute or chronic infection. Immunology 72:508–513
33. Cliffe LJ, Humphreys NE, Lane TE, Potten CS, Booth C, Grencis RK (2005) Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. Science 308:1463–1465
34. Hasnain SZ, Evans CM, Roy M, Gallagher AL, Kindrachuk KN, Barron L, Dickey BF, Wilson MS, Wynn TA, Grencis RK, Thornton DJ (2011) Muc5ac: a critical component mediating the rejection of enteric nematodes. J Exp Med 208:893–900
35. Maizels RM, Hewitson JP, Smith KA (2012) Susceptibility and immunity to helminth parasites. Curr Opin Immunol 24:459–466
36. Artis D, Potten CS, Else KJ, Finkelman FD, Grencis RK (1999) *Trichuris muris*: host intestinal epithelial cell hyperproliferation during chronic infection is regulated by interferon-gamma. Exp Parasitol 92:144–153
37. Figueiredo CA, Barreto ML, Rodrigues LC, Cooper PJ, Silva NB, Amorim LD, Alcantara-Neves NM (2010) Chronic intestinal helminth infections are associated with immune hyporesponsiveness and induction of a regulatory network. Infect Immun 78:3160–3167
38. Turner JD, Jackson JA, Faulkner H, Behnke J, Else KJ, Kamgno J, Boussinesq M, Bradley JE (2008) Intensity of intestinal infection with multiple worm species is related to regulatory cytokine output and immune hyporesponsiveness. J Infect Dis 197:1204–1212
39. Figueiredo CA, Alcantara-Neves NM, Amorim LD, Silva NB, Carvalho LC, Cooper PJ, Rodrigues LC, Barreto ML (2011) Evidence for a modulatory effect of IL-10 on both Th1 and Th2 cytokine production: the role of the environment. Clin Immunol 139:57–64
40. Reina OM, Schreiber F, Benitez S, Broncano N, Chico ME, Vaca M, Alexander N, Lewis DJ, Dougan G, Cooper PJ (2011) Effects of chronic ascariasis and trichuriasis on cytokine production and gene expression in human blood: a cross-sectional study. PLoS Negl Trop Dis 5:e1157
41. Andolfo I, De FL, Asci R, Russo R, Colucci S, Gorrese M, Zollo M, Iolascon A (2010) Regulation of divalent metal transporter 1 (DMT1) non-IRE isoform by the microRNA Let-7d in erythroid cells. Haematologica 95:1244–1252
42. Pandit KV, Corcoran D, Yousef H, Yarlagadda M, Tzouvelekis A, Gibson KF, Konishi K, Yousem SA, Singh M, Handley D, Richards T, Selman M, Watkins SC, Pardo A, Ben-Yehudah A, Bouros D, Eickelberg O, Ray P, Benos PV, Kaminski N (2010) Inhibition and role of let-7d in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 182:220–229
43. Jungmann P, Figueredo-Silva J, Dreyer G (1991) Bancroftian lymphadenopathy: a histopathologic study of fifty-eight cases from northeastern Brazil. AmJTrop Med Hyg 45:325–331
44. Babu S, Bhat SQ, Pavan KN, Lipira AB, Kumar S, Karthik C, Kumaraswami V, Nutman TB (2009) Filarial lymphedema is characterized by antigen-specific Th1 and th17 proinflammatory responses and a lack of regulatory T cells. PLoS Negl Trop Dis 3: e420
45. Babu S, Blauvelt CP, Kumaraswami V, Nutman TB (2006) Regulatory networks induced by live parasites impair both Th1 and Th2 pathways in patent lymphatic filariasis: implications for parasite persistence. J Immunol 176:3248–3256
46. Metenou S, Dembele B, Konate S, Dolo H, Coulibaly SY, Coulibaly YI, Diallo AA, Soumaoro L, Coulibaly ME, Sanogo D, Doumbia SS, Traore SF, Mahanty S, Klion A, Nutman TB (2010) At homeostasis filarial infections have expanded adaptive T regulatory but not classical Th2 cells. J Immunol 184:5375–5382
47. Wammes LJ, Hamid F, Wiria AE, Wibowo H, Sartono E, Maizels RM, Smits HH, Supali T, Yazdanbakhsh M (2012) Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics. PLoS Negl Trop Dis 6:e1655
48. Korten S, Hoerauf A, Kaifi JT, Buttner DW (2011) Low levels of transforming growth factor-beta (TGF-beta) and reduced suppression of Th2-mediated inflammation in hyperreactive human onchocerciasis. Parasitology 138:35–45

49. Hoerauf A, Kruse S, Brattig NW, Heinzmann A, Mueller-Myhsok B, Deichmann KA (2002) The variant Arg110Gln of human IL-13 is associated with an immunologically hyper-reactive form of onchocerciasis (sowda). Microbes Infect 4:37–42
50. Satoguina J, Mempel M, Larbi J, Badusche M, Loliger C, Adjei O, Gachelin G, Fleischer B, Hoerauf A (2002) Antigen-specific T regulatory-1 cells are associated with immunosuppression in a chronic helminth infection (onchocerciasis). Microbes Infect 4:1291–1300
51. Doetze A, Satoguina J, Burchard G, Rau T, Loliger C, Fleischer B, Hoerauf A (2000) Antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by T(h)3/T(r)1-type cytokines IL-10 and transforming growth factor-beta but not by a T(h)1 to T(h)2 shift. Int Immunol 12:623–630
52. Maizels RM, Balic A, Gomez-Escobar N, Nair M, Taylor MD, Allen JE (2004) Helminth parasites—masters of regulation. Immunol Rev 201:89–116
53. Metenou S, Dembele B, Konate S, Dolo H, Coulibaly SY, Coulibaly YI, Diallo AA, Soumaoro L, Coulibaly ME, Sanogo D, Doumbia SS, Wagner M, Traore SF, Klion A, Mahanty S, Nutman TB (2009) Patent filarial infection modulates malaria-specific type 1 cytokine responses in an IL-10-dependent manner in a filaria/malaria-coinfected population. J Immunol 183:916–924
54. Metenou S, Dembele B, Konate S, Dolo H, Coulibaly YI, Diallo AA, Soumaoro L, Coulibaly ME, Coulibaly SY, Sanogo D, Doumbia SS, Traore SF, Mahanty S, Klion A, Nutman TB (2011) Filarial infection suppresses malaria-specific multifunctional Th1 and Th17 responses in malaria and filarial coinfections. J Immunol 186:4725–4733
55. Babu S, Bhat SQ, Kumar NP, Jayantasri S, Rukmani S, Kumaran P, Gopi PG, Kolappan C, Kumaraswami V, Nutman TB (2009) Human type 1 and 17 responses in latent tuberculosis are modulated by coincident filarial infection through cytotoxic T lymphocyte antigen-4 and programmed death-1. J Infect Dis 200:288–298
56. Marin ND, Paris SC, Velez VM, Rojas CA, Rojas M, Garcia LF (2010) Regulatory T cell frequency and modulation of IFN-gamma and IL-17 in active and latent tuberculosis. Tuberculosis (Edinb) 90:252–261
57. Bueno LL, Morais CG, Araujo FF, Gomes JA, Correa-Oliveira R, Soares IS, Lacerda MV, Fujiwara RT, Braga EM (2010) *Plasmodium vivax*: induction of CD4+ CD25+ FoxP3+ regulatory T cells during infection are directly associated with level of circulating parasites. PLoS One 5:e9623
58. Scholzen A, Mittag D, Rogerson SJ, Cooke BM, Plebanski M (2009) *Plasmodium falciparum*-mediated induction of human CD25Foxp3 CD4 T cells is independent of direct TCR stimulation and requires IL-2, IL-10 and TGFbeta. PLoS Pathog 5:e1000543
59. Wammes LJ, Hamid F, de GB Wiria AE, Sartono E, Maizels RM, Luty AJ, Fillie Y, Brice GT, Supali T, Smits HH, Yazdanbakhsh M (2010) Regulatory T cells in human geohelminth infection suppress immune responses to BCG and *Plasmodium falciparum*. Eur J Immunol 40:437–442
60. Nacher M (2011) Interactions between worms and malaria: good worms or bad worms? Malar J 10:259
61. Elliott A, Yazdanbakhsh M (2012) Troubles never come alone. Curr Opin HIV AIDS. doi:10.1097/COH.0b013e32835268ab
62. Elias D, Akuffo H, Thors C, Pawlowski A, Britton S (2005) Low dose chronic *Schistosoma mansoni* infection increases susceptibility to *Mycobacterium bovis* BCG infection in mice. Clin Exp Immunol 139:398–404
63. Dias AT, de Castro SB, Alves CC, Rezende AB, Rodrigues MF, Machado RR, Fernandes A, Negrao-Correa D, Teixeira HC, Ferreira AP (2011) Lower production of IL-17A and increased susceptibility to *Mycobacterium bovis* in mice coinfected with *Strongyloides venezuelensis*. Mem Inst Oswaldo Cruz 106:617–619
64. Erb KJ, Trujillo C, Fugate M, Moll H (2002) Infection with the helminth *Nippostrongylus brasiliensis* does not interfere with efficient elimination of *Mycobacterium bovis* BCG from the lungs of mice. Clin Diagn Lab Immunol 9:727–730
65. Frantz FG, Rosada RS, Peres-Buzalaf C, Perusso FR, Rodrigues V, Ramos SG, Kunkel SL, Silva CL, Faccioli LH (2010) Helminth coinfection does not affect therapeutic effect of a DNA vaccine in mice harboring tuberculosis. PLoS Negl Trop Dis 4:e700
66. Segura M, Matte C, Thawani N, Su Z, Stevenson MM (2009) Modulation of malaria-induced immunopathology by concurrent gastrointestinal nematode infection in mice. Int J Parasitol 39:1525–1532
67. Helmby H (2009) Gastrointestinal nematode infection exacerbates malaria-induced liver pathology. J Immunol 182:5663–5671
68. Nacher M, Singhasivanon P, Yimsamran S, Manibunyong W, Thanyavanich N, Wuthisen R, Looareesuwan S (2002) Intestinal helminth infections are associated with increased incidence of *Plasmodium falciparum* malaria in Thailand. J Parasitol 88:55–58
69. Le Hesran JY, Akiana J, el HM N, Dia M, Senghor P, Konate L (2004) Severe malaria attack is associated with high prevalence of *Ascaris lumbricoides* infection among children in rural Senegal. Trans R Soc Trop Med Hyg 98:397–399
70. Nacher M, Singhasivanon P, Traore B, Vannaphan S, Gay F, Chindanond D, Franetich JF, Mazier D, Looareesuwan S (2002) Helminth infections are associated with protection from cerebral malaria and increased nitrogen derivatives concentrations in Thailand. AmJTrop Med Hyg 66:304–309
71. Magalhaes RJ, Clements AC (2011) Mapping the risk of anaemia in preschool-age children: the contribution of malnutrition, malaria, and helminth infections in West Africa. PLoS Med 8:e1000438
72. Melo GC, Reyes-Lecca RC, Vitor-Silva S, Monteiro WM, Martins M, Benzecry SG, Alecrim MG, Lacerda MV (2010) Concurrent helminthic infection protects schoolchildren with *Plasmodium vivax* from anemia. PLoS One 5:e11206
73. Humphries D, Mosites E, Otchere J, Twum WA, Woo L, Jones-Sanpei H, Harrison LM, Bungiro RD, Benham-Pyle B, Bimi L, Edoh D, Bosompem K, Wilson M, Cappello M (2011) Epidemiology of hookworm infection in Kintampo North Municipality, Ghana: patterns of malaria coinfection, anemia, and albendazole treatment failure. AmJTrop Med Hyg 84:792–800
74. Fernandez-Nino JA, Idrovo AJ, Cucunuba ZM, Reyes-Harker P, Guerra AP, Moncada LI, Lopez MC, Barrera SM, Cortes LJ, Olivera M, Nicholls RS (2012) Paradoxical associations between soil-transmitted helminths and *Plasmodium falciparum* infection. Trans R Soc Trop Med Hyg. doi:10.1016/j.trstmh.2012.07.012
75. Webb EL, Mawa PA, Ndibazza J, Kizito D, Namatovu A, Kyosiimire-Lugemwa J, Nanteza B, Nampijja M, Muhangi L, Woodburn PW, Akurut H, Mpairwe H, Akello M, Lyadda N, Bukusuba J, Kihembo M, Kizza M, Kizindo R, Nabulime J, Ameke C, Namujju PB, Tweyongyere R, Muwanga M, Whitworth JA, Elliott AM (2011) Effect of single-dose anthelmintic treatment during pregnancy on an infant's response to immunisation and on susceptibility to infectious diseases in infancy: a randomised, double-blind, placebo-controlled trial. Lancet 377:52–62
76. Kirwan P, Jackson AL, Asaolu SO, Molloy SF, Abiona TC, Bruce MC, Ranford-Cartwright L, O' Neill SM, Holland CV (2010) Impact of repeated four-monthly anthelmintic treatment on *Plasmodium* infection in preschool children: a double-blind placebo-controlled randomized trial. BMC Infect Dis 10:277
77. Brutus L, Watier L, Hanitrasoamampionona V, Razanatsoarilala H, Cot M (2007) Confirmation of the protective effect of *Ascaris lumbricoides* on *Plasmodium falciparum* infection: results of a randomized trial in Madagascar. AmJTrop Med Hyg 77:1091–1095

78. Tristao-Sa R, Ribeiro-Rodrigues R, Johnson LT, Pereira FE, Dietze R (2002) Intestinal nematodes and pulmonary tuberculosis. Rev Soc Bras Med Trop 35:533–535
79. Elias D, Britton S, Aseffa A, Engers H, Akuffo H (2008) Poor immunogenicity of BCG in helminth infected population is associated with increased in vitro TGF-beta production. Vaccine 26:3897–3902
80. Zevallos K, Vergara KC, Vergara A, Vidal C, Garcia HH, Evans CA (2010) Tuberculin skin-test reactions are unaffected by the severity of hyperendemic intestinal helminth infections and co-infections. AmJTrop Med Hyg 83:319–325
81. Webb EL, Ekii AO, Pala P (2012) Epidemiology and immunology of helminth–HIV interactions. Curr Opin HIV AIDS 7:245–253
82. Walson JL, Herrin BR, John-Stewart G (2009) Deworming helminth co-infected individuals for delaying HIV disease progression. Cochrane Database Syst Rev 3:CD006419
83. Webb EL, Kyosiimire-Lugemwa J, Kizito D, Nkurunziza P, Lule S, Muhangi L, Muwanga M, Kaleebu P, Elliott AM (2012) The effect of anthelmintic treatment during pregnancy on HIV plasma viral load: results from a randomized, double-blind, placebo-controlled trial in Uganda. J Acquir Immune Defic Syndr 60:307–313
84. Sangare LR, Herrin BR, John-Stewart G, Walson JL (2011) Species-specific treatment effects of helminth/HIV-1 co-infection: a systematic review and meta-analysis. Parasitology 138:1546–1558
85. Pulendran B, Artis D (2012) New paradigms in type 2 immunity. Science 337:431–435
86. Xu F, Yan S, Li F, Cai M, Chai W, Wu M, Fu C, Zhao Z, Kan H, Kang K, Xu J (2012) Prevalence of childhood atopic dermatitis: an urban and rural community-based study in Shanghai, China. PLoS One 7:e36174
87. Cooper PJ, Chico ME, Rodrigues LC, Ordonez M, Strachan D, Griffin GE, Nutman TB (2003) Reduced risk of atopy among school-age children infected with geohelminth parasites in a rural area of the tropics. J Allergy Clin Immunol 111:995–1000
88. Dagoye D, Bekele Z, Woldemichael K, Nida H, Yimam M, Hall A, Venn AJ, Britton JR, Hubbard R, Lewis SA (2003) Wheezing, allergy, and parasite infection in children in urban and rural Ethiopia. Am J Respir Crit Care Med 167:1369–1373
89. van den Biggelaar AH, van RR, Rodrigues LC, Lell B, Deelder AM, Kremsner PG, Yazdanbakhsh M (2000) Decreased atopy in children infected with *Schistosoma haematobium*: a role for parasite-induced interleukin-10. Lancet 356:1723–1727
90. Yazdanbakhsh M, Kremsner PG, van RR (2002) Allergy, parasites, and the hygiene hypothesis. Science 296:490–494
91. Cooper PJ, Mitre E, Moncayo AL, Chico ME, Vaca MG, Nutman TB (2008) *Ascaris lumbricoides*-induced interleukin-10 is not associated with atopy in schoolchildren in a rural area of the tropics. J Infect Dis 197:1333–1340
92. Flohr C, Tuyen LN, Quinnell RJ, Lewis S, Minh TT, Campbell J, Simmons C, Telford G, Brown A, Hien TT, Farrar J, Williams H, Pritchard DI, Britton J (2010) Reduced helminth burden increases allergen skin sensitization but not clinical allergy: a randomized, double-blind, placebo-controlled trial in Vietnam. Clin Exp Allergy 40:131–142
93. Smits HH, Hammad H, van NM, Soullie T, Willart MA, Lievers E, Kadouch J, Kool M, Kos-van OJ, Deelder AM, Lambrecht BN, Yazdanbakhsh M (2007) Protective effect of *Schistosoma mansoni* infection on allergic airway inflammation depends on the intensity and chronicity of infection. J Allergy Clin Immunol 120:932–940
94. Santiago HC, Leevan E, Bennuru S, Ribeiro-Gomes F, Mueller E, Wilson M, Wynn T, Garboczi D, Urban J, Mitre E, Nutman TB (2012) Molecular mimicry between cockroach and helminth glutathione *S*-transferases promotes cross-reactivity and cross-sensitization. J Allergy Clin Immunol 130:248–256
95. Acevedo N, Sanchez J, Erler A, Mercado D, Briza P, Kennedy M, Fernandez A, Gutierrez M, Chua KY, Cheong N, Jimenez S, Puerta L, Caraballo L (2009) IgE cross-reactivity between *Ascaris* and domestic mite allergens: the role of tropomyosin and the nematode polyprotein ABA-1. Allergy 64:1635–1643
96. Weinmayr G, Genuneit J, Nagel G, Bjorksten B, van HM, Priftanji A, Cooper P, Rijkjarv MA, von ME, Tsanakas J, Forastiere F, Doekes G, Garrido JB, Suarez-Varela MM, Braback L, Strachan DP (2010) International variations in associations of allergic markers and diseases in children: ISAAC Phase Two. Allergy 65:766–775
97. Vereecken K, Kanobana K, Wordemann M, Junco DR, Menocal HL, Ruiz EA, Nunez FA, Rojas RL, Bonet GM, Polman K (2012) Associations between atopic markers in asthma and intestinal helminth infections in Cuban schoolchildren. Pediatr Allergy Immunol 23:332–338
98. Levin ME, Le Souef PN, Motala C (2008) Total IgE in urban Black South African teenagers: the influence of atopy and helminth infection. Pediatr Allergy Immunol 19:449–454
99. Cooper PJ, Chico ME, Vaca MG, Moncayo AL, Bland JM, Mafla E, Sanchez F, Rodrigues LC, Strachan DP, Griffin GE (2006) Effect of albendazole treatments on the prevalence of atopy in children living in communities endemic for geohelminth parasites: a cluster-randomised trial. Lancet 367:1598–1603
100. Bager P, Vinkel HA, Wohlfahrt J, Melbye M (2012) Helminth infection does not reduce risk for chronic inflammatory disease in a population-based cohort study. Gastroenterology 142:55–62
101. Mpairwe H, Webb EL, Muhangi L, Ndibazza J, Akishule D, Nampijja M, Ngom-wegi S, Tumusime J, Jones FM, Fitzsimmons C, Dunne DW, Muwanga M, Rodrigues LC, Elliott AM (2011) Anthelminthic treatment during pregnancy is associated with increased risk of infantile eczema: randomised-controlled trial results. Pediatr Allergy Immunol 22:305–312
102. Djuardi Y, Wammes LJ, Supali T, Sartono E, Yazdanbakhsh M (2011) Immunological footprint: the development of a child's immune system in environments rich in microorganisms and parasites. Parasitology 138:1508–1518
103. Moncayo AL, Vaca M, Oviedo G, Erazo S, Quinzo I, Fiaccone RL, Chico ME, Barreto ML, Cooper PJ (2010) Risk factors for atopic and non-atopic asthma in a rural area of Ecuador. Thorax 65:409–416
104. Croft AM, Bager P, Kumar S (2012) Helminth therapy (worms) for allergic rhinitis. Cochrane Database Syst Rev 4:CD009238
105. Bager P, Arnved J, Ronborg S, Wohlfahrt J, Poulsen LK, Westergaard T, Petersen HW, Kristensen B, Thamsborg S, Roepstorff A, Kapel C, Melbye M (2010) *Trichuris suis* ova therapy for allergic rhinitis: a randomized, double-blind, placebo-controlled clinical trial. J Allergy Clin Immunol 125:123–130
106. Feary JR, Venn AJ, Mortimer K, Brown AP, Hooi D, Falcone FH, Pritchard DI, Britton JR (2010) Experimental hookworm infection: a randomized placebo-controlled trial in asthma. Clin Exp Allergy 40:299–306
107. Wang CC, Nolan TJ, Schad GA, Abraham D (2001) Infection of mice with the helminth *Strongyloides stercoralis* suppresses pulmonary allergic responses to ovalbumin. Clin Exp Allergy 31:495–503
108. Wohlleben G, Trujillo C, Muller J, Ritze Y, Grunewald S, Tatsch U, Erb KJ (2004) Helminth infection modulates the development of allergen-induced airway inflammation. Int Immunol 16:585–596
109. Pinelli E, Brandes S, Dormans J, Gremmer E, van LH (2008) Infection with the roundworm *Toxocara canis* leads to exacerbation of experimental allergic airway inflammation. Clin Exp Allergy 38:649–658
110. Cooper PJ, Chico ME, Guadalupe I, Sandoval CA, Mitre E, Platts-Mills TA, Barreto ML, Rodrigues LC, Strachan DP, Griffin

GE (2011) Impact of early life exposures to geohelminth infections on the development of vaccine immunity, allergic sensitization, and allergic inflammatory diseases in children living in tropical Ecuador: the ECUAVIDA birth cohort study. BMC Infect Dis 11:184
111. Pritchard DI, Blount DG, Schmid-Grendelmeier P, Till SJ (2012) Parasitic worm therapy for allergy: is this incongruous or avant-garde medicine? Clin Exp Allergy 42:505–512
112. Hamid F, Wiria AE, Wammes LJ, Kaisar MM, Lell B, Ariawan I, Uh HW, Wibowo H, Djuardi Y, Wahyuni S, Schot R, Verweij JJ, van RR, May L, Sartono E, Yazdanbakhsh M, Supali T (2011) A longitudinal study of allergy and intestinal helminth infections in semi urban and rural areas of Flores, Indonesia (ImmunoSPIN Study). BMC Infect Dis 11:83
113. Endara P, Vaca M, Chico ME, Erazo S, Oviedo G, Quinzo I, Rodriguez A, Lovato R, Moncayo AL, Barreto ML, Rodrigues LC, Cooper PJ (2010) Long-term periodic anthelmintic treatments are associated with increased allergen skin reactivity. Clin Exp Allergy 40:1669–1677
114. Weinstock JV, Elliott DE (2009) Helminths and the IBD hygiene hypothesis. Inflamm Bowel Dis 15:128–133
115. Sewell D, Qing Z, Reinke E, Elliot D, Weinstock J, Sandor M, Fabry Z (2003) Immunomodulation of experimental autoimmune encephalomyelitis by helminth ova immunization. Int Immunol 15:59–69
116. Stromnes IM, Cerretti LM, Liggitt D, Harris RA, Goverman JM (2008) Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. Nat Med 14:337–342
117. Kroenke MA, Carlson TJ, Andjelkovic AV, Segal BM (2008) IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. J Exp Med 205:1535–1541
118. Correale J, Farez MF (2011) The impact of parasite infections on the course of multiple sclerosis. J Neuroimmunol 233:6–11
119. Correale J, Farez M (2009) Helminth antigens modulate immune responses in cells from multiple sclerosis patients through TLR2-dependent mechanisms. J Immunol 183:5999–6012
120. Correale J, Farez M, Razzitte G (2008) Helminth infections associated with multiple sclerosis induce regulatory B cells. Ann Neurol 64:187–199
121. Summers RW, Elliott DE, Urban JF Jr, Thompson R, Weinstock JV (2005) *Trichuris suis* therapy in Crohn's disease. Gut 54:87–90
122. Summers RW, Elliott DE, Urban JF Jr, Thompson RA, Weinstock JV (2005) *Trichuris suis* therapy for active ulcerative colitis: a randomized controlled trial. Gastroenterology 128:825–832
123. Fleming JO, Isaak A, Lee JE, Luzzio CC, Carrithers MD, Cook TD, Field AS, Boland J, Fabry Z (2011) Probiotic helminth administration in relapsing–remitting multiple sclerosis: a phase 1 study. Mult Scler 17:743–754
124. Broadhurst MJ, Leung JM, Kashyap V, McCune JM, Mahadevan U, McKerrow JH, Loke P (2010) IL-22+ CD4+ T cells are associated with therapeutic *Trichuris trichiura* infection in an ulcerative colitis patient. Sci Transl Med 2:60ra88
125. Sugimoto K, Ogawa A, Mizoguchi E, Shimomura Y, Andoh A, Bhan AK, Blumberg RS, Xavier RJ, Mizoguchi A (2008) IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. J Clin Invest 118:534–544
126. Smythies LE, Sellers M, Clements RH, Mosteller-Barnum M, Meng G, Benjamin WH, Orenstein JM, Smith PD (2005) Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. J Clin Invest 115:66–75
127. Weng M, Huntley D, Huang IF, Foye-Jackson O, Wang L, Sarkissian A, Zhou Q, Walker WA, Cherayil BJ, Shi HN (2007) Alternatively activated macrophages in intestinal helminth infection: effects on concurrent bacterial colitis. J Immunol 179:4721–4731
128. Hunter MM, Wang A, Parhar KS, Johnston MJ, Van RN, Beck PL, McKay DM (2010) In vitro-derived alternatively activated macrophages reduce colonic inflammation in mice. Gastroenterology 138:1395–1405
129. Wolff MJ, Broadhurst MJ, Loke P (2012) Helminthic therapy: improving mucosal barrier function. Trends Parasitol. doi:10.1016/j.pt.2012.02.008
130. Berry JD, Liu K, Folsom AR, Lewis CE, Carr JJ, Polak JF, Shea S, Sidney S, O'Leary DH, Chan C, Lloyd-Jones DM (2009) Prevalence and progression of subclinical atherosclerosis in younger adults with low short-term but high lifetime estimated risk for cardiovascular disease: the coronary artery risk development in young adults study and multi-ethnic study of atherosclerosis. Circulation 119:382–389
131. Tsimikas S, Kiechl S, Willeit J, Mayr M, Miller ER, Kronenberg F, Xu Q, Bergmark C, Weger S, Oberhollenzer F, Witztum JL (2006) Oxidized phospholipids predict the presence and progression of carotid and femoral atherosclerosis and symptomatic cardiovascular disease: five-year prospective results from the Bruneck study. J Am Coll Cardiol 47:2219–2228
132. Hansson GK (2005) Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 352:1685–1695
133. Libby P, Ridker PM, Hansson GK (2011) Progress and challenges in translating the biology of atherosclerosis. Nature 473:317–325
134. Harrison DG, Guzik TJ, Lob HE, Madhur MS, Marvar PJ, Thabet SR, Vinh A, Weyand CM (2011) Inflammation, immunity, and hypertension. Hypertension 57:132–140
135. Jain MK, Ridker PM (2005) Anti-inflammatory effects of statins: clinical evidence and basic mechanisms. Nat Rev Drug Discov 4:977–987
136. Nissen SE, Tuzcu EM, Schoenhagen P, Crowe T, Sasiela WJ, Tsai J, Orazem J, Magorien RD, O'Shaughnessy C, Ganz P (2005) Statin therapy, LDL cholesterol, C-reactive protein, and coronary artery disease. N Engl J Med 352:29–38
137. Ait-Oufella H, Salomon BL, Potteaux S, Robertson AK, Gourdy P, Zoll J, Merval R, Esposito B, Cohen JL, Fisson S, Flavell RA, Hansson GK, Klatzmann D, Tedgui A, Mallat Z (2006) Natural regulatory T cells control the development of atherosclerosis in mice. Nat Med 12:178–180
138. Kassan M, Galan M, Partyka M, Trebak M, Matrougui K (2011) Interleukin-10 released by CD4(+)CD25(+) natural regulatory T cells improves microvascular endothelial function through inhibition of NADPH oxidase activity in hypertensive mice. Arterioscler Thromb Vasc Biol 31:2534–2542
139. Ueshima H, Sekikawa A, Miura K, Turin TC, Takashima N, Kita Y, Watanabe M, Kadota A, Okuda N, Kadowaki T, Nakamura Y, Okamura T (2008) Cardiovascular disease and risk factors in Asia: a selected review. Circulation 118:2702–2709
140. Chan JC, Malik V, Jia W, Kadowaki T, Yajnik CS, Yoon KH, Hu FB (2009) Diabetes in Asia: epidemiology, risk factors, and pathophysiology. JAMA 301:2129–2140
141. Jex AR, Lim YA, Bethony JM, Hotez PJ, Young ND, Gasser RB (2011) Soil-transmitted helminths of humans in Southeast Asia—towards integrated control. Adv Parasitol 74:231–265
142. Stanley RG, Jackson CL, Griffiths K, Doenhoff MJ (2009) Effects of *Schistosoma mansoni* worms and eggs on circulating cholesterol and liver lipids in mice. Atherosclerosis 207:131–138
143. Meir KS, Leitersdorf E (2004) Atherosclerosis in the apolipoprotein-E-deficient mouse: a decade of progress. Arterioscler Thromb Vasc Biol 24:1006–1014
144. Doenhoff MJ, Stanley RG, Griffiths K, Jackson CL (2002) An anti-atherogenic effect of *Schistosoma mansoni* infections in mice associated with a parasite-induced lowering of blood total cholesterol. Parasitology 125:415–421
145. La Flamme AC, Harvie M, Kenwright D, Cameron K, Rawlence N, Low YS, McKenzie S (2007) Chronic exposure to schistosome

eggs reduces serum cholesterol but has no effect on atherosclerotic lesion development. Parasite Immunol 29:259–266
146. Innala L, Moller B, Ljung L, Magnusson S, Smedby T, Sodergren A, Ohman ML, Rantapaa-Dahlqvist S, Wallberg-Jonsson S (2011) Cardiovascular events in early RA are a result of inflammatory burden and traditional risk factors: a five year prospective study. Arthritis Res Ther 13:R131
147. Maradit-Kremers H, Nicola PJ, Crowson CS, Ballman KV, Gabriel SE (2005) Cardiovascular death in rheumatoid arthritis: a population-based study. Arthritis Rheum 52:722–732
148. del Rincon ID, Williams K, Stern MP, Freeman GL, Escalante A (2001) High incidence of cardiovascular events in a rheumatoid arthritis cohort not explained by traditional cardiac risk factors. Arthritis Rheum 44:2737–2745
149. Hot A, Lenief V, Miossec P (2012) Combination of IL-17 and TNFalpha induces a pro-inflammatory, pro-coagulant and pro-thrombotic phenotype in human endothelial cells. Ann Rheum Dis 71:768–776
150. Micha R, Imamura F, von Wyler BM, Solomon DH, Hernan MA, Ridker PM, Mozaffarian D (2011) Systematic review and meta-analysis of methotrexate use and risk of cardiovascular disease. Am J Cardiol 108:1362–1370
151. Mira E, Leon B, Barber DF, Jimenez-Baranda S, Goya I, Almonacid L, Marquez G, Zaballos A, Martinez A, Stein JV, Ardavin C, Manes S (2008) Statins induce regulatory T cell recruitment via a CCL1 dependent pathway. J Immunol 181:3524–3534
152. Hot A, Lavocat F, Lenief V, Miossec P (2012) Simvastatin inhibits the pro-inflammatory and pro-thrombotic effects of IL-17 and TNF-alpha on endothelial cells. Ann Rheum Dis. doi:10.1136/annrheumdis-2012-201887
153. Rhee SG, Woo HA (2011) Multiple functions of peroxiredoxins: peroxidases, sensors and regulators of the intracellular messenger H (2)O(2), and protein chaperones. Antioxid Redox Signal 15:781–794
154. Robinson MW, Hutchinson AT, Dalton JP, Donnelly S (2010) Peroxiredoxin: a central player in immune modulation. Parasite Immunol 32:305–313
155. Kisucka J, Chauhan AK, Patten IS, Yesilaltay A, Neumann C, Van Etten RA, Krieger M, Wagner DD (2008) Peroxiredoxin1 prevents excessive endothelial activation and early atherosclerosis. Circ Res 103:598–605
156. Park JG, Yoo JY, Jeong SJ, Choi JH, Lee MR, Lee MN, Hwa LJ, Kim HC, Jo H, Yu DY, Kang SW, Rhee SG, Lee MH, Oh GT (2011) Peroxiredoxin 2 deficiency exacerbates atherosclerosis in apolipoprotein E-deficient mice. Circ Res 109:739–749
157. Guo X, Yamada S, Tanimoto A, Ding Y, Wang KY, Shimajiri S, Murata Y, Kimura S, Tasaki T, Nabeshima A, Watanabe T, Kohno K, Sasaguri Y (2012) Overexpression of peroxiredoxin 4 attenuates atherosclerosis in apolipoprotein E knockout mice. Antioxid Redox Signal. doi:10.1089/ars.2012.4549
158. Donnelly S, Stack CM, O'Neill SM, Sayed AA, Williams DL, Dalton JP (2008) Helminth 2-Cys peroxiredoxin drives Th2 responses through a mechanism involving alternatively activated macrophages. FASEB J 22:4022–4032
159. Ou X, Thomas GR, Chacon MR, Tang L, Selkirk ME (1995) *Brugia malayi*: differential susceptibility to and metabolism of hydrogen peroxide in adults and microfilariae. Exp Parasitol 80:530–540
160. Kumagai T, Osada Y, Ohta N, Kanazawa T (2009) Peroxiredoxin-1 from *Schistosoma japonicum* functions as a scavenger against hydrogen peroxide but not nitric oxide. Mol Biochem Parasitol 164:26–31
161. Donath MY, Shoelson SE (2011) Type 2 diabetes as an inflammatory disease. Nat Rev Immunol 11:98–107
162. Feinstein R, Kanety H, Papa MZ, Lunenfeld B, Karasik A (1993) Tumor necrosis factor-alpha suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. J Biol Chem 268:26055–26058
163. Hotamisligil GS, Shargill NS, Spiegelman BM (1993) Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259:87–91
164. Hotamisligil GS (2006) Inflammation and metabolic disorders. Nature 444:860–867
165. Aravindhan V, Mohan V, Surendar J, Rao MM, Ranjani H, Kumaraswami V, Nutman TB, Babu S (2010) Decreased prevalence of lymphatic filariasis among subjects with type-1 diabetes. AmJTrop Med Hyg 83:1336–1339
166. Aravindhan V, Mohan V, Surendar J, Muralidhara RM, Pavankumar N, Deepa M, Rajagopalan R, Kumaraswami V, Nutman TB, Babu S (2010) Decreased prevalence of lymphatic filariasis among diabetic subjects associated with a diminished pro-inflammatory cytokine response (CURES 83). PLoS Negl Trop Dis 4:e707
167. Cooke A (2009) Review series on helminths, immune modulation and the hygiene hypothesis: how might infection modulate the onset of type 1 diabetes? Immunology 126:12–17
168. Wu D, Molofsky AB, Liang HE, Ricardo-Gonzalez RR, Jouihan HA, Bando JK, Chawla A, Locksley RM (2011) Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. Science 332:243–247
169. Ricardo-Gonzalez RR, Red EA, Odegaard JI, Jouihan H, Morel CR, Heredia JE, Mukundan L, Wu D, Locksley RM, Chawla A (2010) IL-4/STAT6 immune axis regulates peripheral nutrient metabolism and insulin sensitivity. Proc Natl Acad Sci U S A 107:22617–22622
170. Hewitson JP, Grainger JR, Maizels RM (2009) Helminth immunoregulation: the role of parasite secreted proteins in modulating host immunity. Mol Biochem Parasitol 167:1–11
171. van der Kleij D, Latz E, Brouwers JF, Kruize YC, Schmitz M, Kurt-Jones EA, Espevik T, de Jong EC, Kapsenberg ML, Golenbock DT, Tielens AG, Yazdanbakhsh M (2002) A novel host–parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. J Biol Chem 277:48122–48129
172. Schramm G, Haas H (2010) Th2 immune response against *Schistosoma mansoni* infection. Microbes Infect 12:881–888
173. Everts B, Hussaarts L, Driessen NN, Meevissen MH, Schramm G, van der Ham AJ, van der Hoeven B, Scholzen T, Burgdorf S, Mohrs M, Pearce EJ, Hokke CH, Haas H, Smits HH, Yazdanbakhsh M (2012) Schistosome-derived omega-1 drives Th2 polarization by suppressing protein synthesis following internalization by the mannose receptor. J Exp Med 209:1753–1767
174. Schramm G, Mohrs K, Wodrich M, Doenhoff MJ, Pearce EJ, Haas H, Mohrs M (2007) Cutting edge: IPSE/alpha-1, a glycoprotein from *Schistosoma mansoni* eggs, induces IgE-dependent, antigen-independent IL-4 production by murine basophils in vivo. J Immunol 178:6023–6027
175. Klotz C, Ziegler T, Figueiredo AS, Rausch S, Hepworth MR, Obsivac N, Sers C, Lang R, Hammerstein P, Lucius R, Hartmann S (2011) A helminth immunomodulator exploits host signaling events to regulate cytokine production in macrophages. PLoS Pathog 7:e1001248
176. Robinson MW, Alvarado R, To J, Hutchinson AT, Dowdell SN, Lund M, Turnbull L, Whitchurch CB, O'Brien BA, Dalton JP, Donnelly S (2012) A helminth cathelicidin-like protein suppresses antigen processing and presentation in macrophages via inhibition of lysosomal vATPase. FASEB J. doi:10.1096/fj.12-213876
177. Grainger JR, Smith KA, Hewitson JP, McSorley HJ, Harcus Y, Filbey KJ, Finney CA, Greenwood EJ, Knox DP, Wilson MS, Belkaid Y, Rudensky AY, Maizels RM (2010) Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-beta pathway. J Exp Med 207:2331–2341
178. Hewitson JP, Filbey KJ, Grainger JR, Dowle AA, Pearson M, Murray J, Harcus Y, Maizels RM (2011) *Heligmosomoides polygyrus* elicits a dominant nonprotective antibody response directed against restricted glycan and peptide epitopes. J Immunol 187:4764–4777

chapter 2

Does treatment of intestinal helminth infections influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study)

Does treatment of intestinal helminth infections influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study)

Aprilianto E Wiria[1,2], Margaretta A Prasetyani[1], Firdaus Hamid[2,3], Linda J Wammes[2], Bertrand Lell[4], Iwan Ariawan[5], Hae Won Uh[6], Heri Wibowo[1], Yenny Djuardi[1], Sitti Wahyuni[7], Inge Sutanto[1], Linda May[2], Adrian JF Luty[8], Jaco J Verweij[2], Erliyani Sartono[2], Maria Yazdanbakhsh[2*], Taniawati Supali[1*]

Abstract

Background: Given that helminth infections are thought to have strong immunomodulatory activity, the question whether helminth infections might affect responses to malaria antigens needs to be addressed. Different cross-sectional studies using diverse methodologies have reported that helminth infections might either exacerbate or reduce the severity of malaria attacks. The same discrepancies have been reported for parasitemia.

Methods/Design: To determine the effect of geohelminth infections and their treatment on malaria infection and disease outcome, as well as on immunological parameters, the area of Nangapanda on Flores Island, Indonesia, where malaria and helminth parasites are co-endemic was selected for a longitudinal study. Here a Double-blind randomized trial will be performed, incorporating repeated treatment with albendazole (400 mg) or placebo at three monthly intervals. Household characteristic data, anthropometry, the presence of intestinal helminth and *Plasmodium spp* infections, and the incidence of malaria episodes are recorded. *In vitro* cultures of whole blood, stimulated with a number of antigens, mitogens and toll like receptor ligands provide relevant immunological parameters at baseline and following 1 and 2 years of treatment rounds. The primary outcome of the study is the prevalence of *Plasmodium falciparum* and *P. vivax* infection. The secondary outcome will be incidence and severity of malaria episodes detected via both passive and active follow-up. The tertiary outcome is the inflammatory cytokine profile in response to parasite antigens. The project also facilitates the transfer of state of the art methodologies and technologies, molecular diagnosis of parasitic diseases, immunology and epidemiology from Europe to Indonesia.

Discussion: The study will provide data on the effect of helminth infections on malaria. It will also give information on anthelminthic treatment efficacy and effectiveness and could help develop evidence-based policymaking.

Trial registration: This study was approved by The Ethical Committee of Faculty of Medicine, University of Indonesia, ref:194/PT02.FK/Etik/2006 and has been filed by ethics committee of the Leiden University Medical Center. Clinical trial number:ISRCTN83830814. The study is reported in accordance with the CONSORT guidelines for cluster-randomized studies.

* Correspondence: M.Yazdanbakhsh@lumc.nl; Taniawati@yahoo.com
[1]Department of Parasitology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia
[2]Department of Parasitology, Leiden University Medical Center, Leiden, The Netherlands

Background

Worldwide, more than a billion people are infected by geohelminths, with a majority harboring roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*), and/or hookworms (*Necator americanus* or *Ancylostoma duodenale*) [1-3]. Such helminths modify the immune system to induce predominant production of T-helper-2 (Th2) cytokines (interleukin/IL-4, IL-5, IL-9, IL-10, IL-13), associated with increased levels of immunoglobulin E (IgE) and eosinophilia [4,5]. Another hallmark of chronic helminth infections is their ability to induce a strong regulatory network characterized by T cell hyporesponsiveness and the increased production of suppressive cytokines such as IL-10 and TGF-β [6]. Their ability to induce regulatory responses is thought to be advantageous for both the parasite and the host as it allows the survival of the parasite for extended periods of time within the host while preventing overt pathological reactions that would otherwise be damaging to the host [7]. The latter may explain why such helminth infections rarely result in overt clinical manifestations.

Although the immunological consequences of helminth infections primarily reflect responses directed towards helminth antigens, there may be spill-over effects on responses to unrelated antigens [6,8]. Whereas the marked Th2 polarization may compete with Th1 cytokines to affect the magnitude of a Th1 response to an incoming antigen, a strong regulatory response can dampen immune reactivity of both Th1 and Th2 types to a third party antigen. Not surprisingly, then, it is argued that helminth infections might modulate the human immune response to common co-infections such as malaria, TB and HIV/AIDS [9-11].

Malaria itself is one of the most serious infectious diseases, infecting 5-10% of the world's population, with 300-600 million cases and more than 2 million deaths annually [12]. In areas of high malaria transmission, the burden of disease is borne by infants and young children [13,14], whilst, in areas of lower transmission, primary infection might also occur later in life, causing severe illness [12,15,16].

Co-infection is the norm in nature [9,17], since helminth infections of different species are often endemic in the same communities that are exposed to infection with plasmodia [18]. The question of whether helminth infections affect the course of malaria has been addressed in various reports [19] in the past few years. Only a small number of papers report on population studies and most of them were cross-sectional in nature, which in contrast to longitudinal studies might not be able to demonstrate the actual dynamics of infection. An early study by Murray and colleagues (1977) in a malnourished population at Anjouan, Comoros Islands suggest that *A. lumbricoides* might suppress malaria symptoms [20]. Since then studies of co-infections have shown helminths to either exacerbate [21] or reduce [22-24] the severity of malaria. The reasons for conflicting data on the effect of helminth co-infection on malaria disease outcomes could be due to differences in study design, study groups, and possibly, most importantly, the helminth species investigated [18,25].

Although helminth infections are considered as pathogens, it is clear that their course of infection is relatively free of overt clinical manifestations and in recent years several reports have shown their suppressive effect on diseases such as allergy [3,26], autoimmunity [27], and inflammatory bowel disease [28], highlighting a possible beneficial effect on inflammation [29]. To assess the influence of helminth infections on inflammatory diseases taking into account environmental and genetic influences in a longitudinal setting, the ImmunoSPIN project http://www.immunospin.org has been initiated. As part of the project ImmunoSPIN, the helminth-malaria sub project (ImmunoSPIN-Malaria) was designed to determine whether and how helminth infections may affect the course of a malaria infection. This study is a double-blind randomized trial of placebo and anthelminthic treatment to elucidate the impact of helminth infections on malaria longitudinally in an area where helminth infections and malaria coexist. It is expected to provide new information and contribute to our understanding of how regulatory responses that may be induced by chronic helminth infections affect inflammatory conditions, especially on malaria infection.

This paper presents the rationale of the ImmunoSPIN-Malaria study that, at the clinical and biological level, aims to discern plausible and meaningful interactions between these infections.

Methods/Design

Study area and population

Nangapanda is a sub-district of the Ende district, Flores Island, Indonesia and is situated in a coastal area with a population of about 22000 (Figure 1). Situated near the equator (8°45'S, 121°40'E)[30], it is characterized by a uniform high temperature, in the range of 23-33.5°C, with humidity of 86-95%. Average yearly rain fall is 1.822 mm with about 82 rainy days, especially from November to April, with the peak in December until March. Malaria is reported to be highly prevalent in this area [31]. Preliminary surveys conducted in 2005 and 2006 found this area to be endemic for geohelminths (*A. lumbricoides*, hookworms and *T. trichiura*) and malaria parasites (*P. falciparum* and *P. vivax*). The sub-district is divided into villages of which those located near the primary health centre (Puskesmas), Ndeturea,

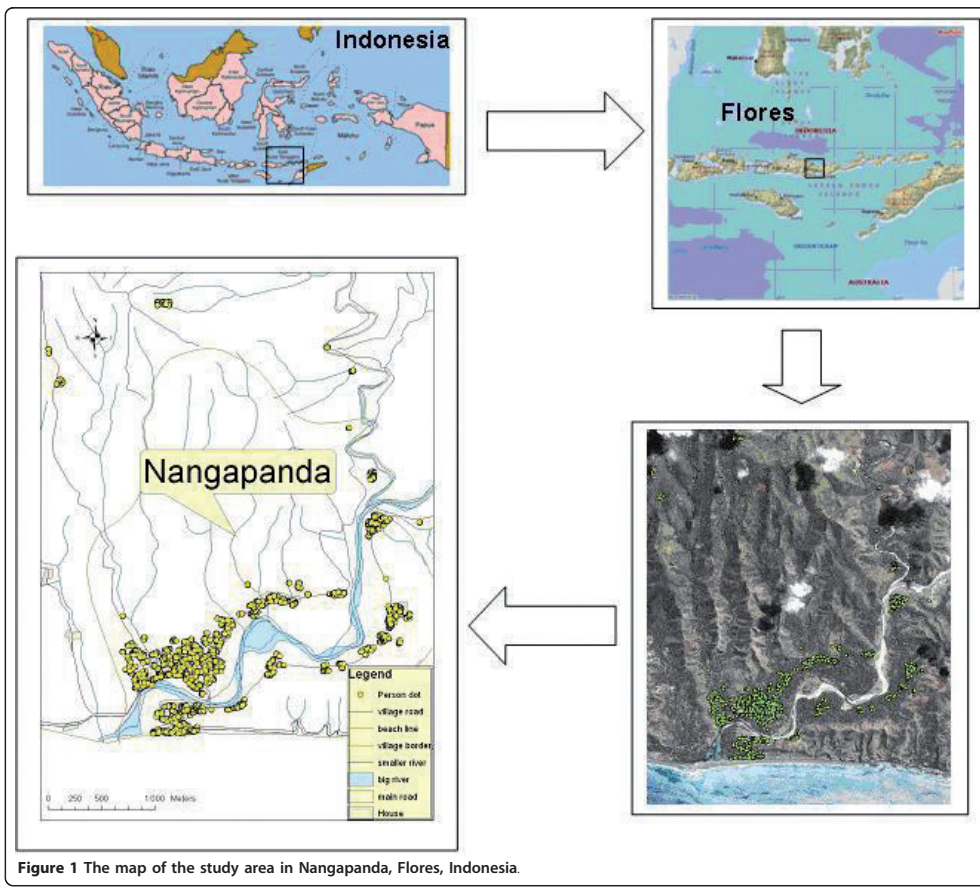


Figure 1 The map of the study area in Nangapanda, Flores, Indonesia.

Ndorurea1, and Ndorurea are the focus of ImmunoSPIN-Malaria study.

The majority in the study area is Ende tribe and migrants from neighbouring district of Bajawa that have populated the island for more than 300 years. Most individuals work as farmers and grow their own food. Recently also government employees and individuals owning small businesses moved to the area. Individuals with Chinese and Arabic ethnicity have come to the area about 200 years ago and most of them, run business corporations with other tribes. There are very few other tribes from outside of Flores and most of them were brought from outside by marriage with Flores natives. The Nangapanda population is dynamic and individuals shift address within the area as well as move in and out of the area for purposes of studying, marriage, or searching for jobs.

Study design, data and sample collection

Baseline mapping of houses by Global Positioning System (GPS) system has allowed maps to be generated using ArcGIS 9,1 software (ESRI, USA). In order to perform spatial analyses based on individual data, geographical coordinates were assigned to each individual as well as centroid coordinates to households.

Community workers were recruited and trained to do questionnaires, follow-ups, malaria surveys (finger prick), and distribute treatment or placebo, as well as doing health promotion among the population. Each community worker will be in charge of a certain number of households that they visit monthly for recording movement and active follow-up of malaria symptoms, and three monthly for finger pricking and distributing the treatment.

The study is designed as a double-blind randomized trial with two arms. One arm is treatment with albendazole (single dose of 400 mg) [32], while the other arm is treatment with matching placebo (both tablets from PT Indofarma Pharmaceutical, Bandung, Indonesia). The treatment is provided every three months for a period of two years (a total of 8 treatments) to everyone, except children below two years of age, pregnant women or severely ill persons. Computer aided block randomization by household, using Random Allocation Software [33] will be assigned to the treated and placebo groups. The treatment is coded as A or B and the codes are concealed from investigators and patients. Labels with the study subject ID are printed from a computer database and attached to the appropriate strip of treatment by a separate team located in Jakarta without the involvement of the study investigators. An interim analysis will be performed by the monitoring committee, 1 year after treatment to test for any adverse effects that retention of anthelminthic treatment might have on the growth of children and on the incidence of malaria episodes (in contrast to the hypothesis being tested). If the trial continues, the final unblinding of the codes will take place two years after treatment.

On approval of individuals in the area, peripheral blood will be collected from a subset of individuals over 4 years of age that were randomly selected for whole blood culture based on households. The assay will be done at baseline, 1 and 2 years after treatment. For those who are not included in whole blood assays, malaria evaluation by finger pricking will be obtained aiming to include the whole population in the study area.

Every household receives a card identifying all family members. This card is used when community workers visit homes and when family members visit the Puskesmas. Data on socioeconomic status and health status is collected at baseline and 2 years after treatment using questionnaires in Bahasa Indonesia. Monthly data such as birth, death and migration will be recorded. Newborns and individuals who enter the study area will be registered with a newly assigned ID number as well as their geographical coordinates. Active follow-up includes a questionnaire with questions on clinical complaints in general and clinical malaria in particular and will be conducted monthly by community workers. In addition to the questionnaire, blood slides will be collected at three monthly intervals to test for plasmodia. For examination of intestinal helminths, stool samples will be collected yearly. Samples will be used for microscopy and PCR analyses. Passive follow-up will be held in collaboration with the Puskesmas that keeps clinical records of individuals that visit either for consultation or for overnight admission, including information on diagnosis and treatment. If malaria is suspected, two blood slides will be collected: one to be examined by the Puskesmas staff and another for re-examination by the research team in Jakarta at the Department of Parasitology, University of Indonesia (UI).

All blood samples (serum, cell pellet, plasma, and whole blood), blood culture supernatants, as well as stool samples for PCR, will be kept at -20°C in a temperature recorded freezer which is checked twice a day and will be sent to Jakarta on dry ice for storage -20°C or -80°C.

Outcomes and case definitions

The study aims to determine whether and how helminth infections may affect the course of infection with malaria parasites and disease outcome. Therefore we will monitor disease in individuals treated with albendazole compared to placebo. The primary outcome is the prevalence of infection with *P. falciparum* and *P. vivax* up to 2 years after treatment, the secondary outcome is the incidence and severity of malaria recorded as a result of passive and active follow-up up to 2 years after treatment. The tertiary outcome is the inflammatory cytokine responses to *P. falciparum* antigens 1 and 2 years after treatment.

Helminth and plasmodia infections will be defined by the presence of parasites detected by microscopic examination of stool and blood samples respectively and will be confirmed by molecular (PCR-based) methods [34-38]. A malaria case is defined as an individual with typical malaria symptoms and a blood slide containing plasmodia asexual forms. Typical malaria case definitions: fever (oral temperature ≥ 37.5°C) and/or history of fever in the past 48 hours with a positive slide (*P. vivax* ≥ 250/ul, *P. falciparum* ≥ 1000/ul). Asymptomatic carriers have a blood slide positive for *P. falciparum* and/or *P. vivax* asexual forms, as well as positive result by PCR and no concomitant clinical symptoms. Suspected malaria cases are defined as individuals who report symptoms typical of malaria but for whom no slide is available at the time of presentation with symptoms.

Parasitological examination

Stool examination by microscopy

The Harada Mori method will be carried out on fresh stool samples to detect hookworm larvae. A certain amount of each stool sample is preserved in formalin (4%) and kept at room temperature for microscopic examination. The formol-ether acetate concentration method [39] is performed on the formalin preserved stool samples followed by microscopical examination for intestinal helminth infections, as well as protozoan infections. For hookworm detection, an amount of fresh

stool sample is incubated using filter paper soaked by distilled water inside sealed plastic tubes according to the Harada Mori method [40].

Stool examination by real-time PCR

For DNA isolation from stool, approximately 100 mg unpreserved faeces (that was kept at -20°C) are suspended in 200 µl PBS containing 2% polyvinylpolypyrolidone (PVPP; Sigma, Steinheim, Germany). DNA isolation and setup of the PCR reactions are performed using a custom-made Hamilton robot platform (made in Germany). After heating for 10 min at 100°C suspensions are treated with sodium dodecylsulphate-proteinase K for 2 h at 55°C. DNA is isolated using QIAamp DNA-easy 96-well plates (QIAgen, Venlo, The Netherlands) [36]. Within the isolation lysis buffer, 10^3 PFU/ml Phocine herpes virus 1 (PhHV-1) is added to serve as an internal control [41].

A. duodenale, N. americanus (hookworm), A. lumbricoides, S. stercoralis real-time PCR (ANAS-PCR) [or just "helminth rt-PCR"]

Sequences of the *A. duodenale*-, *N. americanus*-, and *S. stercoralis*-specific primers and probes are used as described previously [37,38] with some modifications in fluorophore- and quencher-chemistry. Minor groove binding (MGB) probes are replaced by XS probes (Biolegio, Malden, The Netherlands) and to accommodate the specific fluorophor combination of the CFX real-time PCR system (Bio-Rad laboratories, USA) the *A. duodenale* specific XS-probe is labelled with Texas Red and the *S. stercoralis*-specific probe is labelled with Quasar-705. The *A. lumbricoides*-specific primers and probe are chosen using Primer Express software (Applied Biosystems, Foster City, CA), from the internal transcribed-spacer-1 (ITS1) sequence of *A. lumbricoides* (GenBank accession ALJ000895). The *A. lumbricoides*-specific primers, Alum96F 5'-GTAATAGCAGTCGGCGGTTTCTT-3' and Alum183R 5'-GCCCAACATGCCACCTATTC-3' amplify an 87-bp fragment of the ITS1 sequence and the XS-probe Alum124T Yakima Yellow-5'-TTGGCGGA-CAATTGCATGCGAT-3'-XS is used to detect the *A. lumbricoides*-specific product. The real-time PCRs were optimized first as monoplex assays with 10-fold dilution series of *A. duodenale*, *N. americanus*, *A. lumbricoides*, and *S. stercoralis* DNA, respectively. The monoplex real-time PCRs were thereafter compared with the multiplex PCR with the PhHV internal control. The cycle threshold (Ct) values obtained from testing the dilution series of each pathogen in both the individual assay and the multiplex assay were similar, and the same analytical sensitivity was achieved. The multiplex ANAS PCR showed 100% specificity when tested against 145 DNA controls derived from a wide range of intestinal microorganisms [35].

Amplification reactions are performed in white PCR plates in a volume of 25 µl with PCR buffer (HotstarTaq master mix, QIAgen, Germany), 5 mM $MgCl_2$, 2.5 µgram Bovine Serum Albumin (Roche Diagnostics Nederland B.V., Almere, the Netherlands), 5 pmol of each *A. duodenale*-specific primer, and of each *N. americanus*-specific primer, 2 pmol of each *A. lumbricoides*-specific primer, 2.5 pmol of each *S. stercoralis*-specific primer and 3.75 pmol of each PhHV-1-specific primer, 1.25 pmol of each *N. americanus*-specific XS-probe, *A. lumbricoides*-specific XS-probe, *S. stercoralis*-specific double-labelled probe, and PhHV-1-specific double-labelled probe, and 2.5 pmol of the *A. duodenale*-specific XS-probe, and 5 µl of the DNA sample.

Amplification consists of 15 min at 95°C followed by 50 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Amplification, detection, and analysis are performed with the CFX real-time detection system (Bio-Rad laboratories). The PCR output from this system consists of a cycle-threshold (Ct) value, representing the amplification cycle in which the level of fluorescent signal exceeds the background fluorescence, and reflecting the parasite-specific DNA load in the sample tested. Negative and positive control samples are included in each amplification run.

The amplification is considered to be hampered by faecal inhibitory factors if the expected cycle threshold (Ct) value of 33 in the PhHV-specific PCR is increased by more than 3.3 cycles.

Blood examination by microscopy

To detect *P. falciparum, P. vivax, P. malariae*, and *P. ovale*, blood slides (thick and thin) are stained with Giemsa [42] followed by microscopic examination [43].

Blood examination by real-time PCR

DNA was isolated from 200 µl blood with QIAamp DNA-easy 96-well plates according to the manufacturer's recommendations. DNA isolation and setup of the PCR reactions are performed using a custom-made Hamilton robot platform.

P. falciparum, P. vivax, P. ovale, and P. malariae (malaria) real-time PCR

Sequences of the *Plasmodium*-specific primers and the *P. falciparum, P. vivax, P. ovale, and P. malariae*-specific probes are used as described previously[34,44] with some modifications in the fluorophore- and quencher-chemistry. Minor groove binding (MGB) probes are replaced by XS probes and to accommodate the specific fluorophor combination of the CFX system the *P. falciparum*-specific XS-probe is labelled with Yakima Yellow, the *P. ovale*-specific XS-probe is labelled with Texas Red and the *P. malariae*-specific probe is labelled with Quasar-705. An additional *P. ovale*-type 2 XS-probe (Texas Red 5'-TCCAAAAGGAATTTTCTTATT-3'-XSQ) is used for sensitive detection of the *P. ovale* genetic variant type 2 (GenBank accession X99790/J001527).

Amplification reactions of each DNA sample are performed in white PCR plates, in a volume of 25 µl with

PCR buffer (HotstarTaq master mix), 5 mmol/l MgCl2, 12.5 pmol of each Plasmodium-specific primer and 15 pmol of each PhHV-1-specific primer, 1.5 pmol of each *P. falciparum, P. vivax-, P. malariae*-specific XS-probes, and PhHV-1-specific Cy5 double-labelled detection probe, and 2.5 pmol of each *P. ovale*-specific XS-probes (Biolegio), and 5 μl of the DNA sample were used.

Amplification, detection, and analysis are performed as described for the faecal PCR.

Immunological Measurements

Whole blood culture and cytokine measurements

Blood samples (6 ml) are drawn into Vacutainers (BD, Franklin Lakes, NJ, USA) containing sodium heparin as anticoagulant. Within 6 hours, blood cultures are set up according to methods that were optimized and tested under field conditions during pilot studies. The heparinized blood is diluted 1:4 with RPMI 1640 medium (Invitrogen, Breda, The Netherlands) (supplemented with 2 mM glutamate, 1 mM pyruvate, 100 IU penicillin and 100 μg/ml streptomycin) and cultured in 96 well round bottomed plates in 37°C with 5% CO_2. Stimulations were performed with medium/control, PHA (2 μg/ml, Wellcome Diagnostics, Darford, UK), LPS (1 ng/ml Sigma-Aldrich, Zwijndrecht, The Netherlands), Pam3Cys (100 ng/ml, Cayla-InvivoGen Europe, Toulouse, France), PolyIC (50 μg/ml, Cayla-InvivoGen Europe, Toulouse, France), *Ascaris* antigen (20 μg/ml, as prepared by van Riet E et al [45]), iRBC (1×10^6, prepared by Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands[46]), uRBC (1×10^6, prepared by Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands [46]). Supernatants are collected on day 1 (unstimulated control, LPS, Pam3Cys) and day 3 (unstimulated control, PHA, Ascaris, iRBC, uRBC, PolyIC). Cytokine concentrations in supernatants are assessed by means of immunobead-based multiplex assays. This is an assay that permits simultaneous quantification of multiple cytokines in a small sample volume. Panels of capture antibody-coated beads and labeled detection antibodies are purchased from Bio Source (Camarillo, California, USA). The cytokines measured will represent pro- and anti-inflammatory, Th1 and Th2 cytokines: TNF-α and IL-10 from day 1 supernatants as well as IL-2, IL-5, IL-10, IFN-γ, and TNF-α for day 3 supernatants. Analysis will be performed on a Liquichip 200® Workstation (Qiagen, Venlo, The Netherlands) using Liquichip analyzer software (Qiagen, Venlo, The Netherlands).

Total IgE

Total IgE will be measured as described previously [47]. Briefly, maxisorp plates (Thermo Fisher Scientific, Roskilde, Denmark) are coated overnight with 100 μl/well rabbit anti-human IgE (Dako, Glostrup, Denmark) at 1/1400 dilution in 0.1 M carbonate buffer. Plates are blocked with 100 μl/well phosphate buffered saline (PBS) containing 5% bovine serum albumin (BSA, Albumin Fraction V, Boehringer, Mannheim, Germany). Sera to be tested are diluted 1:200 in PBS containing 5% fetal calf serum (FCS, Greiner Bio-One, Alphen a/d Rijn, Netherlands). A positive standard serum containing human IgE (NIBSC, Potters Bar, UK) is diluted down 1/3 in a series from 90 IU/ml until a final concentration 0.12 IU/ml on each plate and incubated for 1 hour at room temperature. After washing step, IgE biotinylated goat anti-human IgE antibody (1/1000 (Vector Laboratories, Burlingame, CA, USA)) is added followed by Streptavidin Alkaline Phosphatase conjugate (1/3000 (Boehringer, Mannheim, Germany)). The colour is developed by addition of para-nitrophenylphosphate substrate (p-NPP (Boehringer, Mannheim, Germany)) diluted in diethanolamine buffer (DEA, 0.5 mM Mg CL2, 0.1 M DEA, pH 9.6 (Merck, Darmstadt, Germany) and optical density is measured at 405 nm.

Statistical analyses

A database using MS Access is developed for this study. At baseline, we will analyze whether helminth infected subjects are at a higher risk of having *P. falciparum* or *P. vivax* infections in terms of presence of infection and intensity. The effect of anthelminthic treatment will be assessed 1 and 2 years after treatment by analyzing the prevalence ratios as well as the incidence ratios of malaria infection (clinical cases and parasitemia). In addition, treatment will be compared with placebo for reduction of helminth infection and is based on an intention to treat principle, in anticipation of individual movement between treatment groups. Cytokine profiles in response to parasite antigens will be analyzed for levels of pro- and anti-inflammatory cytokines.

The characterization of immune responses to helminth infections, malaria infections and co-infections will be assessed prior to treatment using linear regression [48]. As cytokine levels are non-normally distributed, we will use log-transformed cytokine data for all analyses regarding effect of helminth treatment on cytokine profile and susceptibility to plasmodium infection. For these analyses, multilevel modeling will be used and the use of longitudinal data will take into account repeated measurements [48]. Any bias related to selection of participants and outcome of treatment will be assessed by comparing individuals that are lost to follow-up and individuals that are not lost and will be compared on the basis of their baseline characteristics, age, gender, village, and socioeconomic status and parasitic infections. A similar assessment will also be undertaken to compare the characteristics of individuals in the treatment and placebo groups at inclusion into the

study. Chi-square analyses will be used to test proportions.

Multiple regression analysis will be used to determine 1) the association between helminth infections (either all helminths or individual species) with malaria parasites (either all or individual species); 2) the effect of decreasing helminth infection on plasmodia parasitaemia and incidence of malaria cases and 3) the effect of decreasing helminth infection on immunological responses to malaria antigen. All analyses will be adjusted for confounders such as socioeconomic status, body mass index, age and sex. During the study any other confounders that are identified will be used in our analyses.

Power calculation

Unpublished microscopy data from the area showed the combined prevalence of *P. falciparum* and *P. vivax* was 12% in the target population and the helminth infection (*A. lumbricoides*, Hookworm, *T. trichiura*) in the population was 60%. As study activities such as active follow-up and prompt treatment will have an effect on the prevalence, we expect the malaria prevalence to decrease to an estimated 6%. Based on these findings a power calculation (given an alpha of 0.05 and a power of 0.80) gave a sample size of 749 study subjects needed, in order to detect a 50% reduction or increase in the prevalence of plasmodia after 2 years of anthelminthic treatment. The effect of treatment will presumably be present in helminth carriers only and as an estimated 60% will be carriers of helminths during study period, giving an estimated 1248 subjects in each study arm will be needed. Allowing for an estimated 20% lost to follow-up due to movement and refusal, we would need to include 1495 participants in each arm.

Ethical consideration and trial registration, information, recruitment and consent

This study was approved by The Ethical Committee of Faculty of Medicine, University of Indonesia, ref: 194/PT02.FK/Etik/2006 and registered as clinical trial ref: ISRCTN83830814 and has been filed by ethics committee of the Leiden University Medical Center. The study is reported in accordance with the CONSORT guidelines for cluster-randomized studies [49].

The regional health authorities in Ende, the regional capital, were informed of the study and gave their agreement and support. Socialization took place over a two year period prior to the study involving staff at the public health centre (Puskesmas) and 50 community workers, including training for follow-up of study subjects keeping the community well-informed and well-engaged. Through many organized sessions, the village heads were involved in passing on information about the study, including the benefits and risks involved. The longitudinal nature was explained and information sheets and consent forms (in Bahasa Indonesia) were distributed. Inhabitants of the area were invited to participate in the study, consented either by written signature or thumb print and were informed that they may withdraw from the study at any time, for any reason and without consequences. For children below 15 years old of age also parent consent was obtained. Probable illness by burden of helminth infection will be taken into account by three study doctors that will be present in the area as well as by collaboration with the Puskesmas. Severe cases will be treated directly. The medical doctors will also give support and treatment to the Puskesmas. After completion of the study the whole population will be adequately treated for helminth infections [32,50].

Description of the population recruited

The study has so far provided data that are shown in a flow chart given in Figure 2. During registration (January 2007 - April 2008) a total of 4650 individuals were registered in 753 households. At baseline in (April 2008) 3854 of the registered population are residing in the village in 734 household. For the immunological studies 250 households were randomly selected within 734 registered households, to allow for 1065 eligible individuals (Figure 2).

Table 1 shows age-stratified migration patterns. Most migration is for seeking employment or education opportunities and most are male. The median age of the residents is 20 years while this is 19 for those who move out of the area. The age pyramid is shown in Figure 3 and is typical for low to middle income countries [51].

The traditional source of income in the area is farming and fishing while some individuals engage in jobs at government offices with a few in the private sector (Figure 4). The education level of the majority over 15 years of age is elementary school (40.6%) followed by senior high school (27,2%), and junior high school (18.9%) while 5.4% has college or University degrees. Around 5.5% is illiterate, either not educated at all or dropped out from elementary school (Figure 5).

Discussion

Since this large community-based trial provides an important possibility to undertake a series of evaluations on the effect of helminth infections on malaria as well as the control of helminth and malaria at the community level, our study could help develop evidence-based policymaking. This study is unique in that it will provide data on anthelminthic treatment efficacy and effectiveness in a defined large population in a developing country. In conclusion the ImmunoSPIN helminth-malaria study is the first and currently the only longitudinal

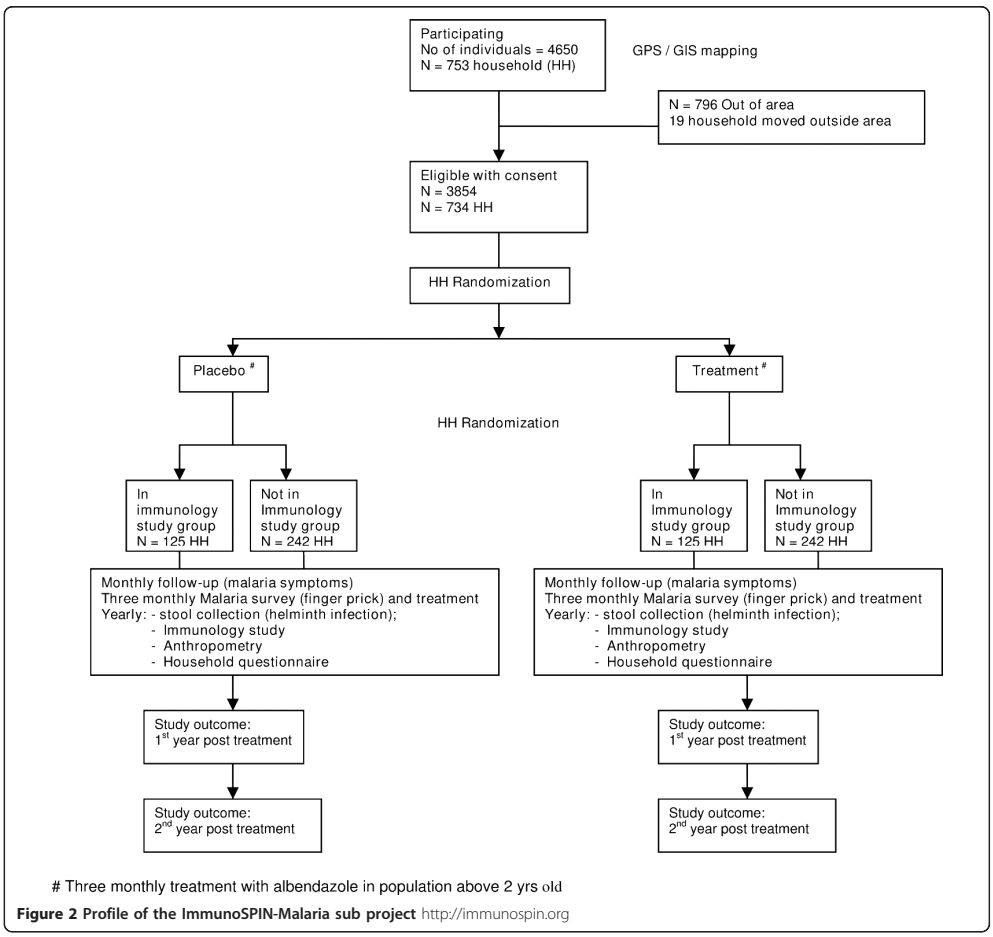


Figure 2 Profile of the ImmunoSPIN-Malaria sub project http://immunospin.org

Table 1 Age distribution of the study population who have stayed or moved out of the study area in 16 months of period*

Age group (years)	Staying			Moved		
	Male (n = 1740)	Female (n = 2087)	Total (n = 3827)	Male (n = 431)	Female (n = 365)	Total (n = 796)
0-4	175 (10%)	221 (10.6%)	396 (10.3%)	10 (2.3%)	11 (3%)	21 (2.6%)
5-14	491 (28.2%)	467 (22.4%)	958 (25%)	49 (11.4%)	47 (12.9%)	96 (12.1%)
15-24	353 (20.3%)	412 (19.7%)	765 (20%)	248 (57.5%)	224 (61.4%)	472 (59.3%)
25-34	191 (11%)	305 (14.6%)	496 (13%)	71 (16.5%)	53 (14.5%)	124 (15.6%)
35-44	207 (11.9%)	284 (13.6%)	491 (12.8%)	37 (8.6%)	15 (4.1%)	52 (6.5%)
45-54	160 (9.2%)	198 (9.5%)	358 (9.4%)	8 (1.8%)	8 (2.2%)	16 (2%)
55-64	94 (5,4%)	119 (5.7%)	213 (5.6%)	2 (0.5%)	3 (0.9%)	5 (0.6%)
> = 65	69 (4%)	81 (3.9%)	150 (3.9%)	6 (1.4%)	4 (1%)	10 (1.3%)

* Data from 4623 of 4650, because 27 individuals was not known for their age or birthday.

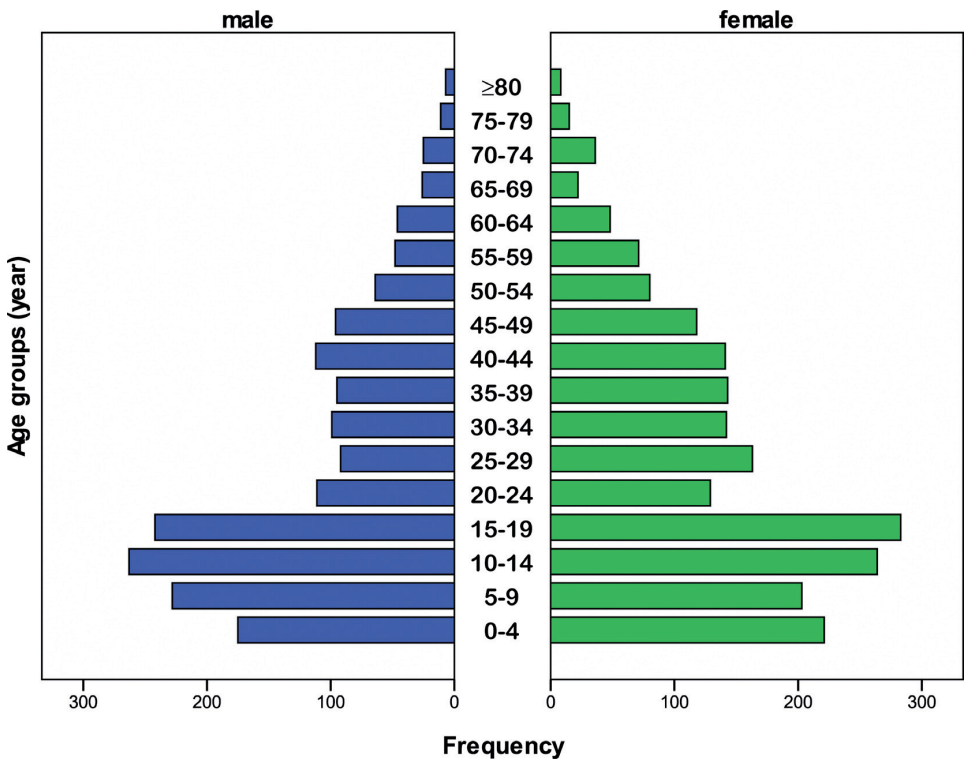


Figure 3 Age pyramid of individuals living in Nangapanda, Flores.

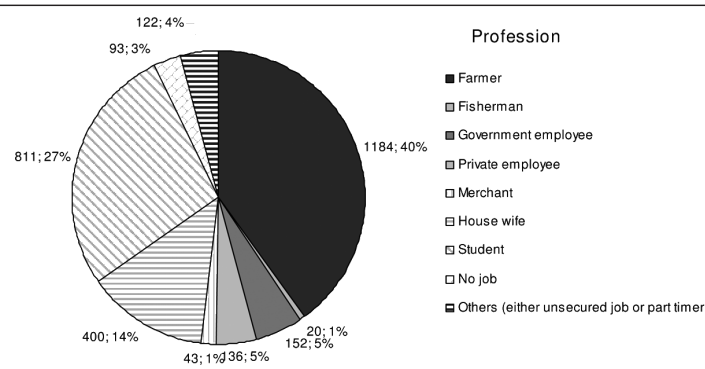


Figure 4 Job distribution. At baseline, profession was assessed for everyone above 15 years of age in the study area (n = 2961).

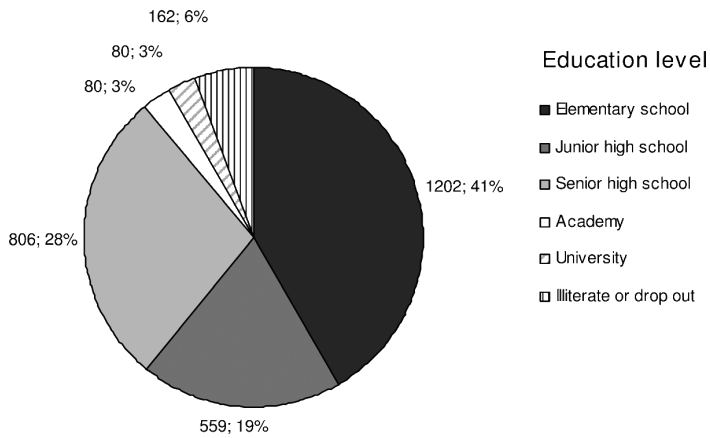


Figure 5 Education level. At baseline, education was assessed for everyone above 15 years of age in the study area (n = 2961).

study of helminth and malaria co-infections in Indonesia. The study has received enthusiastic support from the authorities in Ende. At the same time, the study facilitates the transfer of state of the art technologies in immunology, molecular biology, epidemiology and statistics to Indonesia.

Abbreviations
IgE: immunoglobulin E; IL: interleukin;

Acknowledgements
This study is funded by The Royal Netherlands Academy of Arts and Science (KNAW), Ref.KNAW-05-PP-35, European Commission contracts INCO-CT-2006-031714 and INCO-CT-2006-032436
The authors thank Drg Dominggus Minggu Mere as the former Head of Ende Health District for his support to initiate the study. Health staff in district as well as in the Puskesmas Primary Health center and community workers, Aurelius I Data as data entry person, Markus Rubu and Maksima as field worker, mostly help from UI team (Maria Kaisar, Sudirman, Suwarto, Heni Sitompul, Rosidi) and LUMC team (Yvonne Kruize), Awal Setiawan and Agus Rahmat (NAMRU-Two) who help and assist the mapping process, and last most of all inhabitants of Nangapanda (Ndeturea, Ndorurea1, Ndorurea).

Author details
[1]Department of Parasitology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia. [2]Department of Parasitology, Leiden University Medical Center, Leiden, The Netherlands. [3]Department of Microbiology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia. [4]Medical Research Unit, Albert Schweitzer Hospital, Lambaréné, Gabon; Department of Parasitology, Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany. [5]Department of Biostatistics, School of of Public Health, University of Indonesia, Jakarta, Indonesia. [6]Department of Biostatistics, Leiden University Medical Centre, Leiden, The Netherlands. [7]Department of Parasitology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia. [8]Department of Medical Microbiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.

Authors' contributions
AEW Medical doctor in charge of the field study, involve in setting up, supervising gathering of data, clinical care, and follow up of the study population
MAP Medical doctor in charge of the field study, involve in setting up, supervising gathering of data, clinical care, and follow up of the study population
FH Medical doctor in charge of the field study, involve in setting up, supervising gathering of data, clinical care, and follow up of the study population
LW Medical doctor in charge of the field study, involve in setting up, supervising gathering of data, clinical care, and follow up of the study population
BL Medical doctor who is the advisor on databases, epidemiological and statistical aspects of the study
IA Medical doctor who is the advisor on databases, epidemiological and statistical aspects of the study
HWU Statistician who is developing methods to analyze the complex data generated during the lifetime of the project
HW Parasitologist and field study expert who is in charge of the process of data selection, storage, safeguarding randomization, and privacy of the study subjects
YD Medical doctor who advises on the immunological aspects of the study
SW Medical doctor who is supervising of study set up
IS Medical doctor who is a specialist on malaria and advises on clinical malaria and in the study
LM Immunoepidemiologist who is advising on databases maintenance, epidemiological, statistical, and immunological aspects of the study
AJFL Immunologist who specializes in malaria immunology and advises on malaria responses in the study
JJV Molecular parasitologist who is involved in the molecular diagnosis of parasitic infections
ES Immunoparasitologist who is involved in coordinating the study and advising on parasitological and immunological aspects of the study
MY Immunologist who has developed the study and is the Dutch coordinator of the ImmunoSPIN program
TS Parasitologist who has developed the study and is the Indonesian coordinator of the ImmunoSPIN program
All authors read and approved the final paper.

Competing interests
The authors declare that they have no competing interests.

Received: 18 December 2009 Accepted: 25 March 2010
Published: 25 March 2010

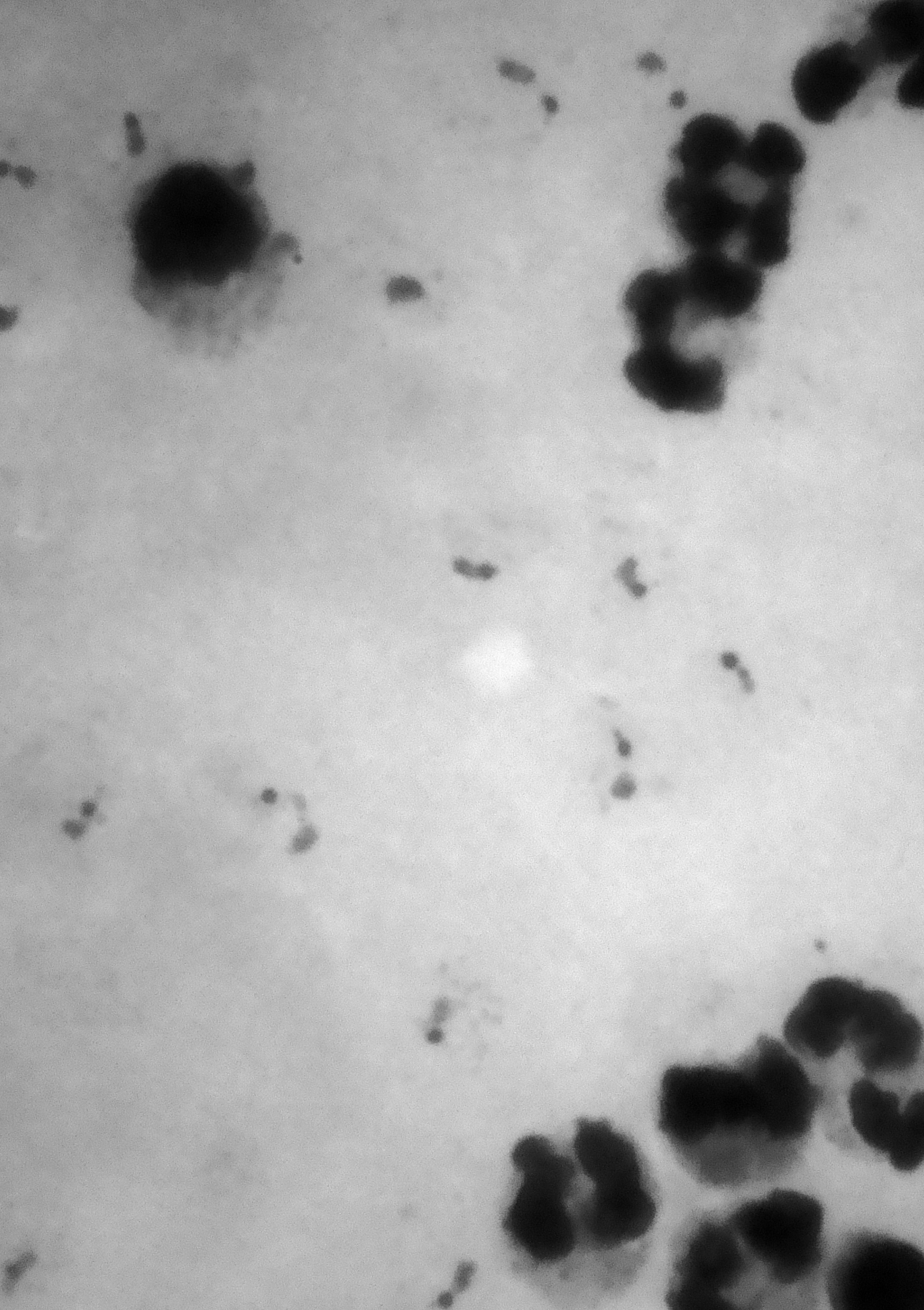
References

1. Brooker S, Miguel EA, Moulin S, Luoba AI, Bundy DA, Kremer M: **Epidemiology of single and multiple species of helminth infections among school children in Busia District, Kenya.** *East Afr Med J* 2000, **77**:157-161.
2. Brooker S, Singhasivanon P, Waikagul J, Supavej S, Kojima S, Takeuchi T, *et al*: **Mapping soil-transmitted helminths in Southeast Asia and implications for parasite control.** *Southeast Asian J Trop Med Public Health* 2003, **34**:24-36.
3. Cooper PJ: **Interactions between helminth parasites and allergy.** *Curr Opin Allergy Clin Immunol* 2009, **9**:29-37.
4. Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D, *et al*: **Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm.** *Lancet* 2006, **367**:1521-1532.
5. Diaz A, Allen JE: **Mapping immune response profiles: the emerging scenario from helminth immunology.** *Eur J Immunol* 2007, **37**:3319-3326.
6. Yazdanbakhsh M, van den Biggelaar BA, Maizels RM: **Th2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease.** *Trends Immunol* 2001, **22**:372-377.
7. Maizels RM, Pearce EJ, Artis D, Yazdanbakhsh M, Wynn TA: **Regulation of pathogenesis and immunity in helminth infections.** *J Exp Med* 2009, **206**:2059-2066.
8. Maizels RM, Yazdanbakhsh M: **Immune regulation by helminth parasites: cellular and molecular mechanisms.** *Nat Rev Immunol* 2003, **3**:733-744.
9. Hartgers FC, Obeng BB, Boakye D, Yazdanbakhsh M: **Immune responses during helminth-malaria co-infection: a pilot study in Ghanaian school children.** *Parasitology* 2008, **135**:855-860.
10. Brutus L, Watier L, Briand V, Hanitrasoamampionona V, Razanatsoarilala H, Cot M: **Parasitic co-infections: does Ascaris lumbricoides protect against Plasmodium falciparum infection?** *Am J Trop Med Hyg* 2006, **75**:194-198.
11. Boraschi D, Abebe AM, Aseffa A, Chiodi F, Chisi J, Del Prete G, *et al*: **Immunity against HIV/AIDS, Malaria, and Tuberculosis during Co-Infections with Neglected Infectious Diseases: Recommendations for the European Union Research Priorities.** *PLoS Negl Trop Dis* 2008, **2**:e255.
12. Schofield L, Grau GE: **Immunological processes in malaria pathogenesis.** *Nat Rev Immunol* 2005, **5**:722-735.
13. Brooker S, Clements AC, Hotez PJ, Hay SI, Tatem AJ, Bundy DA, *et al*: **The co-distribution of Plasmodium falciparum and hookworm among African schoolchildren.** *Malar J* 2006, **5**:99.
14. Brooker S, Akhwale W, Pullan R, Estambale B, Clarke SE, Snow RW, *et al*: **Epidemiology of plasmodium-helminth co-infection in Africa: populations at risk, potential impact on anemia, and prospects for combining control.** *Am J Trop Med Hyg* 2007, **77**:88-98.
15. Nacher M, Treeprasertsuk S, Singhasivanon P, Silachamroon U, Vannaphan S, Gay F, *et al*: **Association of hepatomegaly and jaundice with acute renal failure but not with cerebral malaria in severe falciparum malaria in Thailand.** *Am J Trop Med Hyg* 2001, **65**:828-833.
16. Nacher M, Singhasivanon P, Treeprasertsuk S, Chantachum Y, Vannaphan S, Traore B, *et al*: **Association of splenomegaly with cerebral malaria and decreased concentrations of reactive nitrogen intermediates in Thailand.** *Am J Trop Med Hyg* 2001, **65**:639-643.
17. Booth M, Graham A, Viney M: **Parasitic co-infections: challenges and solutions.** *Parasitology* 2008, **135**:749.
18. Booth M: **The role of residential location in apparent helminth and malaria associations.** *Trends Parasitol* 2006, **22**:359-362.
19. Druilhe P, Tall A, Sokhna C: **Worms can worsen malaria: towards a new means to roll back malaria?** *Trends Parasitol* 2005, **21**:359-362.
20. Murray MJ, Murray AB, Murray MB, Murray CJ: **Parotid enlargement, forehead edema, and suppression of malaria as nutritional consequences of ascariasis.** *Am J Clin Nutr* 1977, **30**:2117-2121.
21. Le Hesran JY, Akiana J, Ndiaye eH, Dia M, Senghor P, Konate L: **Severe malaria attack is associated with high prevalence of Ascaris lumbricoides infection among children in rural Senegal.** *Trans R Soc Trop Med Hyg* 2004, **98**:397-399.
22. Nacher M, Singhasivanon P, Silachamroon U, Treeprasertsuk S, Vannaphan S, Traore B, *et al*: **Helminth infections are associated with protection from malaria-related acute renal failure and jaundice in Thailand.** *Am J Trop Med Hyg* 2001, **65**:834-836.
23. Nacher M, Singhasivanon P, Treeprasertsuk S, Vannaphan S, Traore B, Looareesuwan S, *et al*: **Intestinal helminths and malnutrition are independently associated with protection from cerebral malaria in Thailand.** *Ann Trop Med Parasitol* 2002, **96**:5-13.
24. Nacher M, Singhasivanon P, Traore B, Vannaphan S, Gay F, Chindanond D, *et al*: **Helminth infections are associated with protection from cerebral malaria and increased nitrogen derivatives concentrations in Thailand.** *Am J Trop Med Hyg* 2002, **66**:304-309.
25. Nacher M: **Comment on: Severe malaria attack is associated with high prevalence of Ascaris lumbricoides infection among children in rural Senegal.** *Trans R Soc Trop Med Hyg* 2005, **99**:161-163.
26. Cooper PJ: **Intestinal worms and human allergy.** *Parasite Immunol* 2004, **26**:455-467.
27. Sakaguchi S: **Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses.** *Annu Rev Immunol* 2004, **22**:531-562.
28. Reddy A, Fried B: **An update on the use of helminths to treat Crohn's and other autoimmunune diseases.** *Parasitol Res* 2009, **104**:217-221.
29. Maizels RM, Yazdanbakhsh M: **T-cell regulation in helminth parasite infections: implications for inflammatory diseases.** *Chem Immunol Allergy* 2008, **94**:112-123.
30. **Indonesia Latitude and Longitude.** [http://www.mapsofworld.com/lat_long/indonesia-lat-long.html].
31. **The official site of Ende District, East Nusa Tenggara.** [http://www.endekab.go.id].
32. Keiser J, Utzinger J: **Efficacy of current drugs against soil-transmitted helminth infections: systematic review and meta-analysis.** *JAMA* 2008, **299**:1937-1948.
33. Saghaei M: **Random allocation software for parallel group randomized trials.** *BMC Med Res Methodol* 2004, **4**:26.
34. Adegnika AA, Verweij JJ, Agnandji ST, Chai SK, Breitling LP, Ramharter M, *et al*: **Microscopic and sub-microscopic Plasmodium falciparum infection, but not inflammation caused by infection, is associated with low birth weight.** *Am J Trop Med Hyg* 2006, **75**:798-803.
35. ten Hove RJ, Verweij JJ, Vereecken K, Polman K, Dieye L, van Lieshout L: **Multiplex real-time PCR for the detection and quantification of Schistosoma mansoni and S. haematobium infection in stool samples collected in northern Senegal.** *Trans R Soc Trop Med Hyg* 2008, **102**:179-185.
36. Verweij JJ, Pit DS, van Lieshout L, Baeta SM, Dery GD, Gasser RB, *et al*: **Determining the prevalence of Oesophagostomum bifurcum and Necator americanus infections using specific PCR amplification of DNA from faecal samples.** *Trop Med Int Health* 2001, **6**:726-731.
37. Verweij JJ, Canales M, Polman K, Ziem J, Brienen EA, Polderman AM, *et al*: **Molecular diagnosis of Strongyloides stercoralis in faecal samples using real-time PCR.** *Trans R Soc Trop Med Hyg* 2009, **103**:342-346.
38. Verweij JJ, Brienen EA, Ziem J, Yelifari L, Polderman AM, van Lieshout L: **Simultaneous detection and quantification of Ancylostoma duodenale, Necator americanus, and Oesophagostomum bifurcum in fecal samples using multiplex real-time PCR.** *Am J Trop Med Hyg* 2007, **77**:685-690.
39. Allen AV, Ridley DS: **Further observations on the formol-ether concentration technique for faecal parasites.** *J Clin Pathol* 1970, **23**:545-546.
40. Sutanto I, Ismid IS, Sjarifuddin PK: *Texbook of Parasitology Jakarta: Faculty of Medicine University of Indonesia (FKUI)* Faculty of Medicine University of Indonesia (FKUI), IV 2008.
41. Niesters HG: **Clinical virology in real time.** *J Clin Virol* 2002, **25(Suppl 3)**: S3-12.
42. Petithory JC, Ardoin F, Ash LR: **Rapid and inexpensive method of diluting Giemsa stain for diagnosis of malaria and other infestations by blood parasites.** *J Clin Microbiol* 2005, **43**:528.
43. Trape JF: **Rapid evaluation of malaria parasite density and standardization of thick smear examination for epidemiological investigations.** *Trans R Soc Trop Med Hyg* 1985, **79**:181-184.
44. Muller-Stover I, Verweij JJ, Hoppenheit B, Gobels K, Haussinger D, Richter J: **Plasmodium malariae infection in spite of previous anti-malarial medication.** *Parasitol Res* 2008, **102**:547-550.
45. van Riet E, Wuhrer M, Wahyuni S, Retra K, Deelder AM, Tielens AG, *et al*: **Antibody responses to Ascaris-derived proteins and glycolipids: the role of phosphorylcholine.** *Parasite Immunol* 2006, **28**:363-371.
46. Hartgers FC, Obeng BB, Kruize YC, Dijkhuis A, McCall M, Sauerwein RW, *et al*: **Responses to malarial antigens are altered in helminth-infected children.** *J Infect Dis* 2009, **199**:1528-1535.

47. Terhell AJ, Stolk WA, Haarbrink M, Mangali A, Van Oortmarssen GJ, Yazdanbakhsh M: **Regulation of anti-filarial IgE by infection pressure.** *Parasitology* 2002, **124**:509-519.
48. Uh HW, Hartgers FC, Yazdanbakhsh M, Houwing-Duistermaat JJ: **Evaluation of regression methods when immunological measurements are constrained by detection limits.** *BMC Immunol* 2008, **9**:59.
49. Moher D, Schulz KF, Altman DG: **The CONSORT statement: revised recommendations for improving the quality of reports of parallel-group randomised trials.** *Lancet* 2001, **357**:1191-1194.
50. Olsen A, Namwanje H, Nejsum P, Roepstorff A, Thamsborg SM: **Albendazole and mebendazole have low efficacy against Trichuristrichiura in school-age children in Kabale District, Uganda.** *Trans R Soc Trop Med Hyg* 2009, **103(5)**:443-6.
51. **Health Situation in the South-East Asia Region.** 1998 [http://www.searo.who.int/en/Section1243/Section1382/Section1386/Section1898_9248.htm].

Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-2334/10/77/prepub

doi:10.1186/1471-2334-10-77
Cite this article as: Wiria *et al.*: Does treatment of intestinal helminth infections influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study). *BMC Infectious Diseases* 2010 **10**:77.



chapter 3

The Effect of Three-Monthly Albendazole Treatment on Malarial Parasitemia and Allergy: A Household-Based Cluster-Randomized, Double-Blind, Placebo-Controlled Trial

The Effect of Three-Monthly Albendazole Treatment on Malarial Parasitemia and Allergy: A Household-Based Cluster-Randomized, Double-Blind, Placebo-Controlled Trial

Aprilianto E. Wiria[1,2☯], Firdaus Hamid[2,3☯], Linda J. Wammes[2☯], Maria M. M. Kaisar[1,2], Linda May[2], Margaretta A. Prasetyani[1,2], Sitti Wahyuni[4], Yenny Djuardi[1,2], Iwan Ariawan[5], Heri Wibowo[1], Bertrand Lell[6,7], Robert Sauerwein[8], Gary T. Brice[9], Inge Sutanto[1], Lisette van Lieshout[2], Anton J. M. de Craen[10], Ronald van Ree[11], Jaco J. Verweij[2¤a], Roula Tsonaka[12], Jeanine J. Houwing-Duistermaat[12], Adrian J. F. Luty[8¤b], Erliyani Sartono[2], Taniawati Supali[1]*, Maria Yazdanbakhsh[2]*

1 Department of Parasitology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia, **2** Department of Parasitology, Leiden University Medical Center, Leiden, The Netherlands, **3** Department of Microbiology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia, **4** Department of Parasitology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia, **5** Department of Biostatistics, School of Public Health, University of Indonesia, Jakarta, Indonesia, **6** Medical Research Unit, Albert Schweitzer Hospital, Lambaréné, Gabon, **7** Department of Parasitology, Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany, **8** Department of Medical Microbiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, **9** Naval Medical Research Unit 2, Jakarta, Indonesia, **10** Department of Gerontology and Geriatrics, Leiden University Medical Center, Leiden, The Netherlands, **11** Department of Experimental Immunology and Department of Otorhinolaryngology, Academic Medical Center, Amsterdam, The Netherlands, **12** Department of Medical Statistics and Bioinformatics, Leiden University Medical Center, Leiden, The Netherlands

Abstract

Background: Helminth infections are proposed to have immunomodulatory activities affecting health outcomes either detrimentally or beneficially. We evaluated the effects of albendazole treatment, every three months for 21 months, on STH, malarial parasitemia and allergy.

Methods and Findings: A household-based cluster-randomized, double-blind, placebo-controlled trial was conducted in an area in Indonesia endemic for STH. Using computer-aided block randomization, 481 households (2022 subjects) and 473 households (1982 subjects) were assigned to receive placebo and albendazole, respectively, every three months. The treatment code was concealed from trial investigators and participants. Malarial parasitemia and malaria-like symptoms were assessed in participants older than four years of age while skin prick test (SPT) to allergens as well as reported symptoms of allergy in children aged 5–15 years. The general impact of treatment on STH prevalence and body mass index (BMI) was evaluated. Primary outcomes were prevalence of malarial parasitemia and SPT to any allergen. Analysis was by intention to treat. At 9 and 21 months post-treatment 80.8% and 80.1% of the study subjects were retained, respectively. The intensive treatment regiment resulted in a reduction in the prevalence of STH by 48% in albendazole and 9% in placebo group. Albendazole treatment led to a transient increase in malarial parasitemia at 6 months post treatment (OR 4.16(1.35–12.80)) and no statistically significant increase in SPT reactivity (OR 1.18(0.74–1.86) at 9 months or 1.37 (0.93–2.01) 21 months). No effect of anthelminthic treatment was found on BMI, reported malaria-like- and allergy symptoms. No adverse effects were reported.

Conclusions: The study indicates that intensive community treatment of 3 monthly albendazole administration for 21 months over two years leads to a reduction in STH. This degree of reduction appears safe without any increased risk of malaria or allergies.

Trial Registration: Controlled-Trials.com ISRCTN83830814

Citation: Wiria AE, Hamid F, Wammes LJ, Kaisar MMM, May L, et al. (2013) The Effect of Three-Monthly Albendazole Treatment on Malarial Parasitemia and Allergy: A Household-Based Cluster-Randomized, Double-Blind, Placebo-Controlled Trial. PLoS ONE 8(3): e57899. doi:10.1371/journal.pone.0057899

Editor: Steffen Borrmann, Kenya Medical Research Institute - Wellcome Trust Research Programme, Kenya

Received October 5, 2012; **Accepted** January 28, 2013; **Published** March 19, 2013

Funding: This study was funded by The Royal Netherlands Academy of Arts and Science (KNAW), Ref.KNAW-05-PP-35, European Commission contracts INCO-CT-2006-031714 and INCO-CT-2006-032436, Glofal FP6-2003-FOOD-2-B, and the Prof. Dr. P.C. Flu Foundation. No funding bodies had any role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: M.Yazdanbakhsh@lumc.nl (MY); taniawati@yahoo.com (TS)

¤a Current address: Laboratory for Medical Microbiology and Immunology, St. Elisabeth Hospital, Tilburg, The Netherlands
¤b Current address: Institut de Recherche pour le Développement UMR IRD/UPD 216, Mère et enfant face aux infections tropicales, Faculté de Pharmacie, Paris, France

☯ These authors contributed equally to this work.

*Supplementary Appendix S1 acquired new page numbers.

Introduction

Soil transmitted helminths (STH) (hookworms, *Ascaris lumbricoides* and *Trichuris trichiura*) establish chronic infections in a large proportion of the world population.[1] Major intervention programs using mass drug administration (MDA) to control STH have been launched.[2] However, STH infections seem to persist in the targeted populations raising concern over the development of drug resistance.[3] It is therefore important to conduct well-designed studies that allow evidence-based decisions to be made to maximize effective STH control toward elimination.

While there is no doubt that STH are associated with morbidities in billions of people worldwide, there is also increasing awareness that helminth infections might, like bacterial commensals, play an important role in shaping human health.[4] Helminths may contribute to immunologic and physiologic homeostasis. The immune system is thought to have evolved to operate optimally in the face of helminth-induced immune regulation, and that any disturbance of this long evolutionary co-existence between humans and helminth parasites might be associated with the emergence of pathological conditions[5] possibly involving outcomes of exposure to other pathogens or the development of inflammatory diseases.

In many parts of the world helminths and malarial parasites are co-endemic raising the question of what impact helminth infections may have on the plasmodial parasites that cause malaria. The results have been conflicting in this regard. In some studies a positive association has been reported between helminths and malarial parasitemia while in others, this has been refuted or in yet others a negative association has been shown between helminths and the severity of the clinical outcomes of malaria (reviewed by Nacher).[6]

An increase in the prevalence of allergies has been reported worldwide, in particular in the urban areas of low- to middle-income countries.[7] Although majority of cross-sectional studies have reported inverse associations between helminth infections and allergies[8,9], two randomized trials with albendazole, have shown conflicting results. One in Ecuador, based on school randomization, reported no change in either SPT reactivity to allergens or allergic symptoms after one year of albendazole treatment[10] while another in Vietnam, in which the randomization unit was individual schoolchildren, showed increased SPT reactivity after one year of albendazole treatment, but consistent

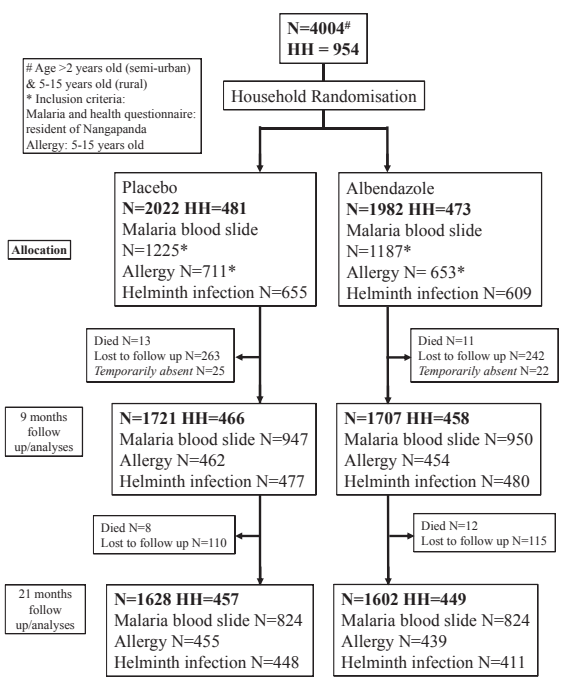


Figure 1. Trial Profile. HH: Household. Lost to follow up implies that the participants have no data from this time point onward. Temporarily absent implies that the participants have no data at this time point but have data available at other time point.
doi:10.1371/journal.pone.0057899.g001

with the Ecuadorean study, clinical allergy did not change significantly.[11] It has been suggested that anthelminthic treatment of longer duration might be needed to reveal the modulatory effect of helminths.[12,13]

In the light of global deworming initiatives, it is important to assess the effectiveness of and to monitor the risks associated with anthelminthic treatment regiments. There is as yet no report of a household-based cluster-randomized double-blind placebo-controlled trial of repeated anthelminthic administration in a community that would be expected to more effectively reduce transmission of STH by decreasing household cross-contamination.

In an area where STH and malaria are co-endemic on Flores Island, Indonesia, we conducted a household cluster-randomized trial of three-monthly albendazole treatment over a two year study period in a whole community to assess benefits and risks associated with this anthelmintic treatment. Specifically we assessed its impact on STH, malarial parasitemia and allergy.

Methods

Study population and design

This trial was conducted in two villages in the Ende District of Flores Island, Indonesia (Appendix S1, p2) as described in detail elsewhere.[14,15] The treatment was based on household and given to all household members except those less than two years old or pregnant (the Indonesian national program guideline). Directly observed treatment was given three monthly during the trial period (June 2008 to July 2010, with treatment starting in Sept 2008). The primary outcomes were prevalence of malarial parasitemia and SPT reactivity to allergens. Additional outcomes were treatment effect on STH and BMI as well as malaria-like and allergy symptoms.

We measured malaria outcomes in Nangapanda only. Malaria was not endemic in Anaranda. Artemisinin-combination therapy (ACT) treatment and treated bed net distribution were not implemented during our study period.[16,17]

Allergy outcomes were measured, in both villages, in school-age children (5–15 years old) as this group is particularly at risk of developing allergy and asthma[18] and is the target population of global deworming programs.

The study was approved by the Ethical Committee of the Medical Faculty, University of Indonesia (ethical clearance ref: 194/PT02.FK/Etik/2006) and filed by the Committee of Medical Ethics of the Leiden University Medical Center. The trial was registered as clinical trial (Ref: ISRCTN83830814). Prior to the study, written informed consent was obtained from participants or from parents/guardians of children. The study is reported in accordance with the CONSORT guidelines for cluster-randomized studies. The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and Protocol S1.

Randomization and masking

The population was randomized by IA using computer aided block randomization at household level utilising Random Allocation software to receive albendazole (single dose of 400 mg) or a matching placebo (both tablets from PT Indofarma Pharmaceutical, Bandung, Indonesia). The treatment code was concealed from trial investigators and participants. The un-blinding of treatment codes occurred after all laboratory results had been entered into the database (August 2011).

Table 1. Baseline characteristics.

	N	Placebo	N	Albendazole
Age (mean in years, SD)	2022	25.7 (18.7)	1982	25.8 (18.7)
Sex (female, n, %)	2022	1090 (53.9)	1982	1042 (52.6)
Area (rural, n, %)	2022	260 (12.9)	1982	253 (12.8)
BMI >19 years old (mean, SD)	575	22.3 (4.0)	582	21.8 (3.6)
Z score of BMI ≤ 19 years old (mean, SD)	427	−1.20 (1.2)	386	−1.37 (1.3)
Parasite infection (n, %)				
Helminth (any *spp*)	655	571 (87.2)	609	533 (87.5)
Hookworm[1]	683	509 (74.5)	629	486 (77.3)
N. americanus[1]	683	503 (73.7)	629	481 (76.5)
A. duodenale[1]	683	44 (6.4)	629	41 (6.5)
A. lumbricoides[1]	683	238 (34.9)	629	209 (33.2)
S. stercoralis[1]	683	7 (1.0)	629	18 (2.9)
T. trichiura[2]	953	258 (27.1)	852	237 (27.8)
Malarial parasitemia (any *spp*)[2]	1225	60 (4.9)	1187	52 (4.4)
P. falciparum	1225	32 (2.6)	1187	28 (2.4)
P. vivax	1225	26 (2.1)	1187	18 (1.5)
P. malariae	1225	2 (0.2)	1187	7 (0.6)
Malarial parasitemia (any *spp*)[1]	772	195 (25.3)	739	200 (27.1)
P. falciparum	772	106 (13.7)	739	112 (15.2)
P. vivax	772	102 (13.2)	739	93 (12.6)
P. malariae	772	10 (1.3)	739	18 (2.4)
Skin prick reactivity (n, %)				
Any allergen	711	190 (26.7)	653	163 (25.0)
House dust mite	711	88 (12.4)	653	75 (11.5)
Cockroach	711	163 (22.9)	653	140 (21.4)
Specific IgE, kU/L (median, IQR)				
House dust mite	452	0.8 (0.3–2.6)	431	0.8 (0.2–2.4)
Cockroach	452	1.5 (0.4–5.7)	431	1.9 (0.5–5.0)

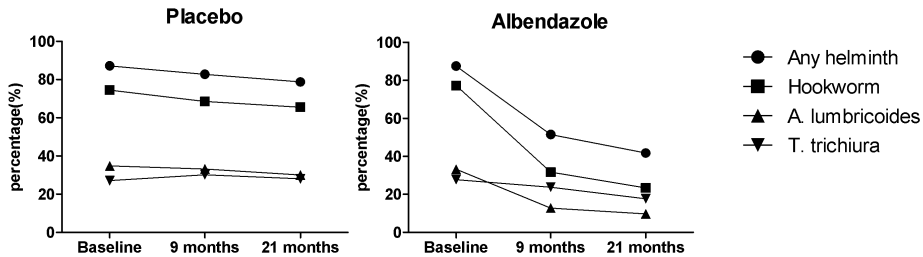
[1]diagnosed by PCR; [2]diagnosed by microscopy.
The number of positives (n) of the total population examined (N).
doi:10.1371/journal.pone.0057899.t001

Procedures

Trained community workers measured fever, administered monthly malaria-like symptoms questionnaire which was based on WHO definitions[19] and took finger-prick blood for the three-monthly malarial parasitemia survey. Subjects with fever (≥37.5°C) or additional malaria-like symptoms (headache, fatigue and nausea) at the time of visits were referred to the local primary health centre (puskesmas). Thick and thin Giemsa-stained blood smears were read at University of Indonesia. At baseline, 9 months and 21 months after the first round of treatment blood was collected for PCR-based detection of *Plasmodium spp*. (Appendix S1, p2), a method that is more sensitive than microscopy.[20]

Regarding allergy outcomes, skin prick tests (SPT) with allergens were performed on school-age children in Nangapanda and Anaranda and clinical symptoms of allergy were recorded. House dust mite (*Dermatophagoides pteronyssinus* and *D. farinae*; kindly provided by Paul van Rijn from HAL Allergy Laboratories, Leiden, The Netherlands) and cockroach (*Blatella germanica*; Lofarma, Milan, Italy) were used for SPT which was considered positive with 3 mm cut off.[14] The SPT was performed by one investigator. IgE with specificity for aeroallergens (*D. pteronyssinus*

A. Percentage of helminth infected subjects in placebo and albendazole treatment arms



B. Effect of albendazole treatment on reduction in the intensity as well as percentage of subjects positive for hookworm and *Ascaris* infection as determined by PCR

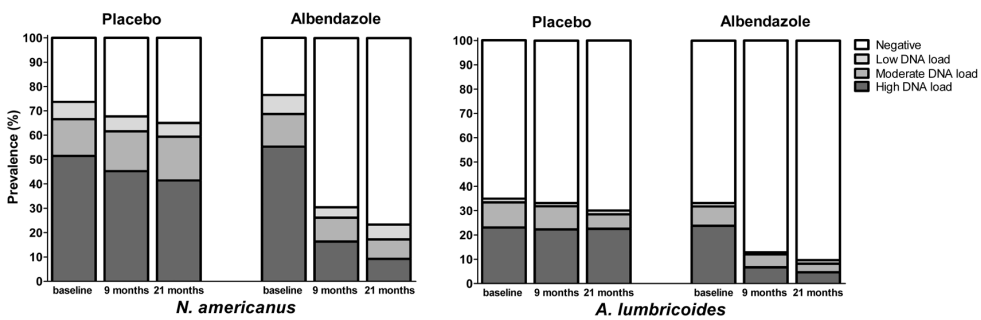


Figure 2. A) Percentage of helminth infected subjects in placebo and albendazole treatment arms. The presence of hookworms (by PCR), *Ascaris lumbricoides* (by PCR) and *Trichuris trichiura* (by microscopy) or any of these helminth infections in subjects who provided stool samples at baseline, 9 and 21 months post treatment (numbers are given in table S1A in Appendix S1). **B) Effect of albendazole treatment on reduction in the intensity as well as percentage of subjects positive for hookworm and *Ascaris* infection as determined by PCR.** Negative is when no helminth specific DNA was found. Positive Ct- values were grouped into three categories: Ct<30.0, 30.0≤Ct<35.0 and ≥35.0 representing a high, moderate and low DNA load, respectively.
doi:10.1371/journal.pone.0057899.g002

and *B. germanica*) was measured in plasma using an ImmunoCAP 250 system (Phadia, Uppsala, Sweden) following the manufacturer's instructions. All measurements were conducted in one laboratory in the Netherlands. Symptoms of asthma and atopic dermatitis were recorded using a modified visually-assisted version of the International Study of Asthma and Allergy in Childhood (ISAAC) questionnaire as reported before.[14]

Yearly stool samples were collected on a voluntary basis. *Trichuris* was detected by microscopy and a multiplex real-time PCR was used for detection of hookworms (*Ancylostoma duodenale*, *Necator americanus)*, *Ascaris lumbricoides*, and *Strongyloides stercoralis* DNA as detailed before[15] (Appendix S1, p2). Very few subjects were infected with *S. stercoralis* and therefore this infection was not included in analyses.

Body weight and height were measured using the National Heart Lung and Blood Institute practical guidelines (scale and microtoise from SECA GmBH & Co, Hamburg, Germany).

Power calculation

Sample size estimation was based on the expected change in primary outcomes taking into account a power of 90% and a significance level of <0.05 with a loss to follow-up of 20%. Based on previous observations we expected to find a decrease in malarial parasitemia prevalence and an increase in SPT reactivity after anthelminthic treatment. Based on a prevalence of about 10% and a risk ratio (RR) of 0.60 we aimed to include 2412 people in the malaria assessments. In a pilot study we found SPT to *D. pteronyssinus* to be around 15%, and expected that due to treatment the prevalence would increase. In order to find a RR of 1.5 we aimed to include at least 1418 children.

Statistical analyses

For children ≤19 years, BMI age-standardized z-scores were calculated according to WHO references.[21] The IgE data were log-transformed to obtain normally distributed variable. To assess treatment effects generalized linear mixed models were used which provide a flexible and powerful tool to derive valid inference while

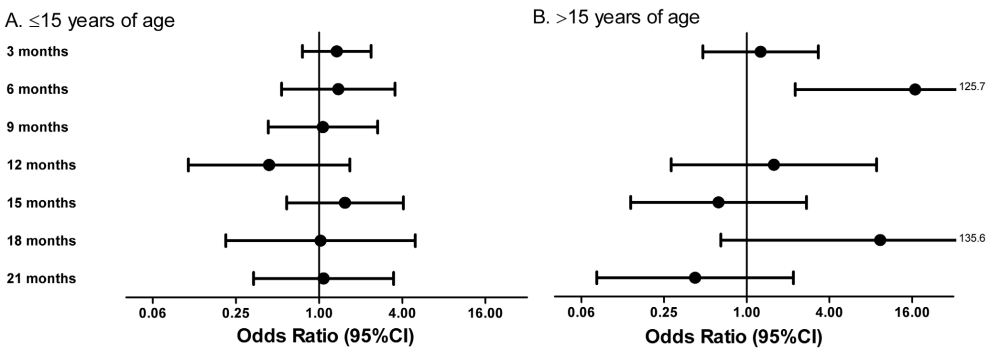


Figure 3. Effect of albendazole treatment on malarial parasitemia based on two age categories. Malarial parasitemia A) ≤15 and B) >15 years of age. The risk of malarial parasitemia after albendazole treatment compared to placebo is shown as odds ratio with 95% CI. The reference line is set at 1, indicating that symbols at the right of this line represent an increased risk, while symbols at the left of the line would predict decreased risk of malarial parasitemia. Note: at 9 month time point in those >15 years of age, the OR is ∞.
doi:10.1371/journal.pone.0057899.g003

capturing the data correlations induced by clustering within households and repeated evaluations in time of the same subject. Parameter estimates for treatment effects at 9 and 21 months and 95% confidence intervals are reported. The reported p-values are obtained using likelihood ratio tests by comparing the model with and without the treatment effect. Unless stated otherwise all outcomes were adjusted for area (the two study villages in Ende District: Nangapanda or Anaranda) as covariate in the model. For the continuous outcomes (linear mixed-effects models[22] were used with three random effects, namely to model clustering within households a random household specific intercept was used and to model correlation within subjects a random subject specific intercept and slope were used. For the binary outcomes a logistic model was used with random household effects and random subject effects. All models were fitted using the lme4 package (Appendix S1, p6-7).[23] For each fever and additional malaria-like symptoms, total number of events and person months are computed for each treatment arm. Hazard ratios for effect of treatment were calculated with Cox regression with robust SE to allow for within-household clustering (STATA 11).

Results

At baseline, 954 households with 4004 subjects were registered. Randomization of households resulted in 1982 people assigned to albendazole treatment and 2022 people to placebo (473 and 481 houses respectively). At baseline 87·3% of the individuals were infected with one or more helminth species. The baseline

Table 2. Effect of three-monthly albendazole treatment on malaria outcomes: Percentage of subjects with malarial parasitemia.

	P. falciparum		*P. vivax*		*P. malariae*	
	Placebo	**Albendazole**	**Placebo**	**Albendazole**	**Placebo**	**Albendazole**
	n/N (%)	**n/N (%)**	**n/N (%)**	**n/N (%)**	**n/N (%)**	**n/N (%)**
Malarial parasitemia by microscopy						
0 month	32/1225 (2.6)	28/1187 (2.4)	26/1225 (2.1)	18/1187 (1.5)	2/1225 (0.2)	7/1187 (0.6)
3 months	41/897 (4.6)	46/910 (5.1)	17/897 (1.9)	22/910 (2.4)	1/897 (0.1)	6/910 (0.7)
6 months	8/815 (1.0)	20/794 (2.5)	4/815 (0.5)	9/794 (1.1)	0	0
9 months	14/947 (1.5)	7/950 (0.7)	4/947 (0.4)	5/950 (0.5)	1/947 (0.1)	1/950 (0.1)
12 months	9/834 (1.1)	9/813 (1.1)	4/834 (0.5)	2/813 (0.2)	0	0
15 months	14/773 (1.8)	13/772 (1.7)	3/773 (0.4)	4/772 (0.5)	1/773 (0.1)	3/772 (0.4)
18 months	3/815 (0.4)	10/803 (1.2)	1/815 (0.1)	1/803 (0.1)	1/815 (0.1)	1/803 (0.1)
21 months	6/824 (0.7)	11/824 (1.3)	6/824 (0.7)	0	3/824 (0.4)	1/824 (0.1)
Malarial parasitemia by PCR						
0 month	106/772 (13.7)	112/739 (15.2)	102/772 (13.2)	93/739 (12.6)	10/772 (1.3)	18/739 (2.4)
9 months	35/656 (5.3)	56/627 (8.9)	56/656 (8.5)	50/627 (8.0)	7/656 (1.1)	9/627 (1.4)
21 months	21/584 (3.6)	31/553 (5.6)	24/584 (4.1)	27/553 (4.9)	10/584 (1.7)	5/553 (0.9)

The number of positives (n) of the total population examined (N).
doi:10.1371/journal.pone.0057899.t002

Table 3. Effect of three-monthly albendazole treatment on malaria outcomes: Malarial parasitemia by microscopy

	Placebo	Albendazole	OR (95%CI) *
	n/N (%)	n/N (%)	
Malarial parasitemia (any *spp*)			
3 months	59/897 (6.6)	72/910 (7.9)	1.54 (0.75–3.16)
6 months	12/815 (1.5)	29/794 (3.7)	4.16 (1.35–12.80)
9 months	19/947 (2.0)	13/950 (1.4)	0.57 (0.16–2.04)
12 months	13/834 (1.6)	10/813 (1.2)	0.62 (0.12–3.15)
15 months	18/773 (2.3)	20/772 (2.6)	1.17 (0.18–7.65)
18 months	5/815 (0.6)	12/803 (1.5)	1.84 (0.12–29.03)
21 months	15/824 (1.8)	12/824 (1.5)	0.26 (0.01–6.59)

The number of positives (n) of the total population examined (N). *Odds ratio and 95% confidence interval are based on mixed effects logistic regression models. OR's and 95% CI are shown for the separate time points on malarial parasitemia. The p-value is generated from the modeled data for the combined effect of albendazole treatment over time, which is significant (P = 0.0064) and might result from the effect of 6 months post treatment time point.
doi:10.1371/journal.pone.0057899.t003

characteristics were similar between the treatment arms (table 1). The overall trial profile is shown in figure 1, and figure S1A, S1B, S1C (p13–15) in Appendix S1 separately for malaria, allergy and helminth outcomes. The analysis was intention-to-treat and involved all participants as assigned randomly at the start of the trial. During the study, in the albendazole arm 61 people moved to a house that was assigned to placebo while in the placebo arm 62 people moved to a house that was assigned to albendazole. The 44 subjects who died during the trial, included 4 people below the age of 20, 3 between 20 and 40 and the rest above 40 years of age, and were equally distributed between the treatment arms. At 9 months post-treatment full compliance was 77.8% for albendazole treatment and 78.0% for placebo. This was 63.1% and 62.5% respectively at 21 months.

Table 4. Effect of three-monthly albendazole treatment on malaria outcomes: Malarial parasitemia by PCR.

	Placebo	Albendazole	OR (95% CI)
	n/N (%)	n/N (%)	
Malaria (any spp)			
9 months	95/656 (14.5)	103/627 (16.4)	1.13 (0.77–1.64)
21 months	53/584 (9.1)	59/553 (10.7)	1.09 (0.68–1.76)
P. falciparum			
9 months	35/656 (5.3)	56/627 (8.9)	2.82 (1.29–6.15)
21 months	21/584 (3.6)	31/553 (5.6)	1.63 (0.63–4.22)
P. vivax			
9 months	56/656 (8.5)	50/627 (8.0)	0.84 (0.41–1.71)
21 months	24/584 (4.1)	27/553 (4.9)	1.40 (0.56–3.52)
P. malariae			
9 months	7/656 (1.1)	9/627 (1.4)	0.34 (0.04–2.79)
21 months	10/584 (1.7)	5/553 (0.9)	0.04 (0.00–0.39)

The number of positives (n) of the total population examined (N). Odds ratio and 95% confidence interval based on logistic mixed models. The statistically significant results are given in bold. The p-values are generated from the modeled data for the combined effect of albendazole treatment over time for each of the species separately, which were significant for *P. falciparum* (P = 0.029) and *P. malariae* (P = 0.016).
doi:10.1371/journal.pone.0057899.t004

This intensive treatment with albendazole resulted in a reduction but not elimination of STH. There was a decrease both after 9 (OR (95% CI) = 0.07 (0.04–0.11) and 21 months (0.05 (0.03–0.08)) of treatment ($p<0.0001$). Albendazole had the largest effect on hookworm followed by *Ascaris* while the effect on *Trichuris* was less pronounced (figure 2A and table S1 in Appendix S1 p8). Treatment also led to statistically significant reduction in the intensity of hookworm and *Ascaris* infection as determined by PCR (figure 2B). The fact that the stool sampling was on a voluntary basis could have created a selection bias. Analyzing baseline characteristics of subjects providing stool samples and those who did not at 9 months follow up, showed no differences in helminth prevalence, age and sex. Although at 21 months post treatment, sex and helminth prevalence were not different, age was slightly but significantly higher in subjects who provided stool samples mean age in years (SD) = 29.9 (20.4) vs 24.3 (17.5), p = 0.006)

The overall percentage of subjects with malarial parasitemia, irrespective of treatment arm, decreased over the trial period (table 2). However, when the data were modelled to assess the effect of albendazole treatment over time, there was a significant (P = 0.0064) increase, which might result from the transient four-fold increased risk of malarial parasitemia (OR 4.16 (1.35–12.80)) (table 3) at 6 months after initiation of treatment (after 2 doses of albendazole). The effect of anthelminthic treatment was assessed in those younger than 15 years of age who would be the prime target of the global deworming programs. The transient increase in parasitemia was only seen in the older (>15 years) age group (figure 3). Malarial parasites were also assessed by PCR, at 9 and 21 months after initiation of treatment and revealed that albendazole had no effect when all *Plasmodium* species were considered together, but when analyzed separately there was a significant increase in the percentage of subjects positive for *P. falciparum* at 9 months post-treatment (table 4). There was no difference in the incidence of fever and additional malaria-like symptoms between the two treatment arms (table S2 in Appendix S1 p10).

The proportion of subjects with SPT reactivity was 353/1364 (25.9%) at baseline. Albendazole treatment had no statistically significant effect on SPT to any allergen (table 5), but it was noted that there was an incremental increase in the risk of being SPT positive to any allergen at 9 months and 21 months post initiation of treatment. Moreover, additional analysis on allergens separately, showed a significantly higher SPT to cockroach at 21 months after treatment (1.63 (1.07–2.50)) (table 6). The levels of IgE to allergens showed that albendazole treatment had no effect (table 6). No effect of treatment was seen on symptoms of asthma or atopic dermatitis (table S3 in Appendix S1 p11).

No significant change in BMI was observed in children or in adults (table S4 in Appendix S1 p12). Moreover, there was no adverse effect of treatment reported.

Discussion

This household-based clustered-randomized, double-blind, placebo-controlled trial shows that administering a total of seven single doses of albendazole, at three-monthly intervals, to a population living in an area of Indonesia where STH are highly prevalent, leads to decreased prevalence of helminth infections which although statistically significant, can be taken as an incomplete reduction. The results show a transient increase in

Table 5. Effect of three-monthly albendazole treatment on allergy outcomes: Skin prick test to any allergens.

	Placebo	Albendazole	OR (95%CI) *
	n/N (%)	n/N (%)	
SPT to any allergen			
9 months	80/462 (17.3)	82/454 (18.1)	1.18 (0.74–1.86)
21 months	145/455 (31.9)	161/439 (36.7)	1.37 (0.93–2.01)

The number of positives (n) of the total population examined (N). *Odds ratio and 95% confidence interval are based on mixed effects logistic regression models. OR's and 95% CI are shown for the separate time points on SPT to any allergen. The p-value is generated from the modeled data for the effect of albendazole treatment overtime and no significant effects were found (P>0.05).
doi:10.1371/journal.pone.0057899.t005

malarial parasitemia in the albendazole- compared with the placebo-treated arm in the first six months after initiation of treatment. Albendazole treatment had no statistically significant effect on the designated co-primary outcome, skin prick test reactivity to allergens.

The clinical data collected of fever and additional malaria-like symptoms, were not affected by the deworming. Clinical signs of asthma and atopic dermatitis were also not affected by albendazole treatment.

The prevalence of infection was high (>60%), which reflects the situation in many areas that are being targeted by the global deworming programs. Using a three-monthly treatment regimen which represents an extreme scenario for helminth control strategy, percentage of subjects positive for STH was reduced by 39% compared to placebo. It should be noted that in our study the sensitive PCR method has been used. The reduction in the proportion of subjects infected with hookworm and *Ascaris* was more pronounced than for *Trichuris* infections, confirming the findings using a single dose of albendazole.[24] Subjects who provided stool samples at 21 months were slightly but significantly older than those who did not. Given that hookworm infection is more prevalent in older subjects, this may have contributed to the poor deworming achieved by albendazole. The reduction achieved in worm loads, did not have any beneficial effect on BMI. Observational studies have reported that helminth infections affect growth; however randomized trials have not been consistent.[25,26] In this regard, our study would support the outcome of a recent Cochrane review of no beneficial effect of deworming programs on nutritional indicators [27] even though it can be argued that in our study the suboptimal reduction in the STH would not allow any beneficial effect of anthelmintic in terms of BMI to be seen in the community. Importantly, the fact that the effect of such an intensive deworming strategy in a community is incomplete, needs to be considered in the agenda for the control and elimination of helminth diseases of humans.[28]

Most studies on the effect of helminth infections on malarial parasitemia and clinical malaria episodes have used cross-sectional designs and have been inconclusive.[6] Longitudinal studies of anthelminthic treatment have also reported conflicting results.[29,30] A small study conducted in Madagascar[29] has reported an increase in malarial parasitemia in levimasole treated subjects, older than 5 years of age, while in Nigeria [30], albendazole treatment of pre school children was associated with lower *P. falciparum* infection and anemia, however, the lost to follow up in this study was very high. The question whether albendazole treatment during pregnancy could affect health outcomes in the offspring, was addressed in a recent report from Uganda.[31] It was found that the incidence of malaria up to one years of age was not different in the offspring of mothers born to those treated with albendazole or placebo. Our study, reports the results of a community wide randomized-controlled trial that used three-monthly malarial parasitemia data obtained by microscopy. A significantly higher percentage of subjects positive for malarial parasites in the albendazole compared to the placebo arm was seen but this seemed to be a transient effect and restricted to individuals older than 15 years of age, an age group that is not the main target of the current deworming programs. The question

Table 6. Effect of three-monthly albendazole treatment on allergy outcomes: Skin prick test and specific IgE to aeroallergens.

	Placebo	Albendazole	
Skin prick test reactivity*	**n/N (%)**	**n/N (%)**	**OR (95% CI)**
House dust mite			
9 months	36/462 (7.8)	35/454 (7.7)	1.31 (0.52–3.27)
21 months	77/455 (16.9)	76/439 (17.3)	1.37 (0.62–3.02)
Cockroach			
9 months	60/462 (13.0)	65/454 (14.3)	1.27 (0.75–2.15)
21 months	112/455 (24.6)	139/439 (31.7)	1.63 (1.07–2.50)
Specific IgE**	N (Median, IQR)	N (Median, IQR)	β (95% CI)
House dust mite			
9 months	391 (0.46, 0.16–2.35)	381 (0.46, 0.14–1.98)	1.01 (0.91–1.12)
21 months	339 (0.82, 0.27–3.29)	334 (0.65, 0.20–2.69)	0.93 (0.81–1.06)
Cockroach			
9 months	391 (1.47, 0.30–5.01)	381 (1.55, 0.44–4.40)	1.04 (0.93–1.16)
21 months	339 (1.83, 0.47–5.44)	334 (1.64, 0.42–4.82)	0.98 (0.85–1.14)

The number of positives (n) of the total population examined (N). *Odds ratio and 95% confidence interval based on logistic mixed models; **β (beta) and 95% confidence interval based on generalized linear mixed models from the log-transformed IgE. The values shown are back-transformed. The p-values are generated from the modeled data for the effect of albendazole treatment overtime and no significant effects were found (P>0.05).
doi:10.1371/journal.pone.0057899.t006

arises as to why this effect was only seen in those >15 years of age. This could be due to the fact that *Ascaris* infection is lower in older age and therefore more easily cleared. It has been suggested that *Ascaris* is the spesies of helminth that has the most effect on malarial parasitemia and diseases.[6] Therefore by removing *Ascaris* in older age, we might be seeing a more profound effect on malarial parasitemia.

Using PCR, which enables detection of sub-microscopic infections at species level, it was also concluded that albendazole did not affect overall malarial parasitemia. When malaria species were analyzed separately, the percentage of subjects infected with *P. falciparum* but not with *P. vivax* increased significantly in the first 9 months post-treatment in the albendazole-treated arm, which is contrary to our hypothesis that anthelminthic treatment would reduce prevalence of malarial parasitemia.[32] It was expected that by decreasing STH, the immune hyporesponsiveness would be reversed and this would be associated with stronger immune effector responses to malaria parasites. One of the possible explanations for the enhanced malarial parasitemia would be that with a reduction in STH, there is increased nutrient availability for other co infections and their growth.

It has been suggested that there are different malaria outcomes with different species of helminths; *Ascaris* being associated with protection regarding parasitemia and severity of malaria while hookworm with higher incidence of malaria.[6] Our study was not powered to conduct a stratified analysis, and with the overall gradual decrease in malaria in the study area during our study, the numbers of subjects positive for malaria parasites are too few for an ad hoc analysis.

The findings concerning allergy outcomes, although not significant, are in line with our hypothesis that anthelminthic treatment would increase SPT reactivity. The risk of SPT reactivity increased incrementally with longer treatment and raises the question whether even longer deworming periods are needed for more pronounced effects on allergic outcomes. In support of this, a recent study reported that 15–17 years of ivermectin treatment for onchocerciasis control in Ecuador led to a significant increase in SPT reactivity to allergens.[12] In the same country, one year of anthelmintic treatment in schoolchildren did not lead to any change in SPT.[10] The question whether different species of helminths might affect allergic outcomes to a different degree, remains unanswered. It is interesting that a one year anthelmintic treatment in Vietnam where hookworm infection was the prominent species, as in our study, resulted in a significant increase in SPT positivity in schoolchildren. This is in contrast to what was seen in Ecuador where *Ascaris lumbricoides* was the most prevalent species. One common feature of the anthelmintic trials seems to be that clinical symptoms of allergy do not change with deworming. However, an important trial in pregnant women in Uganda has shown an increased risk of infantile eczema in infants of mothers treated with anthelminthics compared to those that received placebo.[33] This could indicate that exposure to worms in early life, might affect allergic outcomes more profoundly than when helminths are removed later in life.[34]

One of the limitations of this trial is the overall decrease in malarial parasitemia during the two year study period, most probably caused by actively referring subjects with malaria-like symptoms to puskesmas. Therefore further studies in areas highly endemic for malaria are needed. Treatment in the trial did result in a significant reduction in percentage of subjects infected with STH, but this reduction was incomplete. It is therefore possible that the community was insufficiently dewormed. However, our primary aim was to measure the possible effect of deworming programmes on malaria or allergy. We conclude that despite transient increase in malarial parasitemia as a result of albendazole treatment, there were no clinically relevant changes to outcome measures 21 months after treatment was initiated.

In conclusion, an extremely intensive anthelminthic treatment in a community where STH are highly endemic, does not lead to elimination but reduces both prevalence and intensity of helminths. Such MDA regiment appears safe and does not lead to any significant change with respect to malaria infections or allergies. However, it is worrying that such vigorous community treatment does not have a more pronounced effect on STH burden. Better integrated control strategies would be needed to deworm and subsequently assess whether the risk for malaria infections or allergies change.

Supporting Information

Appendix S1 Here we describe additional details on methods: study area and procedure, data collection on clinical symptoms, and detailed description of statistical models used. We provide tables on the effect of three monthly albendazole treatment on helminth infections, on reported fever and malaria-like symptoms, on reported clinical symptoms of allergy, and on body mass index. In addition, trial profiles of the separate outcomes are provided: malarial parasitemia, skin prick test, and helminth infection.
(DOC)

Checklist S1 CONSORT Checklist.
(DOC)

Protocol S1 Trial Protocol.
(DOC)

Acknowledgments

The authors thank the staff from Puskesmas Nangapanda, Ende health authorities, the community field workers and most of all the study participants from Nangapanda and Anaranda, Flores, Indonesia. We also thank Serge Versteeg, from Amsterdam Medical Center, for IgE measurements and Paul van Rijn from HAL Allergy for providing the study with the allergens used in skin prick testing.

Author Contributions

Contributed to the setting up the field study: GTB IS. Led the work on PCR detection of parasites: JJV LL. Contributed to parasitological investigation: MMMK. Did the randomization: IA. Contributed to safeguarding randomization codes and privacy of the study subjects: HW. Contributed to the setting up of the data base and monitoring: BL LM. Advised the allergy study: SW. Conceived and designed the experiments: MY TS. Performed the experiments: AEW FH LJW. Analyzed the data: MY JJH RT AJFL AEW FH LJW LM YD ES TS. Contributed reagents/materials/analysis tools: RR RS. Wrote the paper: MY JJH AJMC AJFL AEW FH LJW LM YD ES TS.

References

1. Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, et al. (2006) Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet 367: 1521–1532.
2. Utzinger J (2012) A research and development agenda for the control and elimination of human helminthiases. PLoS Negl Trop Dis 6: e1646.
3. Lustigman S, Prichard RK, Gazzinelli A, Grant WN, Boatin BA, et al. (2012) A research agenda for helminth diseases of humans: the problem of helminthiases. PLoS Negl Trop Dis 6: e1582.
4. Allen JE, Maizels RM (2011) Diversity and dialogue in immunity to helminths. Nat Rev Immunol 11: 375–388.

5. Maizels RM, Yazdanbakhsh M (2003) Immune regulation by helminth parasites: cellular and molecular mechanisms. Nat Rev Immunol 3: 733–744.
6. Nacher M (2011) Interactions between worms and malaria: Good worms or bad worms? Malar J 10: 259.
7. Bach JF (2002) The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med 347: 911–920.
8. Feary J, Britton J, Leonardi-Bee J (2011) Atopy and current intestinal parasite infection: a systematic review and meta-analysis. Allergy 66: 569–578.
9. Flohr C, Quinnell RJ, Britton J (2009) Do helminth parasites protect against atopy and allergic disease? Clin Exp Allergy 39: 20–32.
10. Cooper PJ, Chico ME, Vaca MG, Moncayo AL, Bland JM, et al. (2006) Effect of albendazole treatments on the prevalence of atopy in children living in communities endemic for geohelminth parasites: a cluster-randomised trial. Lancet 367: 1598–1603.
11. Flohr C, Tuyen LN, Quinnell RJ, Lewis S, Minh TT, et al. (2010) Reduced helminth burden increases allergen skin sensitization but not clinical allergy: a randomized, double-blind, placebo-controlled trial in Vietnam. Clin Exp Allergy 40: 131–142.
12. Endara P, Vaca M, Chico ME, Erazo S, Oviedo G, et al. (2010) Long-term periodic anthelmintic treatments are associated with increased allergen skin reactivity. Clin Exp Allergy 40: 1669–1677.
13. Lau S, Matricardi PM (2006) Worms, asthma, and the hygiene hypothesis. Lancet 367: 1556–1558.
14. Hamid F, Wiria AE, Wammes LJ, Kaisar MM, Lell B, et al. (2011) A longitudinal study of allergy and intestinal helminth infections in semi urban and rural areas of Flores, Indonesia (ImmunoSPIN Study). BMC Infect Dis 11: 83.
15. Wiria AE, Prasetyani MA, Hamid F, Wammes LJ, Lell B, et al. (2010) Does treatment of intestinal helminth infections influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study). BMC Infect Dis 10: 77.
16. Harijanto PN (2010) Malaria treatment by using artemisinin in Indonesia. Acta Med Indones 42: 51–56.
17. Elyazar IR, Hay SI, Baird JK (2011) Malaria distribution, prevalence, drug resistance and control in Indonesia. Adv Parasitol 74: 41–175.
18. Szefler SJ (2008) Advances in pediatric asthma in 2007. J Allergy Clin Immunol 121: 614–619.
19. World Health Organization (2010) Guidelines for the treatment of malaria, 2nd ed. World Health Organization. Available: http://whqlibdoc.who.int/publications/2010/9789241547925_eng.pdf.Accessed 2012 August 1.
20. Adegnika AA, Verweij JJ, Agnandji ST, Chai SK, Breitling LP, et al. (2006) Microscopic and sub-microscopic Plasmodium falciparum infection, but not inflammation caused by infection, is associated with low birth weight. Am J Trop Med Hyg 75: 798–803.
21. WHO Multicentre Growth Reference Study Group (2006) WHO Child Growth Standards: Length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age: Methods and development. Geneva:World Health Organization 312.
22. Laird NM, Ware JH (1982) Random-effects models for longitudinal data. Biometrics 38: 963–974.
23. R-forge website. Available: http://lme4.r-forge.r-project.org/. Accessed 2011 Oct 24.
24. Keiser J, Utzinger J (2008) Efficacy of current drugs against soil-transmitted helminth infections: systematic review and meta-analysis. JAMA 299: 1937–1948.
25. Alderman H, Konde-Lule J, Sebuliba I, Bundy D, Hall A (2006) Effect on weight gain of routinely giving albendazole to preschool children during child health days in Uganda: cluster randomised controlled trial. BMJ 333: 122.
26. Dickson R, Awasthi S, Williamson P, Demellweek C, Garner P (2000) Effects of treatment for intestinal helminth infection on growth and cognitive performance in children: systematic review of randomised trials. BMJ 320: 1697–1701.
27. Taylor-Robinson DC, Maayan N, Soares-Weiser K, Donegan S, Garner P (2012) Deworming drugs for soil-transmitted intestinal worms in children: effects on nutritional indicators, haemoglobin and school performance. Cochrane Database Syst Rev 7: CD000371.
28. Prichard RK, Basanez MG, Boatin BA, McCarthy JS, Garcia HH, et al. (2012) A research agenda for helminth diseases of humans: intervention for control and elimination. PLoS Negl Trop Dis 6: e1549.
29. Brutus L, Watier L, Hanitrasoamampionona V, Razanatsoarilala H, Cot M (2007) Confirmation of the protective effect of Ascaris lumbricoides on Plasmodium falciparum infection: results of a randomized trial in Madagascar. Am J Trop Med Hyg 77: 1091–1095.
30. Kirwan P, Jackson AL, Asaolu SO, Molloy SF, Abiona TC, et al. (2010) Impact of repeated four-monthly anthelmintic treatment on Plasmodium infection in preschool children: a double-blind placebo-controlled randomized trial. BMC Infect Dis 10: 277.
31. Webb EL, Mawa PA, Ndibazza J, Kizito D, Namatovu A, et al. (2011) Effect of single-dose anthelmintic treatment during pregnancy on an infant's response to immunisation and on susceptibility to infectious diseases in infancy: a randomised, double-blind, placebo-controlled trial. Lancet 377: 52–62.
32. Specht S, Hoerauf A (2007) Does helminth elimination promote or prevent malaria? Lancet 369: 446–447.
33. Mpairwe H, Webb EL, Muhangi L, Ndibazza J, Akishule D, et al. (2011) Anthelminthic treatment during pregnancy is associated with increased risk of infantile eczema: randomised-controlled trial results. Pediatr Allergy Immunol 22: 305–312.
34. Djuardi Y, Wammes LJ, Supali T, Sartono E, Yazdanbakhsh M (2011) Immunological footprint: the development of a child's immune system in environments rich in microorganisms and parasites. Parasitology 138: 1508–1518.

Supplementary Appendix S1

Supplementary Methods

Additional information on the study area and procedures

Ende district, an area highly endemic for STH, is situated near the equator (8°45'S, 121°40'E) and it is characterized by a uniform high temperature, in the range of 23-33.5 °C, with humidity of 86-95%. Average yearly rain fall is 1.822 mm with about 82 rainy days, especially from November to April, with the peak in December until March. The semi-urban village of Nangapanda, endemic for malaria, had a population of 3583 and is located in the coastal area with most villagers being farmers and fishermen with some government officers or private sector employees. The rural village Anaranda had 1631 inhabitants and is located 80 km further inland of Nangapanda. There was poor infrastructure and inhabitants generated income mainly from farming.

Regarding the availability of the anthelminthics in the community, there was no deworming campaign in this area during the study period. Pyrantel pamoat (Combantrin®) and dehydropiperazine (Bintang 7 puyer 17®) were the only available anthelminthics in the market. The local primary health centre (Puskesmas) did not provide the current trial study participants by any anthelminthic treatment but referred them to the trial team.

Malaria control, such as by artemisinin-combination therapy (ACT) treatment and insecticide-treated nets (ITN) or long-lasting insecticide-treated nets (LLIN) although planned, were not implemented yet during our study period. This was due to several difficulties faced in some parts of Indonesia, such as instable drug supply, lack of training on definitive diagnosis of malaria by the laboratory staff, as well as insufficient bednet supply and poor compliance (17). Malaria drugs such as chloroquine and quinine were available in the shops, however, little information is available on proper self medication. Therefore, before and during the study period, regular training of field workers was undertaken on how to prevent malaria (use of repellent and bednet, irrigation of breeding places) and how to treat malaria (not to self medicate but to visit puskesmas for diagnosis and treatment). Indoor residual spraying was done by the local health authority for dengue control against an outbreak at the beginning of 2008.

The treatment of suspected malaria cases at the puskesmas was chloroquine and primaquine for *P. vivax,* while for *P. falciparum* sulfadoxine/pyrimethamine was commonly used. Subjects in our study with fever and/or any one of the malaria-like symptoms (see below for detailed description) were referred to the puskesmas for assessment and treatment according to local health center policy.

The anthelmintic treatment and placebo were coded and the code was concealed from trial investigators and participants. The tablets were distributed by trained health workers and the intake was directly observed. Labels with the study subject ID were printed from a computer database and attached to the appropriate strip of treatment by a separate team located in Jakarta without the involvement of the study investigators. In order to assess whether anthelminthic treatment had any adverse effect on the growth of children or on the incidence of allergy, interim analyses were done at one year post-treatment by a monitoring committee. After completion of the study the whole population was treated with albendazole (a single dose of 400mg for three consecutive days).

The malaria slides were read by microscopy at the Department of Parasitology in Jakarta. The quality control for microscopic reading took place in the pilot phase of the project. In cooperation

with NAMRU-2 (US Naval Medical Research Unit-2) two microscopists from our team were trained, inter-observer differences were assessed and following satisfactory training they were certified. At the pilot phase, and throughout the study, PCR was used to monitor the microscopy data with a high degree of agreement between microscopy and PCR. In a random sub-sample at 9 months and 21 months post-treatment we measured malarial parasitemia by PCR.

Primers and the *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*-specific probes were used with some modifications in the fluorophore- and quencher-chemistry. Amplification reactions of each DNA sample are performed in white PCR plates, in a volume of 25 µl with PCR buffer (HotstarTaq master mix), 5 mmol/l MgCl2, 12.5 pmol of each Plasmodium-specific primer and 15 pmol of each PhHV-1-specific primer, 1.5 pmol of each *P. falciparum*, *P. vivax*, *P. malariae*-specific XS- probes, and PhHV-1-specific Cy5 double-labelled detection probe, and 2.5 pmol of each *P. ovale*-specific XS-probes (Biolegio), and 5 µl of the DNA sample were used. Amplification consists of 15 min at 95ºC followed by 50 cycles of 15 s at 95ºC, 30 s at 60ºC, and 30 s at 72ºC. Amplification, detection, and analysis are performed with the CFX96 real-time detection system (Bio-Rad laboratories). The PCR output from this system consists of a cycle-threshold (Ct) value, representing the amplification cycle in which the level of fluorescent signal exceeds the background fluorescence, and reflecting the parasite-specific DNA load in the sample tested. Negative and positive control samples are included in each amplification run.

Stool samples were collected and preserved in 4% formaldehyde for microscopy examination or frozen (-20°C) unpreserved for PCR detection. The formol-ether acetate concentration method was performed on the formalin preserved stool samples followed by microscopic examination for *Trichuris trichiura* infections. As described in detail before(15), DNA was isolated from approximately 100 mg unpreserved feces and examined for the presence of *Ancylostoma duodenale, Necator americanus, Ascaris lumbricoides* and *Strongyloides stercoralis* DNA by the multiplex qPCR. The qPCR output from this system consisted of a Ct value; negative and positive control samples were included in each run of the amplification. Positive Ct- values were grouped into three categories: Ct<30.0, 30.0≤Ct<35.0 and ≥35.0 representing a high, moderate and low DNA load, respectively.

Data collection on clinical symptoms

A year before the study enrolment, community workers were recruited and trained in taking finger-prick blood for the three-monthly malarial parasitemia survey in Nangapanda, observing drug intake, recording adverse treatment effects, as well as measuring fever and administering monthly malaria-like symptoms questionnaire. These questionnaires were based on WHO definitions (19) and were assessed in all individuals that were present at the time of the survey. Subjects with fever (≥37.5°C) or additional malaria-like symptoms (headache, fatigue and nausea) at the time of visits were referred to the puskesmas for treatment according to local standard protocols. The monthly data on fever (≥37.5, using digital thermometer) and additional malaria-like symptoms were collected at baseline September 2008 and in the months Oct 08, Nov 08, Dec 08, Jan 09, Feb 90, March 09, Apr 09, May 09, June 09, Aug 09, Sept 09, Oct 09, Nov 09, Dec 09, Jan 10, Feb 10, March 10 and Apr 10. At baseline, 1396 individuals were assessed in placebo and 1381 in the albendazole arm and at the last timepoint, 1165 and 1181 subjects were followed up in the two groups, respectively. Questionnaire data were available for all timepoints from 45.8% and

47.2% of placebo and albendazole group whereas data for 80% of the timepoints were available from 83.8% and 87.6% of the two groups, respectively. The number of events was recorded in total of 15259 and 15307 person months at risk for placebo and albendazole groups, respectively.

The modified video-assisted (for asthma symptoms) and illustration-assisted (for atopic dermatitis) ISAAC questionnaire, translated to Bahasa Indonesia and back translated for use in our studies within the EU funded project GLOFAL (www.glofal.org), were administered at baseline and at 21 month timepoints. Data were available from 629 in placebo and 635 in albendazole arm at baseline, while these numbers were 460 and 445, respectively, at the 21 month timepoint. These questionnaires were administered to the parents/guardians of subjects who were skin prick tested with allergens: the trial profile is given in supplementary figure 1B. The prevalence of asthma symptoms were obtained from the following questions: (i) has your child ever had asthma? (ii) has your child ever been diagnosed for asthma by a doctor? and (iii) has your child in the past 12 months had wheezing or whistling in the chest?; while the prevalence of atopic dermatitis was obtained from the questions: (i) has your child ever had doctor/paramedic diagnosed allergic eczema and (ii) has your child ever had one or more skin problems accompanied by an itchy rash?

If the answer to one or more of these questions was positive, the subjects were considered to have either asthma or atopic dermatitis symptoms.

Detailed description of the statistical models used

Descriptives were computed for each variable (mean and standard deviation or median and interquartile range for continuous outcomes, numbers and percentages for categorical variables). For children ≤ 19 years, BMI age-standardized z-scores were calculated according to WHO references (21).

Two sources of correlation among observations should be accounted for when modeling these data, namely observations at various timepoints for a subject are correlated due to subject specific effects and observations within households are correlated due to environmental effects shared within households. To model these correlations we used random effects. For subject effects a random intercept and a random slope were used, i.e. each subject has its own intercept and slope, where the latter models the change of the outcome variable over time. Observations within a household also have a shared random intercept. Thus the intercept for an observation of a specific subject from a specific household is the overall mean plus the subject specific effect plus the household effect. By doing so correlation among observations of the same household was modeled since these observations share the same household effect. To assess treatment effects generalized linear mixed models (22) were used where the term "mixed" corresponds to the used random effects. Unless stated otherwise all models included area as covariate in the model to take into account the differences between the two villages. Generalized linear mixed models provide a flexible and powerful tool to derive valid inference while capturing the data correlations induced by clustering within households and repeated evaluations in time of the same subject.

For continuous outcome variables which were measured at 0, 9 and 21 months, treatment effects were modeled at timepoint 9 and 21 months, because treatment started at 0 months and the design is a randomized trial no treatment effect should be present at time 0. We allowed

for different treatment effects at 9 and 21 months. Beta's and 95% confidence intervals are provided for 9 and 21 months. The betas represent the mean difference between the placebo and treatment group. An overall test for treatment effect over time was performed by using a likelihood ratio test which compares the model with and without the treatment effect (2 df test).

For binary outcome variables measured at 9 and 21 months, the logit link was used (mixed effect logistic regression). In these models only the two random intercepts were included and the random subject specific slope was omitted. Odds ratios and 95% confidence intervals are reported. Analogously to continuous outcome variables two degrees of freedom likelihood ratio tests were performed to assess treatment effects over time. Note that the model based odds ratios are different from crude odds ratios directly computed from the sample due to missing observations and due to the presence of random effects and the covariate area in the model. Malarial parasitemia by microscopy was measured at a three monthly basis. To model these data, similar models were used. Specifically at each of the seven timepoints (excluding time zero) a treatment effect was included. The likelihood ratio test for treatment effect over time has therefore 7 degrees of freedom. All generalized linear mixed models were fitted using the lme4 package (Douglas Bates, Martin Maechler and Ben (2011). lme4: Linear mixed-effects models using S4 classes. R package version 0.999375-42. http://CRAN.R-project.org/package=lme4) in R (23).

For each malaria-like symptom (fever, headache, fatigue, and nausea), total number of events and person months were computed for each treatment group. We calculated incidence rates for all events. Symptom episodes within three months of an initial presentation with the same symptom were regarded as part of the same episode. Hazard ratios for effect of treatment were calculated with Cox regression with robust SE to allow for within-subject and within household clustering (STATA 12).

Supplementary Tables

Table S1. Effect of three-monthly albendazole treatment on helminth infection

	Placebo n/N (%)	Albendazole n/N (%)	OR (95% CI)
Helminth infection (any spp)			
9 months	395/477 (82.8)	247/480 (51.4)	0·07 (0·04-0·11)
21 months	353/448 (78.8)	172/411 (41.9)	0·05 (0·03-0·08)
Hookworm[1]			
9 months	359/524 (68.5)	161/508 (31.7)	0·02 (0·01-0·04)
21 months	305/466 (65.5)	99/423 (23.4)	0·01 (0·01-0·03)
A. lumbricoides[1]			
9 months	174/524 (33.2)	65/508 (12.8)	0·24 (0·16-0·36)
21 months	140/466 (30.0)	41/423 (9.7)	0·18 (0·11-0·29)
T. trichiura[2]			
9 months	219/726 (30.2)	160/673 (23.8)	0·58 (0·42-0·80)
21 months	177/633 (28.0)	101/571 (17.7)	0·40 (0·28-0·58)

The number of positives (n) of the total population examined (N).[1]diagnosed by PCR. [2]diagnosed by microscopy. Odds ratio and 95% confidence interval based on logistic mixed models. The p-values are generated from the modeled data for the combined effect of albendazole treatment over time, which were significant (P < 0.001) for any helminth and for each of the species separately.

Table S2. The effect of albendazole on fever and additional malaria like symptoms

	Placebo		Albendazole			
	Events (PM)	Incidence per PM	Events (PM)	Incidence per PM	Unadjusted IRR	Adjusted IRR
Fever	414 (18494)	0.02	429 (18636)	0.02	1.03	1.03
Headache	333 (19067)	0.02	340 (19563)	0.02	1.00	1.00
Fatigue	49 (22362)	0.002	69 (22535)	0.003	1.39	1.41
Nausea	76 (21749)	0.003	55 (22211)	0.002	0.71	0.71
Any symptom	661 (15259)	0.04	690 (15307)	0.05	1.04	1.04

IRR: incidence rate ratio
PM: Person months
Adjusted with age and sex
The p-values are generated from Cox regression of albendazole treatment over time with robust SEs to allow for within-subject and within household clustering and no significant effects were found (P>0.05).

Table S3. Reported clinical symptoms of allergy

	Placebo n/N (%)	Albendazole n/N (%)	OR (95% CI)
Asthma			
21 months	8/461 (1.7)	11/445 (2.5)	1.11 (0.07-17.26)
Atopic dermatitis			
21 months	13/461 (2.8)	9/445 (2.0)	0.57 (0.16-2.02)

The number of positives (n) of the total population examined (N).The p-values are generated from the modeled data for the effect of albendazole treatment after 21 months and no significant effects were found (P>0.05).
At baseline 8/692 (1.2%) and 18/692 (2.6%) in the placebo group reported symptoms of asthma and atopic dermatitis, respectively, while in Albendazole this was 10/635 (1.6%) and 11/635 (1.7%).

Table S4. Effect of three-monthly albendazole treatment on BMI

	Placebo N (Median, IQR)	Albendazole N (Median, IQR)	β (95% CI)
BMI			
9 months	498 (22.42, 19.91 - 25.54)	499 (22.07, 19.96 - 24.56)	-0·10 (-0·29-0·09)
21 months	430 (22.42, 19.68 - 25.56)	425 (21.56, 19.44 - 24.12)	-0·15 (-0·39-0·10)
z-BMI			
9 months	346 (-0.81, -1.44 - -0.13)	334 (-0.96, -1.56 - -0.30)	-0·04 (-0·17-0·09)
21 months	272 (-1.29, -2.21 - -0.56)	269 (-1.57, -2.32 - -0.74)	-0·07 (-0·23-0·10)

The total population examined (N). IQR = Interquartile range. β (beta) and 95% confidence interval based on generalized linear mixed models. The p-values are generated from the modeled data for the combined effect of albendazole treatment over time and no significant effects were found (P>0.05). Baseline data are shown in Table 1 of the manuscript.

Supplementary Figures

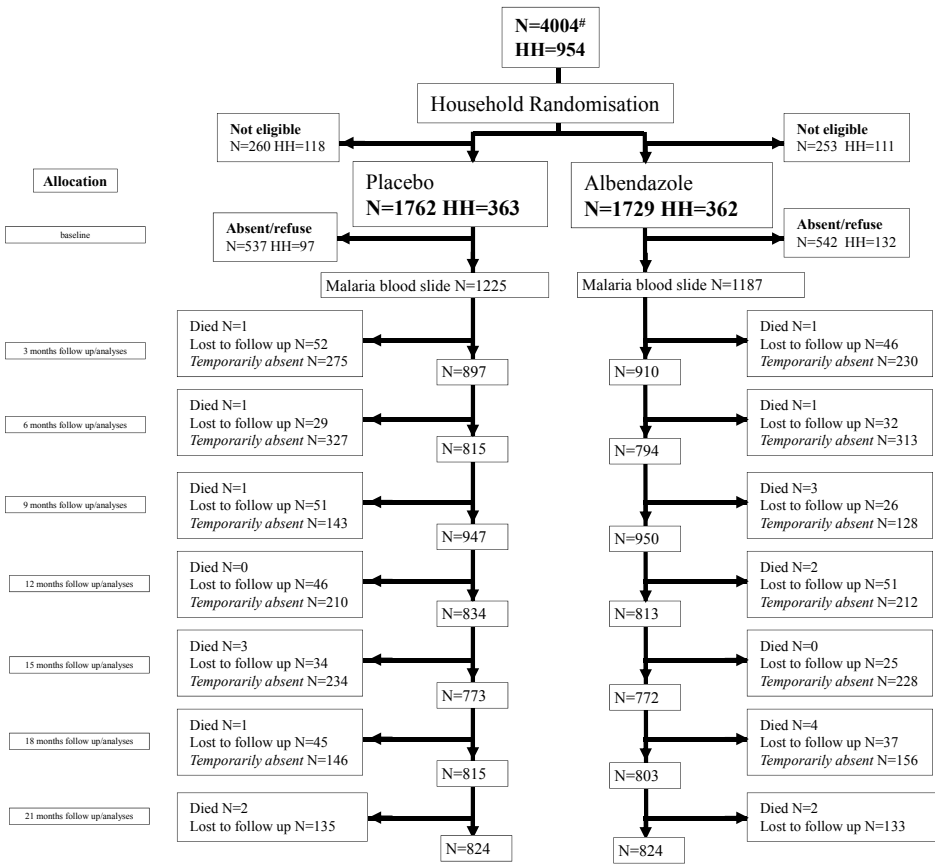


Figure S1A. Profile of trial with malarial parasitemia as outcome in the village of Nangapanda where malaria is endemic

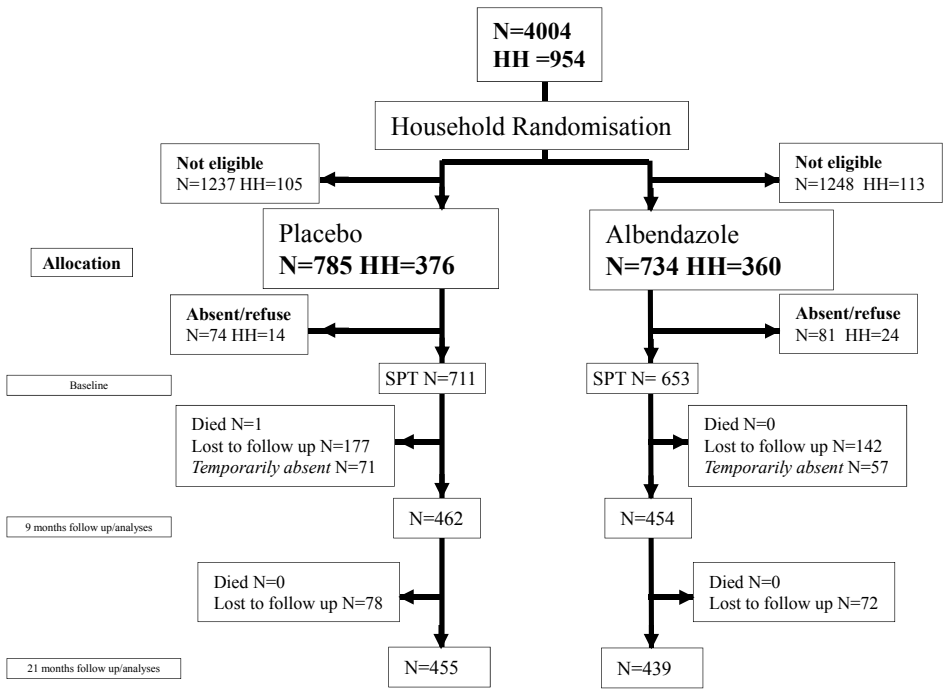


Figure S1B. Profile of trial with skin prick test (SPT) reactivity as outcome in children 5-15 years of age in both Nangapanda and Anaranda

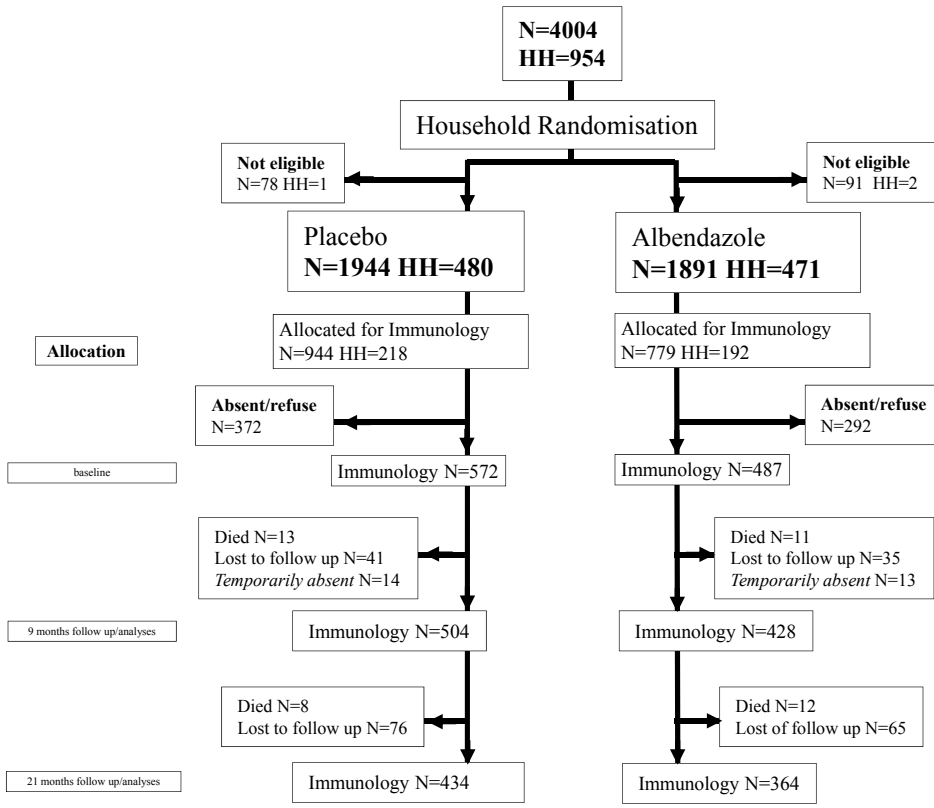


Figure S1C. Profile of trial with helminth infection as outcome in villages of Nangapanda and Anaranda

chapter 4

Three-monthly albendazole treatment alleviates geohelminth-induced immune hyporesponsiveness; results of a double blind placebo-controlled household-randomized trial

Abstract

Background Chronic helminth infections are proposed to induce cellular immune hyporesponsiveness, which secures their long-term survival in their host, but which also may affect immune responses to unrelated antigens. As there are several other causes of immunosuppression, we conducted a household-clustered RCT to evaluate the specific effect of deworming on cellular immune responses in an helminth-endemic area in Indonesia.

Methods Cytokine (IL-2, IL-5, IL-10, IFN-γ and TNF) responses to antigens and mitogens were assessed in 1059 subjects at baseline, 9 and 21 months after three-monthly treatment with either albendazole or placebo.

Results This intensive treatment resulted in significant increase in malaria-specific and mitogen-induced TNF and IFN-γ responses. This effect was not associated with changes in cell counts or BMI.

Conclusions These findings establish unequivocally that helminth infections suppress pro-inflammatory responses, which may help to understand the possible protective effect of helminths on inflammatory diseases in rural areas of the world.

Trial registration: http://www.controlled-trials.com/ISRCTN83830814

Linda J Wammes[1,2,*], Firdaus Hamid[1,2,3,*], Aprilianto E Wiria[1,2,*], Linda May[1], Maria MM Kaisar[1,2], Margaretta A Prasetyani[1,2], Yenny Djuardi[1,2], Iwan Ariawan[4], Heri Wibowo[2], Yvonne CM Kruize[1], Heni Suryani[2], Jaco J Verweij[1], Roula Tsonaka[7], Jeanine J Houwing-Duistermaat[7], Adrian JF Luty[8], Erliyani Sartono[1], Taniawati Supali[2#], Maria Yazdanbakhsh[1#]

*these authors contributed equally

1. Dept Parasitology, Leiden University Medical Center, Leiden, the Netherlands
2. Dept Parasitology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia
3. Dept Microbiology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia
4. Dept Biostatistics, School of Public Health, University of Indonesia, Jakarta, Indonesia
5. Medical Research Unit, Albert Schweitzer Hospital, Lambaréné, Gabon
6. Dept Parasitology, Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany
7. Dept Medical Statistics and Bioinformatics, Leiden University Medical Center, Leiden, the Netherlands
8. Institut de Recherche pour le Développement, UMR 216 Mère et enfant face aux infections tropicales, Paris, France

– Manuscript is submitted –

Introduction

Infection with soil-transmitted helminths (STH) is the most common infectious disease worldwide and affects mostly inhabitants of rural areas in low- and middle-income countries (1). In addition to causing direct worm-associated morbidities, chronic STH infections may magnify poor health conditions common in communities remote from health care facilities, such as anemia, poor nutritional status, stunting and possibly poor cognitive development (1,2).

An important hallmark of chronic helminth infections is cellular hyporesponsiveness, which is thought to allow the long-term survival of these parasites within their host (3,4). Although unresponsiveness in lymphocyte proliferation was already described in the 1970s for individuals with *Schistosoma mansoni* infection or bancroftian filariasis (5,6), the evidence for this has not moved beyond animal models and cross-sectional studies in humans (reviewed by Danilowicz-Luebert et al. (7)). An important drawback to the cross-sectional nature of these studies is that other factors, which are also associated with immune suppression, could bias the results. Individuals infected with helminths may be in a poor nutritional state and shortage of proteins or amino acids can interfere with expression of immune effector molecules. Malnutrition has been specifically associated with decreased cell-mediated immunity, exemplified by atrophy of the thymus and other lymphoid tissues leading to lower T-cell numbers and reactivity (8). It is also known that other microorganisms and parasites can be associated with immune suppression or T-cell exhaustion (9) and therefore coinfections could act as confounders (10).

The consequences of immunosuppression are manifold and could be of major public health importance. Immune hyporesponsiveness, in the presence of helminth infections, can affect responses to unrelated antigens, it could curtail the development of effective immune responses to incoming protozoan, bacterial or viral infections, thereby increasing susceptibility to these pathogens. Similarly, vaccination studies have shown suboptimal responses to childhood vaccinations in subjects infected with STH (11,12). On the other hand, the dampened immune responses associated with helminths might help to prevent immune-induced pathology during coinfections and, possibly, overt reactivity to self- or environmental antigens (13).

A few longitudinal studies have been undertaken to assess the effect of anthelminthic treatment on cellular immune responsiveness, however these were in small number of subjects or specifically targeted children (14-16). Conversely, clinical trials that have experimentally infected humans have mostly not evaluated cellular immune responses and have all been conducted in adults (17,18). Moreover, therapeutic infections are often not long enough to establish a chronic infection, which could be important for the gradual onset of hyporesponsiveness. So far there are no large-scale community-based intervention studies that show helminth infections lead to immune hyporesponsiveness in man.

To disentangle the impact of helminths on the immune system from other influences, we conducted a randomized double blind placebo-controlled trial of three-monthly single dose albendazole treatment in an area where STH are highly endemic. Here we present the results of our trial; the effect of anthelminthic treatment on the peripheral blood cytokine responses of a community in Flores island, Indonesia.

Methods

Study design

This report describes a nested study within the ImmunoSPIN trial (19,20). The trial was conducted in two villages in Ende district, Flores island, Indonesia. The coastal village Nangapanda is located around the main road of Flores and can be characterized as semi-urban, based on the location and the presence of a primary healthcare centre. Anaranda village is located 80 km north of Nangapanda and is more remote from roads, health centres and other facilities. In 2008 the double blind placebo-controlled trial of two year duration was initiated by randomizing all households in the two villages to receive either a single dose of 400 mg albendazole or a matching placebo every three-months over a two year study period (tablets from PT Indofarma Pharmaceutical, Bandung, Indonesia). Treatment allocation was based on household to minimise the risk cross-contamination and therefore reinfection of treated individuals. Treatment was provided to all household members older than two years of age, except for pregnant women (according to Indonesian national guidelines), and intake was observed by field workers. The study was approved by the Ethical Committee of the Medical Faculty, University of Indonesia, Jakarta (ref: 194/PT02.FK/Etik/2006) and has been filed by the ethics committee of the Leiden University Medical Center, the Netherlands. The trial was registered as clinical trial (ISRCTN83830814). Informed consent or parental consent was obtained from all participants.

Study population

The randomisation for the total study was based on 954 households in the two villages, comprising of 4004 individuals, resulting in 2022 (481 houses) and 1982 (473 houses) subjects in placebo and albendazole group, respectively. For the immunological component of this study in Nangapanda, aiming at 1000 participants, 250 households were randomly selected and individuals older than 4 years of age were invited for morning venous blood sampling and assessment of anthropometric parameters. This resulted in the inclusion of 882 individuals from the semi-urban area, of which 858 provided sufficient blood samples for whole blood cultures. In the rural area Anaranda, only children were included since this area was included for our allergy studies (19). 250 children were randomly selected from the total population and children from the same households were also included, leading to a total number of 295 children with whole blood cultures. After exclusion of cytokine data from wells that were suspected of being infected (see below), the number of subjects included at baseline was 839 and 220 for the two respective areas, corresponding to 572 placebo- and 487 albendazole-treated individuals.

Whole blood culture and cytokine measurements

Heparinized blood was diluted 1:4 with RPMI 1640 medium (Invitrogen, Breda, the Netherlands) and cultured in 96-well round-bottomed plates. Cultures were stimulated for 24h to assess innate responses (to lipopolysaccharide (LPS) from *E. coli*, Sigma-Aldrich, Zwijndrecht, the Netherlands), and for 72h to detect adaptive responses (to *Ascaris lumbricoides* antigen, *Plasmodium falciparum*-parasitized red blood cells (PfRBC), uninfected RBC (uRBC) and phytohaemagglutinin (PHA, Wellcome Diagnostics, Darford, UK)) and at each time point unstimulated control wells were included. PfRBC and uRBC were kindly provided by professor Sauerwein from Radboud University Medical Center Nijmegen, the Netherlands and were only used in the semi-urban

area, since the rural area was not endemic for malaria. The cultures were carried out in the field laboratory in Nangapanda and the supernatants were kept at -20°C and transported to Jakarta. There cytokine responses were quantified using Luminex cytokine kits (Biosource, Camarillo, CA, USA) and run on a Liquichip 200® Workstation (Qiagen, Venlo, The Netherlands) equipped with Liquichip analyzer software (Qiagen, Venlo, The Netherlands). TNF and IL-10 were assessed in 24h supernatants and TNF, IFN-γ, IL-2, IL-5 and IL-10 in 72h supernatants. Samples with TNF levels ≥250 pg/mL in unstimulated blood were excluded from the analyses as they are considered unreliable with respect to possible infection in the culture. This value was derived from the data distribution, which indicated outliers to be identified with this cut-off. Cytokine levels that fell below the assay's detectable range were replaced by half of the detection limit.

Stool examination by microscopy and PCR

In order to examine the effect of treatment on helminth prevalence, yearly stool samples were collected. *T. trichiura* was detected by microscopy after formol-ether concentration and 18S-based multiplex real-time PCR was used for the specific amplification and detection of hookworm (*Ancylostoma duodenale*, *Necator americanus)*, *A. lumbricoides*, and *Strongyloides stercoralis* DNA, as described previously (20).

Complete blood counts

Complete blood counts (CBC) and differential counts before and one year after treatment were determined using heparinized blood on a routine cell counter (Coulter® Ac-T™ diff Analyzer, Beckman Coulter Inc., Fullerton, CA, USA), while CBC 2 years after treatment were determined using heparinized and EDTA blood on Sysmex KX-21N hematology analyzer (PT Sysmex Indonesia, Jakarta, Indonesia). Since both heparinized and EDTA blood samples were used at the last time point, 325 samples were tested in parallel analysis. All outcomes were highly comparable except for thrombocyte counts, thus the data of all parameters but thrombocyte counts were pooled.

Statistical analysis

The cytokine data were log transformed (log10(concentration+1)) to obtain normally distributed variables. For children ≤19 years, BMI age-standardized z-scores were calculated according to WHO references (21). To assess treatment effects, generalized linear mixed models were used with addition of three random effects, namely a random household-specific intercept to model clustering within households and a random subject-specific intercept and slope to model correlation within subjects. Linear or logistic mixed-effects models (22) were applied for continuous and binary outcomes, respectively. Parameter estimates for treatment effects at 9 and 21 months and 95% confidence intervals are reported. The reported p-values are obtained using likelihood ratio tests by comparing the model with and without the treatment effect. Unless stated otherwise, all outcomes were adjusted for area by using area as covariate in the model. All models were fitted using the lme4 package (23).

Results

Study population

The baseline characteristics of the study participants are shown in table 1. At baseline 88.7% of the individuals were infected with one or more helminth species, with hookworm

Table 1. Baseline characteristics

	N	Placebo	N	Albendazole
Age (mean in years, SD)	572	25.7 (18.5)	487	24.9 (18.4)
Sex (female, n, %)*	572	328 (57.3)	487	279 (57.3)
Area (rural, n, %)*	572	114 (19.9)	487	106 (21.8)
BMI > 19 years old (mean, SD)	264	22.1 (4.1)	220	22.1 (3.8)
Z score of BMI ≤ 19 years old (mean, SD)	194	-1.15 (1.11)	386	-1.14(1.15)
Parasite infection (n, %)*				
Helminth *(any spp)*	322	286 (88.8)	237	210 (88.6)
Hookworm[1]	335	255 (76.1)	245	192 (78.4)
N. americanus[1]	335	252 (75.2)	245	188 (76.7)
A. duodenale[1]	335	25 (7.5)	245	17 (6.9)
A. lumbricoides[1]	335	105 (31.3)	245	80 (32.7)
S. stercoralis[1]	335	3 (0.9)	245	14 (5.7)
T. trichiura[2]	415	106 (25.5)	310	62 (20.0)
Malarial parasitaemia *(any spp)*[2]	567	24 (4.2)	483	24 (5.0)
P. falciparum	567	16 (2.8)	483	11 (2.3)
P. vivax	567	8 (1.4)	483	10 (2.1)
P. malariae	567	0 (0.0)	483	4 (0.8)
Cytokine production, pg/mL [median, IQR]				
LPS				
TNF-α	554	743 [368-1293]	468	769 [339-1318]
IL-10	554	271 [163-441]	468	256 [158-406]
PHA				
TNF-α	516	100 [50-222]	435	103 [50-214]
IL-10	515	76 [41-129]	435	70 [37-116]
IFN-γ	516	1625 [584-3983]	435	1270 [538-4340]
IL-2	516	23 [0-101]	432	23 [0-92]
IL-5	516	563 [309-840]	435	520 [317-829]
iRBC				
TNF-α	299	18 [4-42]	237	14 [3-38]
IL-10	300	10 [5-19]	238	10 [5-20]
IFN-γ	300	163 [75-388]	239	176 [70-376]
IL-2	300	50 [5-125]	239	40 [5-112]
IL-5	300	14 [5-26]	239	12 [4-23]
Ascaris				
TNF-α (pg/mL)	517	5 [0-15]	438	6 [0-14]
IL-10 (pg/mL)	516	7 [2-15]	438	7 [1-14]
IFN-γ (pg/mL)	516	19 [6-47]	441	21 [7-47]
IL-2 (pg/mL)	497	38 [4-114]	426	36 [0-107]
IL-5 (pg/mL)	515	24 [9-68]	440	24 [9-63]

[1]diagnosed by PCR; [2]diagnosed by microscopy.
*The number of positives (n) of the total population examined (N)

infection being the most prevalent (77.1% of total). The consort diagram of the trial is shown in figure 1; follow-up after 9 months was 88% for both groups and 76% for placebo- and 75% for albendazole-treated groups after 21 months, corresponding to a total loss of 138 and 123 individuals respectively. Six subjects died during the study period, which were all above the age of 35, suggesting non-infectious causes of death. The analysis was intention-to-treat, and involved all participants as assigned randomly at the start of the trial. No significant change in BMI was observed over the 2-year study period in children (analysed by zBMI, p=0.70) or in adults (BMI, p=0.45; data not shown).

Effect of albendazole treatment on helminth prevalence

Treatment with albendazole resulted in a reduction in the prevalence of geohelminths both after 9 (51.9% vs. 84.1% for placebo) and after 21 months (39.2% vs. 80% for placebo) (Table S1). Albendazole had the most effect on hookworm (from 78.4% at baseline to 32.6% at 9 months and 21.6% at 21 months post-treatment) compared to placebo (from 76.1% to 70.5% and 67.0% respectively), followed by on *Ascaris* (albendazole from 32.7%, to 13.3% and 12.2%; placebo from 31.3% to 33.5% and 24.2%), while the effect on *Trichuris* was less pronounced (from 20.0% at baseline to 21.0% at 9 months and 16.2% at 21 months post-treatment, compared to placebo from 25.5% to 31.7% and 28.8%). When assessing the intensity of infection in categories based on cycle threshold values of PCR, it was in particular the high-load infections that were greatly diminished in the treatment group (24).

Effect of albendazole treatment on whole blood cytokine responses

In figure 2, we present the effect of treatment on cytokine responses to *Ascaris* antigens, PfRBC and PHA. The model-estimated treatment effects on cytokines at 9 months (figure 2A) and 21 months (figure 2B) are shown. Regarding helminth (*Ascaris*) antigen-specific cytokine responses, IL-2 responses were significantly enhanced by treatment over the study period (p_{time}=0.018), with significantly higher IL2 in the treated group at 9 months (estimate [95% CI]: 0.17 [0.05–0.28], figure 2A). Neither Th1, nor Th2 responses changed with treatment. In response to *P. falciparum* antigens, there was an increase over time in pro inflammatory cytokine TNF, which was highly significant (p_{time}<0.0001), and IFN-γ (p_{time}=0.036), in the albendazole-treated group. As shown in figure 2A, both TNF and IFN-γ were significantly higher than in the placebo treated group at the 9 months time point (0.37 [0.21-0.53] for TNF and 0.14 [0.03-0.24] for IFN-γ). Moreover, the general adaptive response (cytokine production stimulated by PHA), albendazole treatment significantly increased TNF and IL-10 secretion (p_{time}=0.011 and p_{time}=0.003 respectively) over the trial period. Interestingly, for TNF, albendazole treatment resulted in elevated response at 9 months whereas for IL-10 the response was significantly higher at the later 21 months time point (for TNF 0.14 [0.05–0.24], figure 2A; for IL-10 0.12 [0.05–0.19], figure 2B). At 9 months post-treatment, IFN-γ (0.10 [0.01-0.19]) and IL-2 (0.12 [0.01-0.23]) responses were transiently increased (figure 2A) and at 21 months a significant enhancement of IL-5 production (0.10 [0.01-0.19]) was observed (figure 2B), however these alterations were not significant over the whole trial time period (IFN-γ p_{time}=0.076, IL-2 p_{time}=0.11, IL-5 p_{time}=0.068). Albendazole had no effect on immune responses to LPS (overall p-value for TNF p=0.77, for IL-10 p=0.12, data not shown). Analysis of cytokines in unstimulated whole blood cultures revealed no

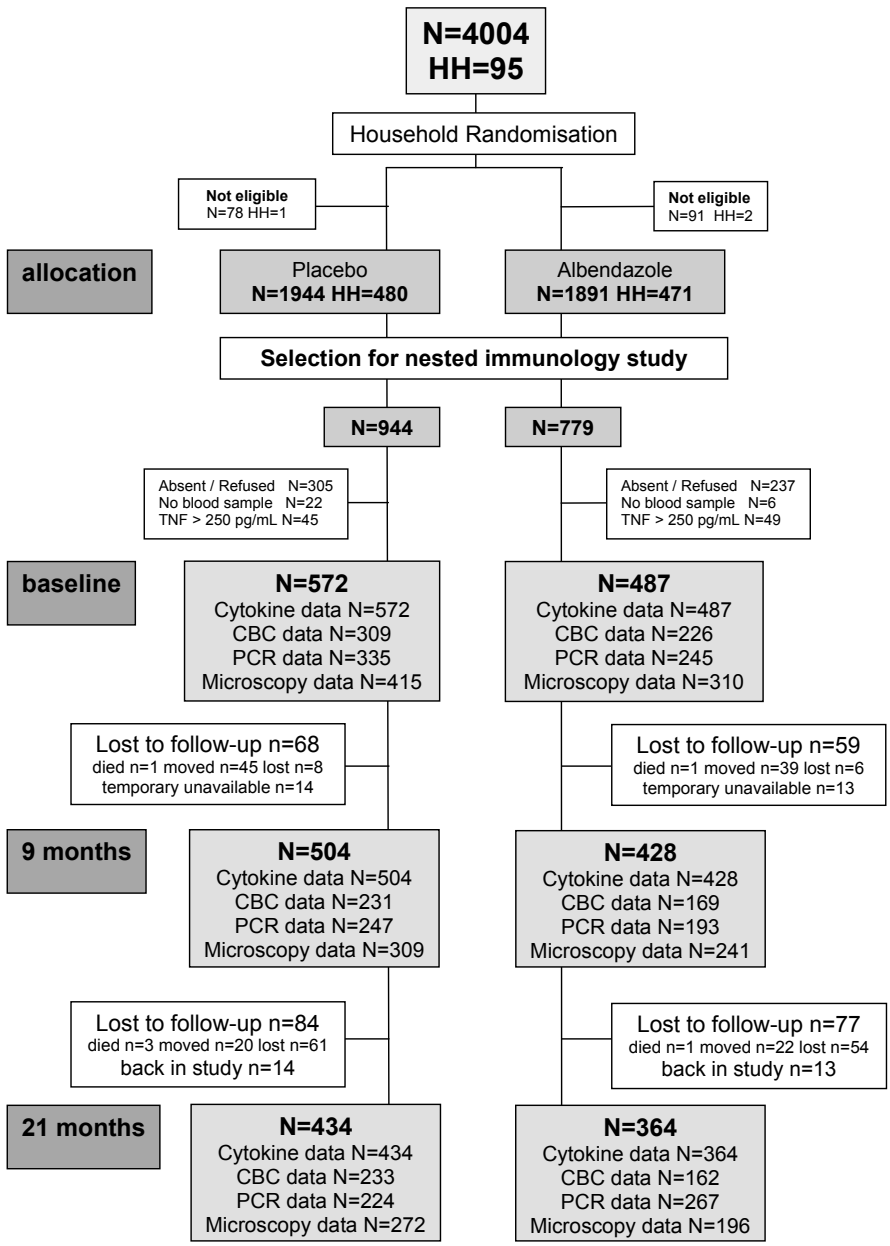


Figure 1. Consort diagram. The current study is nested within the ImmunoSPIN trial, with a total of 4004 individuals in two participating villages. Allocation of placebo and albendazole treatment resulted in 480 and 471 households including 1944 and 1891 in the two groups, respectively. For the immunological studies, a random selection was made and 1723 individuals were invited to participate (n=944 and n=779 respectively). Cytokines were assessed for 1059 subjects, of which 572 in the placebo and 487 in the albendazole arm. After 9 months 504 and 428 and after 21 months 434 and 364 individuals were analyzed, in placebo and albendazole group, respectively. Availability of complete blood counts (CBC) and parasitological data is indicated at the different time points for both groups.

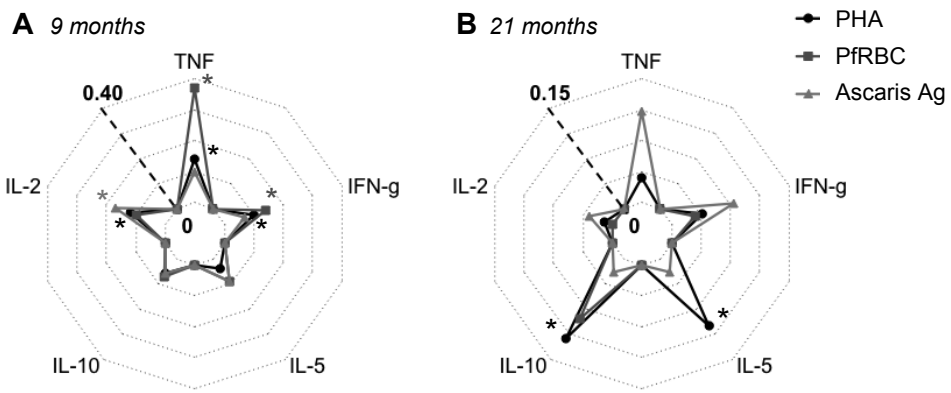


Figure 2. Effect of deworming on cytokine responses to *Ascaris*, PfRBC and PHA TNF, IFN-γ, IL-2, IL-5 and IL-10 concentrations were assessed in supernatants of 72h-stimulated whole blood cultures. The effect of albendazole treatment on cytokine responses to PHA (black circles), PfRBC (dark grey squares) and *Ascaris* (light grey triangles) is shown. The estimates of the treatment effect in the whole study population after 9 (A) and 21 (B) months of albendazole treatment were obtained by general linear mixed models; asterisks with corresponding colors (black for PHA, dark grey for PfRBC, light grey for *Ascaris*) indicate a significant effect.

treatment-related differences (data not shown). When assessing responses to uRBC as control for PfRBC, we found that IFN-γ levels were not different between the treatment arms (p=0.91), however TNF production was increased post-treatment in the albendazole arm, although to a lesser extent than what was seen in response to PfRBC (p=0.018; figure S1).

Increase in proinflammatory responses after treatment in helminth-infected individuals

To determine whether the enhanced cytokine responses could be due to a direct effect of albendazole, we stratified the analysis based on STH infection status at baseline. The enhancement of mitogen- as well as malaria-induced TNF by albendazole treatment was observed in helminth-infected individuals (overall p-values for PHA and PfRBC were p_{time}=0.098 and p_{time}=0.0004 respectively, figure 3A and 3B), but not in uninfected ones (data not shown). Importantly, uRBC-induced TNF responses were not increased in either helminth-infected or uninfected subjects (data not shown). Also in the response to *Ascaris* antigen, enhancement of TNF was observed in the stratified analysis of helminth-positives at baseline (p_{time}=0.044, figure 3C) but not in helminth negatives (data not shown). Moreover, elevated IFN-γ and IL-2 responses to Ascaris were only observed after treatment of the helminth-infected (p_{time}=0.028 and p_{time}=0.006 respectively; not shown).

Increased cytokine responses are not associated with changes in cell counts

The total leukocyte count increased in the albendazole group at 9 months post-treatment and was similar in both groups at 21 months (p=0.035 and p=0.14 respectively; data not shown). However, we observed a negative association of leukocyte counts and both TNF and IFN-γ responses to PfRBC, indicating that increased leukocyte numbers could not be responsible for the enhanced cytokine responses after treatment. When analyzing differential cell counts and proportions, the

lymphocytes and granulocytes did not change after treatment, whereas monocyte proportions and numbers were higher in the albendazole group. No association was found between monocyte numbers and cytokine production in response to any of the stimuli (data not shown). No treatment effect was noted on thrombocyte or erythrocyte counts, hemoglobin levels or hematocrit.

Discussion

This is the first time that helminth-specific and -unrelated cytokine responses have been analyzed in a whole community before and after repeated long-term placebo-controlled anthelminthic treatment. We show that treatment of STH infections increases cytokine

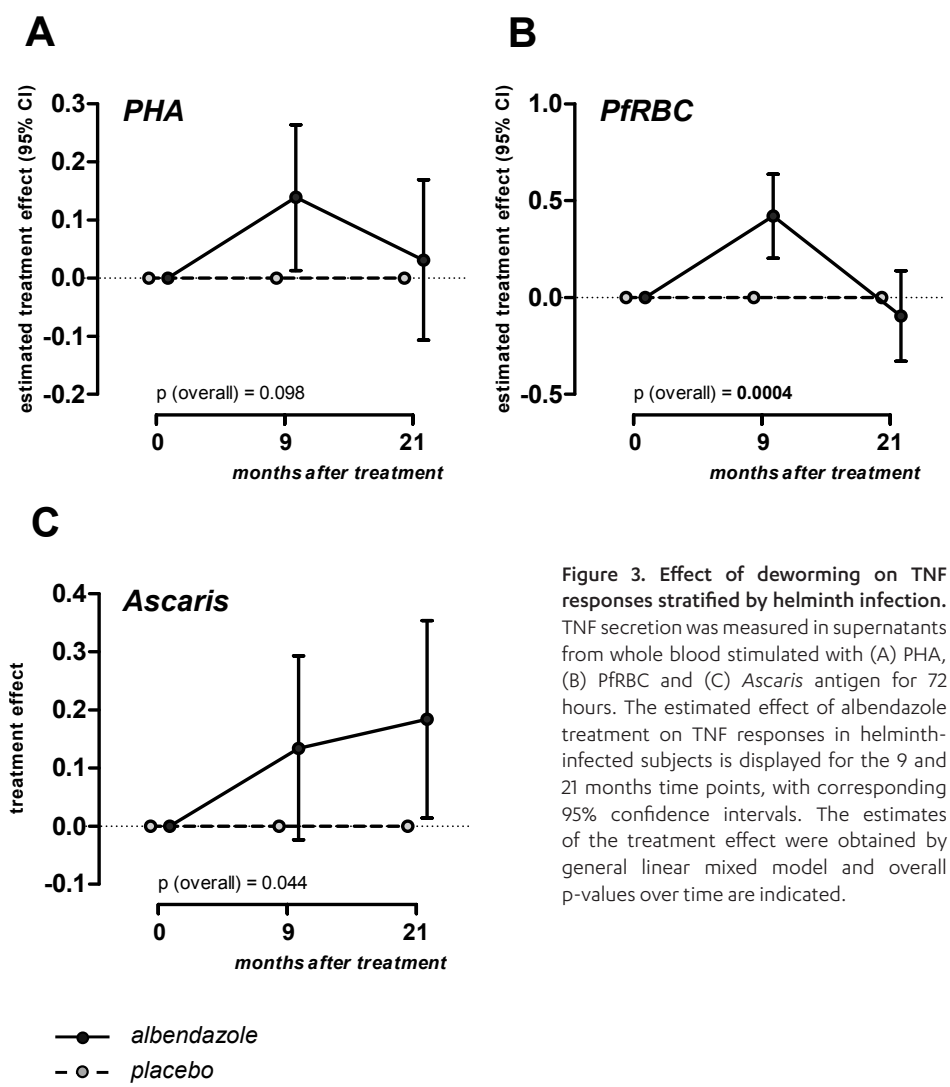


Figure 3. Effect of deworming on TNF responses stratified by helminth infection. TNF secretion was measured in supernatants from whole blood stimulated with (A) PHA, (B) PfRBC and (C) *Ascaris* antigen for 72 hours. The estimated effect of albendazole treatment on TNF responses in helminth-infected subjects is displayed for the 9 and 21 months time points, with corresponding 95% confidence intervals. The estimates of the treatment effect were obtained by general linear mixed model and overall p-values over time are indicated.

responses, with profound effects on helminth-specific and other adaptive immune responses, providing conclusive evidence for helminth-induced immune hyporesponsiveness in humans.

Most pronounced were elevated pro-inflammatory, TNF and IFN-γ, cytokine responses after stimulation with mitogen and malarial antigens. Albendazole is a drug, which might induce production of inflammatory cytokines in a human monocytic cell line (25). Stratifying the analysis for helminth infection status at baseline revealed stronger effects in the helminth-infected group, indicating that the suppression of pro-inflammatory cytokine responses is unlikely to be due to direct effects of albendazole, but can be regarded as a true helminth-induced phenomenon.

Subsequent to the rise in pro-inflammatory responses at 9 months, an interesting finding was the enhancement of IL-5 and IL-10 responses at 21 months post-treatment. Although helminth infections skew the immune system towards type 2 responses, suppression of these responses during helminth infections has been reported before in studies comparing helminth infected and uninfected subjects (26,27). IL-10 upregulation appears particularly surprising; as it has been postulated that helminth-associated inhibition of pro-inflammatory responses is mediated by this suppressory cytokine (28). Increased IL-10 responses after anthelminthic treatment have previously been observed in schistosomiasis (29) and STH infection (16). Whether the increased pro-inflammatory responses in the first year leads to higher IL-10 in the second year to prevent overt inflammation, is not clear from these data. Moreover, it is known that IL-10 can be part of the Th2 response and therefore the increased IL-10 might be a component of the enhanced Th2 response following deworming, leaving the question whether IL-10 originating from other cells is involved in cellular hyporesponsiveness caused by helminth infections. However, the fact that different cytokines appear to all increase in response to antigens and mitogens after anthelminthic treatment seems to indicate that all adaptive immune responses are enhanced after deworming. This would predict that there is a general helminth-mediated hyporesponsiveness which is neither restricted to a particular pro- or anti-inflammatory nor to a Th1 or Th2 response, but might stem from a common general effect such as alternation in cell counts, changes in nutrients essential to functioning of the immune system or suppressory cells and factors which do not involve IL-10.

Importantly, cell counts were affected by reduction in helminths, but did not show any correlation with cytokine responses, excluding the possibility that the general increase in responsiveness is due to higher numbers of cytokine-secreting cells. As immune responses can be enhanced by improved energy resources, we assessed BMI, and fasting glucose level (not shown), as proxies for nutritional status but these parameters were not affected by deworming.

The three-monthly albendazole treatment over a two-year period was not effective in eliminating all helminth infections. Treatment efficacy was particularly low for *Trichuris*, shown in earlier studies that used single or double doses of albendazole and / or mebendazole (30,31). Here we show that even 7 doses of albendazole over a 21 month period is not sufficiently effective against *Trichuris* infection. By using a household-clustered design for randomization, repeated treatments and observed intake, we had expected a more effective reduction in transmission of STH. For better deworming results, more intensive treatment or inclusion of environmental control would be needed. However, it is clear that even a reduction in helminth infections in the community can lead to alleviation of immune hyporesponsiveness and that a more effective deworming, might result in even more pronounced immunological effects.

There is an increasing awareness that helminths might play an essential role in the development and homeostasis of the human immune system (32,33). In areas where chronic helminth infections are persistent, the immune system may have evolved to operate optimally in the face of helminth-induced downmodulation; any disturbance of the long evolutionary coexistence between humans and helminths might be associated with the emergence of pathological conditions (34). The question of what the clinical consequences of the enhanced adaptive immune responses are following deworming will need to be addressed next. So far, our trial of three-monthly albendazole treatment over a 21-month period did not show clear clinical changes (24). Although we found a transient increase in malarial parasitemia at 6 months post treatment, the time point after the rainy season, this could not be confirmed during the further follow-up period, as the prevalence of malaria decreased drastically in the study area. With respect to allergy, skin prick test (SPT) reactivity was assessed in school children in our study cohort. This revealed a specific increase in SPT reactivity to cockroach allergens after two years of anthelminthic treatment, but no effect on allergy symptoms (24). The effects of anthelminthic treatment on other infectious or inflammatory diseases should probably be investigated over a longer period, since the immunomodulatory effects of helminths are likely to have developed over several years of chronic infection. In the case of allergic diseases this is illustrated by a recent publication, showing an increase in allergen SPT reactivity and possibly eczema symptoms after more than 15 years of ivermectin treatment (35), whereas studies with a shorter treatment course failed to show increased SPT reactivity or symptoms (14,15).

Several clinical trials are underway to evaluate the possible beneficial effect of helminth infection or their excretory products on the symptoms and prevalence of inflammatory diseases (36,37), while at the same time efforts are made to control STH by implementing regular treatment programs in developing countries (38). These studies, if conducted within appropriate time frames, should be able to answer the question what clinical consequences of human helminth infections are. Given our results that there are major effects on immune responses following deworming, it will be important to include immunological measurements in future deworming programs in order to understand causation and predict clinical outcomes.

References

1. Hotez, P.J., *et al*. Helminth infections: the great neglected tropical diseases. *The Journal of clinical investigation* 118, 1311-1321 (2008).
2. Bethony, J., *et al*. Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *Lancet* 367, 1521-1532 (2006).
3. Allen, J.E. & Maizels, R.M. Diversity and dialogue in immunity to helminths. *Nature reviews. Immunology* 11, 375-388 (2011).
4. van Riet, E., Hartgers, F.C. & Yazdanbakhsh, M. Chronic helminth infections induce immunomodulation: consequences and mechanisms. *Immunobiology* 212, 475-490 (2007).
5. Ottesen, E.A., Hiatt, R.A., Cheever, A.W., Sotomayor, Z.R. & Neva, F.A. The acquisition and loss of antigen-specific cellular immune responsiveness in acute and chronic schistosomiasis in man. *Clinical and experimental immunology* 33, 37-47 (1978).
6. Ottesen, E.A., Weller, P.F. & Heck, L. Specific cellular immune unresponsiveness in human filariasis. *Immunology* 33, 413-421 (1977).
7. Danilowicz-Luebert, E., O'Regan, N.L., Steinfelder, S. & Hartmann, S. Modulation of specific and allergy-related immune responses by helminths. *Journal of biomedicine & biotechnology* 2011, 821578 (2011).
8. Chandra, R.K. Nutrition and the immune system from birth to old age. *European journal of clinical nutrition* 56 Suppl 3, S73-76 (2002).
9. Wherry, E.J. T cell exhaustion. *Nature immunology* 12, 492-499 (2011).
10. Stelekati, E. & Wherry, E.J. Chronic bystander infections and immunity to unrelated antigens. *Cell host & microbe* 12, 458-469 (2012).
11. Cooper, P.J., *et al*. Human infection with Ascaris lumbricoides is associated with suppression of the interleukin-2 response to recombinant cholera toxin B subunit following vaccination with the live oral cholera vaccine CVD 103-HgR. *Infection and immunity* 69, 1574-1580 (2001).
12. Elias, D., *et al*. Effect of deworming on human T cell responses to mycobacterial antigens in helminth-exposed individuals before and after bacille Calmette-Guerin (BCG) vaccination. *Clinical and experimental immunology* 123, 219-225 (2001).
13. Rook, G.A. Review series on helminths, immune modulation and the hygiene hypothesis: the broader implications of the hygiene hypothesis. *Immunology* 126, 3-11 (2009).
14. Cooper, P.J., *et al*. Repeated treatments with albendazole enhance Th2 responses to Ascaris Lumbricoides, but not to aeroallergens, in children from rural communities in the Tropics. *The Journal of infectious diseases* 198, 1237-1242 (2008).
15. Flohr, C., *et al*. Reduced helminth burden increases allergen skin sensitization but not clinical allergy: a randomized, double-blind, placebo-controlled trial in Vietnam. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 40, 131-142 (2010).
16. Wright, V.J., *et al*. Early exposure of infants to GI nematodes induces Th2 dominant immune responses which are unaffected by periodic anthelminthic treatment. *PLoS neglected tropical diseases* 3, e433 (2009).
17. Bourke, C.D., *et al*. Trichuris suis ova therapy for allergic rhinitis does not affect allergen-specific cytokine responses despite a parasite-specific cytokine response. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 42, 1582-1595 (2012).
18. Gaze, S., *et al*. Characterising the mucosal and systemic immune responses to experimental human hookworm infection. *PLoS pathogens* 8, e1002520 (2012).
19. Hamid, F., *et al*. A longitudinal study of allergy and intestinal helminth infections in semi urban and rural areas of Flores, Indonesia (ImmunoSPIN Study). *BMC infectious diseases* 11, 83 (2011).
20. Wiria, A.E., *et al*. Does treatment of intestinal helminth infections influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study). *BMC infectious diseases* 10, 77 (2010).
21. WHO. WHO Child Growth Standards: Lenght/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age: Method and development. *Geneva: World Health Organization*, 312 (2006).
22. Laird, N.M. & Ware, J.H. Random-effects models for longitudinal data. *Biometrics* 38, 963-974 (1982).

23. R-Forge. lme4 - Mixed-effects models project. (2011).
24. Wiria, A.E., *et al*. The effect of three-monthly albendazole treatment on malarial parasitemia and allergy: A household-based cluster-randomized, double-blind, placebo-controlled trial. *PLoS One* 8, e57899 (2013).
25. Mizuno, K., Toyoda, Y., Fukami, T., Nakajima, M. & Yokoi, T. Stimulation of pro-inflammatory responses by mebendazole in human monocytic THP-1 cells through an ERK signaling pathway. *Archives of toxicology* 85, 199-207 (2011).
26. Grogan, J.L., Kremsner, P.G., Deelder, A.M. & Yazdanbakhsh, M. Antigen-specific proliferation and interferon-gamma and interleukin-5 production are down-regulated during Schistosoma haematobium infection. *The Journal of infectious diseases* 177, 1433-1437 (1998).
27. Wammes, L.J., *et al*. Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics. *PLoS neglected tropical diseases* 6, e1655 (2012).
28. Couper, K.N., Blount, D.G. & Riley, E.M. IL-10: the master regulator of immunity to infection. *J Immunol* 180, 5771-5777 (2008).
29. van den Biggelaar, A.H., Borrmann, S., Kremsner, P. & Yazdanbakhsh, M. Immune responses induced by repeated treatment do not result in protective immunity to Schistosoma haematobium: interleukin (IL)-5 and IL-10 responses. *The Journal of infectious diseases* 186, 1474-1482 (2002).
30. Namwanje, H., Kabatereine, N.B. & Olsen, A. Efficacy of single and double doses of albendazole and mebendazole alone and in combination in the treatment of Trichuris trichiura in school-age children in Uganda. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 105, 586-590 (2011).
31. Speich, B., *et al*. Efficacy and safety of nitazoxanide, albendazole, and nitazoxanide-albendazole against Trichuris trichiura infection: a randomized controlled trial. *PLoS neglected tropical diseases* 6, e1685 (2012).
32. Elliott, D.E. & Weinstock, J.V. Helminth-host immunological interactions: prevention and control of immune-mediated diseases. *Annals of the New York Academy of Sciences* 1247, 83-96 (2012).
33. Hoerauf, A. Microflora, helminths, and the immune system-who controls whom? *The New England journal of medicine* 363, 1476-1478 (2010).
34. Maizels, R.M. & Yazdanbakhsh, M. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nature reviews. Immunology* 3, 733-744 (2003).
35. Endara, P., *et al*. Long-term periodic anthelminthic treatments are associated with increased allergen skin reactivity. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 40, 1669-1677 (2010).
36. Falcone, F.H. & Pritchard, D.I. Parasite role reversal: worms on trial. *Trends in parasitology* 21, 157-160 (2005).
37. Harnett, W. & Harnett, M.M. Helminth-derived immunomodulators: can understanding the worm produce the pill? *Nature reviews. Immunology* 10, 278-284 (2010).
38. Utzinger, J. A research and development agenda for the control and elimination of human helminthiases. *PLoS neglected tropical diseases* 6, e1646 (2012).

Table S1. Prevalence of helminth infections at post-treatment timepoints

		N	any spp n (%)	Hookworm[1] n (%)	A. lumbricoides[1] n (%)	T.trichiuria[2] n (%)
9 months	Placebo	227	191 (84.1%)	160 (70.5%)	76 (33.5%)	72 (31.7%)
	Albendazole	181	94 (51.9%)	59 (32.6%)	24 (13.3%)	38 (21.0%)
21 months	Placebo	215	171 (80.0%)	144 (67.0%)	52 (24.2%)	62 (28.8%)
	Albendazole	148	58 (39.2%)	32 (21.6%)	18 (12.2%)	24 (16.2%)

[1]diagnosed by PCR. [2]diagnosed by microscopy.

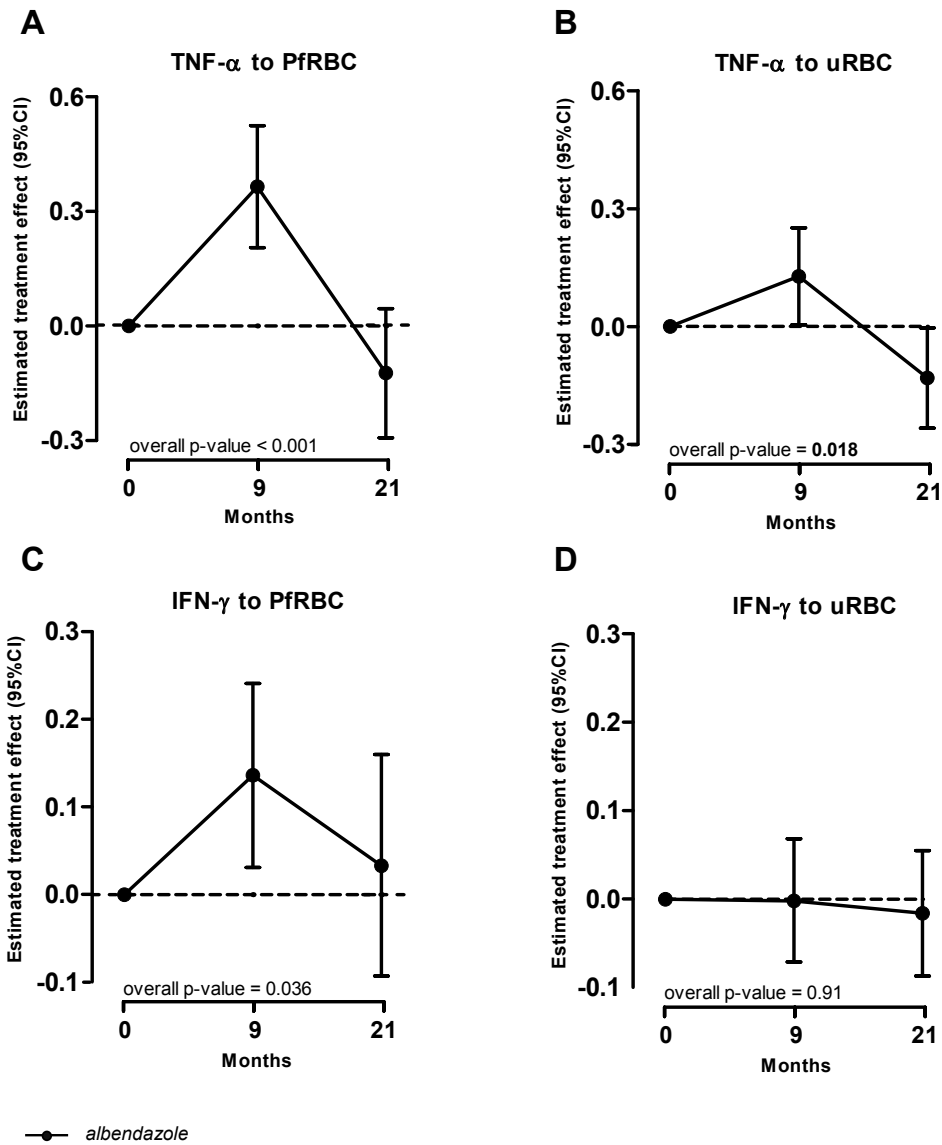


Figure S1. Effect of deworming on TNF and IFN-γ responses to PfRBC and uRBC. TNF (A, B) and IFN-γ (C, D) responses were measured after 72h of stimulation with *Plasmodium falciparum*-infected and -uninfected RBC (PfRBC (A, C) and uRBC (B, D), respectively). The estimated effect of albendazole treatment on TNF responses in helminth-infected subjects is displayed for the 9 and 21 months time points, with corresponding 95% confidence intervals. The estimates of the treatment effect were obtained by general linear mixed model and overall p-values over time are indicated.

chapter 5

Relationship between Carotid Intima Media Thickness and Helminth Infections on Flores Island, Indonesia

Relationship between Carotid Intima Media Thickness and Helminth Infections on Flores Island, Indonesia

Aprilianto Eddy Wiria[1,2]*, Linda J. Wammes[2], Firdaus Hamid[2,3], Olaf M. Dekkers[4,6], Margaretta A. Prasetyani[1,2], Linda May[2], Maria M. M. Kaisar[1,2], Jaco J. Verweij[2¤], Jouke T. Tamsma[5], Felix Partono[1], Erliyani Sartono[2], Taniawati Supali[1☯], Maria Yazdanbakhsh[2☯], Johannes W. A. Smit[6,7☯]

1 Department of Parasitology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia, **2** Department of Parasitology, Leiden University Medical Center, Leiden, The Netherlands, **3** Department of Microbiology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia, **4** Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, The Netherlands, **5** Vascular Medicine, Department of Endocrinology and General Internal Medicine, Leiden University Medical Center, Leiden, The Netherlands, **6** Department of Endocrinology and General Internal Medicine, Leiden University Medical Center, Leiden, The Netherlands, **7** Department of General Internal Medicine, Radboud University Medical Center, Nijmegen, The Netherlands

Abstract

Objective: To examine the association between helminth infections and atherosclerosis.

Background: Chronic helminth infection, which can lead to poor nutritional status and anti-inflammatory response, might protect against the development of atherosclerosis.

Methods: A cross-sectional study was performed in Flores, Indonesia, an area highly endemic for soil-transmitted helminths (STH). Stool samples from 675 participants aged 18–80 years were collected and screened for *Trichuris trichiura* by microscopy and for *Ascaris lumbricoides*, *Necator americanus, Ancylostoma duodenale, and Strongyloides stercoralis* by qPCR. We collected data on body mass index (BMI), waist to hip ratio (WHR), blood pressure, fasting blood glucose (FBG), lipid, high sensitive C-reactive protein (hs-CRP), total immunoglobulin-E (TIgE) and *Escherichia coli* lipopolysaccharide stimulated cytokines (tumor necrosis factor and interleukin-10). In a subset of 301 elderly adults (≥40 years of age) carotid intima media thickness (cIMT) was measured.

Results: Participants with any STH infection had lower BMI (kg/m2) (mean difference −0.66, 95%CI [−1.26, −0.06]), WHR (−0.01, [−0.02, −0.00]), total cholesterol (mmol/L) (−0.22, [−0.43, −0.01]) and LDL-cholesterol (mmol/L) (−0.20, [−0.39, −0.00]) than uninfected participants. After additional adjustment for BMI the association between helminth infection and total cholesterol (mean difference −0.17, 95%CI [−0.37, 0.03]) as well as LDL-cholesterol (−0.15, [−0.33, 0.04]) was less pronounced. BMI, WHR, and total cholesterol were negatively associated with number species of helminth co-infections. Participants with high TIgE, an indicator of exposure to helminths, had lower FBG, TC, and HDL. The association between TIgE and TC and HDL remained significant after adjustment with BMI. No clear association was found between STH infection or TIgE and mean cIMT.

Conclusions: This cross-sectional study presents evidence that helminth infections were negatively associated with risk factors for cardiovascular disease, an association at least partially mediated by an effect on BMI. The significance of this finding needs to be determined.

Citation: Wiria AE, Wammes LJ, Hamid F, Dekkers OM, Prasetyani MA, et al. (2013) Relationship between Carotid Intima Media Thickness and Helminth Infections on Flores Island, Indonesia. PLoS ONE 8(1): e54855. doi:10.1371/journal.pone.0054855

Editor: David Joseph Diemert, The George Washington University Medical Center, United States of America

Received July 23, 2012; **Accepted** December 17, 2012; **Published** January 24, 2013

Funding: This study was funded by The Royal Netherlands Academy of Arts and Science (KNAW), Ref.KNAW-05-PP-35, European Commission contracts INCO-CT-2006-031714 and INCO-CT-2006-032436, and the Prof. Dr. P.C. Flu Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: a.e.wiria@lumc.nl

¤ Current address: Laboratory for Medical Microbiology and Immunology, St. Elisabeth Hospital, Tilburg, The Netherlands

☯ These authors contributed equally to this work.

Introduction

Mortality from cardiovascular diseases (CVD) accounts for 30% of total global deaths [1]. CVD is no longer a disease of Western countries exclusively since 80% of all CVD deaths worldwide take place in developing countries. In many Asian countries, rapid socioeconomic development has led to a shift in infrastructure, technology and food supply that promotes over nutrition and sedentary lifestyle [2,3]. The relationship between a disturbed energy balance resulting from decreased physical activity or excess consumption of high-energy foods and CVD has long been acknowledged, but there is now abundant evidence that inflammation plays a role in chronic non-

communicable diseases, including CVD. Indeed, in CVD elevated levels of several inflammation-related markers such as interleukin 6 (IL-6), IL-8, tumor necrosis factor (TNF), and C-reactive protein (CRP) [4] have been reported. One particular modifier of the pathogenesis of CVD in non-western societies may be related to differences in infectious pressure between rural and urban societies.

Although helminth infections, vary in their lifecycle and clinical impact in humans, they appear to share the ability to decrease inflammation and subsequently the development of inflammatory diseases which may include CVD [5]. We and others have shown that various helminth infections, which are endemic in many non-western societies, induce regulatory T cells (Treg) in order to ensure their survival within an immune competent host [6–9]. Helminths, such as schistosomes, that establish a systemic infection or soil-transmitted helminths (STH), which are restricted to the intestine, are also known to reduce energy intake and to be associated with poor nutritional status [10,11]. Interestingly, in a study with apoE−/− mice, the development of atherosclerotic lesions was reduced by approximately 50% in animals with *S. mansoni* infections [12] whereas lipid-lowering effects were mediated by factors released from *S. mansoni* eggs [13]. Moreover, in an animal model of helminth infection with *Nippostrongylus brasiliensis*, a gastrointestinal nematode, with a lifecycle similar to hookworm in humans, it has been shown that infection is associated with beneficial effects of reducing traditional CVD risk factors such as obesity and serum lipid levels [14]. So far, to our knowledge, no studies have been published on the association between STH infections and atherosclerosis in humans.

Carotid intima media thickness (cIMT), is a marker for subclinical atherosclerosis [15] and is strongly associated with risk of CVD [16]. Assessment of cIMT is widely used in large-scale observational and experimental research. We set out to study the relationship between helminth infections CVD risk factors and cIMT as marker for atherosclerosis in an area endemic for STH on Flores Island, Indonesia.

Materials and Methods

Study Objectives

The primary objective of the study was to investigate the association between helminth infections and cIMT as marker for subclinical atherosclerosis. Our hypothesis was that, since helminths might protect against CVD, cIMT is lower in subjects with helminth infections than in subjects without helminth infections. The secondary objective was to study the association between helminth infections as well as total immunoglobulin E (TIgE), an indicator of exposure to helminths [17], and conventional CVD risk factors, including body mass index (BMI), waist hip ratio (WHR), blood pressure (BP), fasting blood glucose (FBG), serum lipid profile and serum markers of inflammation.

Study Population

The study area is the semi urban area of Nangapanda on Flores Island in Indonesia [18,19]. The area of Nangapanda is endemic for STH infections but not for filarial nematodes [9]. In this area, a large project is being conducted on the relationship between helminth infections and the immune system (ImmunoSPIN study [18,19]). For the current study, a cross sectional representative sample was included from all inhabitants aged 18 years and above. Data were collected between May and August 2009.

Study Design

From 2799 inhabitants from the Nangapanda area who participated in the ImmunoSPIN project, 691 were randomly selected to participate in the present cross-sectional study and invited to provide data on BMI, WHR, BP, and blood sampling for fasting glucose measurements, lipid profiles, TIgE, hs-CRP and whole blood culture to stimulate cytokine production. Data on helminth infections, BP, BMI and WHR ratio were available from 675 subjects included in the present analysis. In 595 of these subjects, laboratory measurements were performed. Carotid artery IMT was measured in a subset of 301 adult participants above 40 years of age.

The study was approved by The Ethical Committee of Faculty of Medicine, University of Indonesia, ref: 194/PT02.FK/Etik/2006 with addendum ref: 96/PT02.FK/Etik/2010 and registered as clinical trial ref: ISRCTN83830814 and was filed by the Leiden University Medical Center Committee of Medical Ethics (CME). Because of the high rate of illiteracy amongst elderly participants, either written or verbal informed consent was obtained from each participant.

Clinical and Laboratory Assessment

Anthropometric measurement of body weight (SECA 761, SECA GMBH & Co. Kg., Hamburg, Germany), height (SECA 206, SECA GMBH & Co. Kg., Hamburg, Germany), waist and hip circumference (WC and HC) (SECA 203, SECA GMBH & Co. Kg., Hamburg, Germany) were obtained using the NHLBI practical guidelines (NHLBI web). Three blood pressure (BP) measurements (left arm, sitting upright position, after resting 5 minutes) were taken from each subject, using a digital Omron sphygmomanometer (705IT HEM-759P-E2, OMRON Healthcare Europe BV, The Netherlands), and calibrated using a Riester nova-presameter®-Desk model mercury sphygmomanometer (Gerhard Glufke Rudolf Riester GmbH & Co, Jungingen, Germany) and a 3M™ Littmann® Classic II S.E. Stethoscope (3M, St. Paul, Minnesota, USA). The average of three systolic/diastolic BP measurements was used. Abnormal BMI is ≥25 kg/m^2 and the Asian modified abnormal waist hip ratio (WHR) is >0.9 (men) and >0.8 (women) [20]. Abnormal blood pressure was considered as hypertension when BP≥140/90 mmHg.

All participants were instructed to be fasting before venous sampling. FBG was analyzed using Breeze®2 glucose meter (Bayer Health Care LLC, Basel, Switzerland). Lipid profile was measured using commercial enzymatic kits for total cholesterol (TC), high density lipoprotein-cholesterol (HDL-c) and triglycerides (TG) (Roche Molecular Biochemicals, Indianapolis, USA) and determined using ELISA reader (LabSystem Multiscan, MHC347, Helsinki, Finland). Low density lipoprotein-cholesterol (LDL-c) was calculated by using the Friedwald calculation [21]. High sensitive C-reactive protein (hs-CRP) level was measured using MSD® 96-Well MULTI-ARRAY® CRP Assay (Meso Scale Discovery, Gaithersburg, USA). TIgE level was measured by an ELISA as described in detail previously [18,19].

Helminth Status

Stool samples were collected and preserved in 4% formaldehyde for microscopy examination or frozen (−20°C) unpreserved for PCR detection. The formol-ether acetate concentration method was performed on the formalin preserved stool samples followed by microscopy examination for *Trichuris trichiura* infections [19]. As described in detail before [19] the DNA of *Ancylostoma duodenale*, *Necator americanus*, *Ascaris lumbricoides* and *Strongyloides stercoralis* were isolated from approximately 100 mg unpreserved faeces and were examined by the multiplex qPCR. The qPCR output from this

system consisted of a cycle-threshold (CT) value, representing the amplification cycle in which the level of fluorescent signal exceeds the background fluorescence, and reflecting the parasite-specific DNA load in the sample tested. Negative and positive control samples were included in each run of the amplification. We defined a positive case for *T. trichiura* by the egg findings and for *A. duodenale*, *N. americanus*, *A. lumbricoides* and *S. stercoralis* by parasite-specific DNA findings. Participants were also grouped by number of helminth co-infections.

Carotid Intima Media Thickness

We used ultrasound for measuring cIMT [22]. Quality control and details of the IMT measurement have been described before [23]. cIMT was measured while the participant was lying in a supine position. Measurements were made at 3 different angles of both the right and left common carotid artery at 10 mm proximal of the carotid artery bulb using a mobile device: Mylab®25 ultrasound system with a LA523 13-4 MHz transducer (ESAOTE, S.p.A, Maastricht, The Netherlands). The mean of these 6 measurements was used in the analysis. In order to keep variation minimal, one of the physicians (AEW) performed all intima-media thicknesses measurements on the participants in this study.

Whole Blood Stimulation and Cytokine Measurement

The procedure of whole blood stimulation and cytokines measurement has been described previously [19]. Briefly, heparinized blood within 6 hours of blood draws was diluted 4× and stimulated. Stimulations were performed with control medium or *Escherichia coli* lipopolysaccharide (LPS, 1 ng/ml Sigma-Aldrich, Zwijndrecht, The Netherlands), incubated for 24 hours at 37°C and 5% CO_2. The supernatants were frozen at −20°C and transported to Jakarta where TNF and IL-10 were assessed by means of immunobead-based multiplex assays (Biosource, Camarillo, CA, USA) on a Liquichip 200® Workstation (Qiagen, Venlo, The Netherlands) using Liquichip analyzer software (Qiagen, Venlo, The Netherlands). Samples with TNF levels higher than 250 pg/ml in medium stimulation were excluded from further analyses (2 samples).

Statistical Analysis

Participant characteristics were stratified for helminth infection (uninfected and infected). Linear regression was used to study the associations between helminth infections and also number of helminth species co-infection and risk factors for CVD adjusted for age and sex. Differences between infected and uninfected participants were reported as mean differences with 95% confidence intervals (95% CI). TIgE, cytokines and CRP concentrations were normalized by log-transformation, analyses were performed with these log transformed values but results were presented as geometric means after exponentiation of the values on a logarithmic scale. To assess whether a potential association between helminth infection and CVD risk factors is mediated through an effect on BMI, we performed a second analysis additionally adjusted for BMI. Similar analyses were performed on the cIMT subset participants. Furthermore, in the helminth infected group we tested the association of helminth load per species with CVD risk factors. In addition, as high TIgE is associated with exposure to helminth infections, we tested the association between TIgE and risk factors for CVD as well as cIMT. Subjects were considered to have high or low TIgE based on the value above or below and equal the geometric mean. P values <0.05 were considered to be statistically significant. Statistical analysis was done using SPSS statistics 17.0.2 (SPSS Inc., Chicago, Illinois, USA).

Results

Characteristics of Study Participants

A total of 446 participants infected with at least one helminth were compared to 229 uninfected participants. There were more males in the infected group (37.7%) than in the uninfected group (34.1%), whereas the mean age was similar (45.0 vs. 44.8 years). The most prevalent STH infections were *N. americanus* 348/675 (51.6%), *A. lumbricoides* 149/675 (22.1%) and *T. trichiura* 139/675 (20.6%). The proportion of participants infected with *A. duodenale* 24/675 (3.6%) and with *S. stercoralis* 5/675 (0.7%) was clearly lower. 273 participants were infected with one helminth only, 131 with two helminths and 42 with 3 or more helminths species. As expected, TIgE was higher in individuals infected with STH infection. (Table 1).

Association between Helminth Infection and CVD Risk Factors

Participants with any helminth infection had lower BMI (mean difference −0.66, 95%CI −1.26, −0.06), WHR (−0.01, 95%CI −0.02, −0.00), total cholesterol (−0.22, 95%CI −0.43, −0.01) and LDL-cholesterol (−0.20, 95% CI −0.39, −0.00) than uninfected participants (Table 1). After additional adjustment for BMI the association between helminth infection and total cholesterol (mean difference −0.17, 95%CI −0.37, 0.03) as well as LDL-cholesterol (−0.15, 95%CI −0.33, 0.04) was less pronounced. No clear associations were found between helminth infection and blood pressure, fasting blood glucose and triglycerides. No difference in hs-CRP between helminth-infected and –uninfected groups was found.

Next, we analyzed whether the number of helminth species infecting a participant was related to CVD risk factors (data not shown). BMI, WHR, and total cholesterol were negatively associated with the number of helminth infections. Adjustment for BMI attenuated the association between number of infections and total cholesterol. No marked associations were found between number of infections and blood pressure, LDL, HDL, triglycerides or fasting glucose levels. In addition, TNF production in response to LPS stimulation was positively associated with the number of helminth infections with the highest LPS-TNF levels in participants with 3 or more infections. This association remained after adjustment for BMI ($p = 0.03$).

Looking at intensity of infection; in infected participants no associations were found between *N. americanus* load (as measured by qPCR) and BMI, WHR, fasting blood glucose, blood pressure or cholesterol levels. A negative association was found between *A. lumbricoides* load and triglyceride independent of BMI ($P<0.001$).

The Association between Helminth Infections and IMT

IMT was measured in 70% of participants ≥40 years (table 2). In accordance with the whole study population, BMI, WHR, TC and LDL were lower in helminth infected patients. IMT was only marginally lower in infected participants than in uninfected participants (mean difference −4.7, 95% CI −27.7, 18.3). In addition, no relationships between the *number* and load of helminth infections and IMT were found. No differences in IMT were found between *N. americanus* infected participants and non-infected participants either.

The Association between TIgE Level, CVD risk Factors and cIMT

A negative association was found between TIgE and FBG (mean difference −0.31, 95%CI −0.61, −0.02), TC (−0.23,

Table 1. Characteristics of the study population regarding helminth uninfected and infected.

	Helminth uninfected (n = 229)	Helminth infected (n = 446)	Mean difference adjusted for age and sex (95% confidence interval)	Mean difference adjusted for age, sex and BMI (95% confidence interval)
Age (year) (mean, Range)	44.8 (18.2–80.2)	45.0 (18.0–79.5)	–	–
Male (%)	34.1	37.7	–	–
***Trichuris trichiura*[1] (%)**	0	31.2	–	–
***Ascaris lumbricoides*[2] (%)**	0	33.4	–	–
***Necator americanus*[2] (%)**	0	78.0	–	–
***Ancylostoma duodenale*[2] (%)**	0	5.4	–	–
***Strongyloides stercoralis*[2] (%)**	0	1.1	–	–
BMI (Kg/m2) (mean, SD)	23.1 (3.7)	22.5 (3.8)	−0.66 (−1.26, −0.06), p = 0.031	–
WHR (mean, SD)	0.89 (0.07)	0.88 (0.06)	−0.01 (−0.02, −0.00), p = 0.011	–
Systole (mmHg) (mean, SD)	130.9 (22.5)	129.8 (24.4)	−1.18 (−4.56, 2.19), p = 0.49	−0.38 (−3.71, 2.95), p = 0.82
Diastole (mmHg) (mean, SD)	78.5 (12.2)	76.7 (12.6)	−1.76 (−3.71, 0.20), p = 0.078	−1.28 (−3.17, 0.61), p = 0.19
FBG (mmol/L) (mean, SD)	5.9 (1.5)	5.9 (1.6)	−0.05 (−0.31, 0.21), p = 0.71	0.02 (−0.24, 0.27), p = 0.89
TC (mmol/L) (mean, SD)	5.1 (1.2)	4.9 (1.1)	−0.22 (−0.43, −0.01), p = 0.037	−0.17 (−0.37, 0.03), p = 0.098
HDL-c (mmol/L) (mean, SD)	1.6 (0.4)	1.5 (0.4)	−0.02 (−0.09, 0.05), p = 0.49	−0.03 (−0.10, 0.04), p = 0.34
TG (mmol/L) (mean, SD)	1.2 (0.7)	1.3 (0.7)	0.00 (−0.13, 0.13), p = 1.00	0.05 (−0.07, 0.17), p = 0.45
LDL-c (mmol/L) (mean, SD)	3.3 (1.1)	3.1 (1.0)	−0.20 (−0.39, −0.00), p = 0.048	−0.15 (−0.33, 0.04), p = 0.13
TC to HDL-c ratio (mean, SD)	3.5 (1.1)	3.3 (1.0)	−0.09 (−0.27, 0.10), p = 0.38	−0.03 (−0.21, 0.15), p = 0.74
TIgE (IU/ml) (geometric mean [95%CI])*	834.3 (716.5–971.5)	1182.1 (1044.5–1337.8)	1.42 (1.15, 1.75), p = 0.0010	1.40 (1.14, 1.73), p = 0.0016
hs-CRP (mg/l) (geometric mean [95%CI])*	0.5 (0.4–0.6)	0.5 (0.4–0.6)	1.15 (0.74, 1.30), p = 0.90	1.16 (0.77, 1.37), p = 0.85
TNF (pg/ml) (geometric mean [95%CI])*	217.8 (175.3–270.5)	278.6 (243.7–318.4)	1.28 (1.00, 1.64), p = 0.050	1.27 (0.99, 1.64), p = 0.062
IL-10 (pg/ml) (geometric mean [95%CI])*	128.0 (105.4–155.6)	125.5 (111.7–140.0)	0.98 (0.79, 1.22), p = 0.87	0.98 (0.99, 1.22), p = 0.85

1. positive by microscopy.
2. positive by PCR.
Abbreviations: BMI = body mass index, WHR = waist to hips ratio, FBG = fasting blood glucose, TC = total cholesterol, TG = triglyceride, HDL-c = high density lipoprotein cholesterol, LDL-c = low density lipoprotein cholesterol, TIgE = total immunoglobulin E, hs-CRP = high sensitive C reactive protein, TNF = tumor necrosis factor, IL-10 = interleukin 10 cytokines were stimulated for 24 h with *E. coli* lipopolysaccharide (LPS). *Adjusted mean difference for TIgE, hs-CRP and cytokines were anti-log transformed and represent ratio.
doi:10.1371/journal.pone.0054855.t001

95%CI −0.42, −0.03), and HDL (−0.08, 95%CI −0.14, −0.01). The association remained significant after adjustment for BMI for TNF (1.22, 95%CI 0.96, 1.57) and IL-10 (1.27, 95%CI 1.03, 1.57). No difference was found between TIgE and cIMT. In analyses using continuous levels of TIgE, we found a significant association between TIgE and TNF.

Discussion

The objective of this study was to examine the association between helminth infections and atherosclerosis in a population residing in an area highly endemic for STH. The hypothesis being tested is that helminth infections may have a beneficial effect on the development of atherosclerosis, both by influencing conventional CVD risk factors and systemic inflammation. Although this beneficial effect has been illustrated in animal studies [12,13], no human studies on the relationship between STH infections and atherosclerosis have been published before.

We found a negative association between helminth infections and conventional CVD risk factors, including BMI, WHR and serum cholesterol levels, which was independent of age and gender. In addition, with increasing number of helminth species, the negative association with CVD risk factors increased. The association was similar when we analyzed TIgE as a marker of exposure to helminth infections and reflects not only current but also prior exposure to helminth infection [17].

The finding of lower lipid levels in individuals with infections in our study area is similar to what was found in Tsimane [24] or Shipibo [25] population. The lipids measured included HDL, which might seem surprising as decreased HDL level is considered to be a CVD risk factor. Interestingly, the anti-atherogenic properties of HDL might be dependent on the composition of HDL and the context in terms of type of inflammation [26]. It would be important to investigate whether helminth infections affect the type of HDL or its role in cholesterol transport.

With regards to cytokine production, we found a positive association of TNF production in response to LPS stimulation with helminth infections as well as with TIgE. These data suggest that helminths are associated with pro-inflammatory responses. Although higher TNF and pro-inflammatory cytokines have been found in the circulation of helminth infected patients with pathology [27–29], most studies of subjects infected with helminths with no overt pathology, report an anti-inflammatory effect of these parasites on the immune system. However, the before

Table 2. Characteristic of the group with intima media thickness measurement (population 40+).

	Helminth uninfected (n = 140)	Helminth infected (n = 291)	Mean difference adjusted for age and sex (95% confidence interval)	Mean difference adjusted for age, sex and BMI (95% confidence interval)
Age (year) (mean, Range)	53.4 (40.0–80.2)	53.6 (40.5–79.5)	–	–
Male (%)	40.7	45.7	–	–
***Trichuris trichiura*[1] (%)**	0	31.6	–	–
***Ascaris lumbricoides*[2] (%)**	0	28.9	–	–
***Necator americanus*[2] (%)**	0	79.4	–	–
***Ancylostoma duodenale*[2] (%)**	0	5.5	–	–
***Strongyloides stercoralis*[2] (%)**	0	1.4	–	–
IMT (≥40 years old) (μm) (mean, SD)	654 (122)	650 (112)	−4.68 (−27.65, 18.29), p = 0.69	−0.95 (−24.17, 22.27), p = 0.94
BMI (Kg/m2) (mean, SD)	23.2 (3.7)	22.2 (3.8)	−0.91 (−1.65, −0.17), p = 0.017	–
WHR (mean, SD)	0.89 (0.07)	0.88 (0.06)	−0.02 (−0.03, −0.00), p = 0.015	–
Systole (mmHg) (mean, SD)	139.8 (23.4)	136.8 (26.1)	−3.08 (−8.06, 1.91), p = 0.23	−1.52 (−6.43, 3.39), p = 0.54
Diastole (mmHg) (mean, SD)	81.3 (13.7)	78.2 (13.4)	−2.99 (−5.75, −0.24), p = 0.033	−2.12 (−4.77, 0.53), p = 0.12
FBG (mmol/L) (mean, SD)	6.1 (1.8)	6.1 (1.9)	−0.01 (−0.41, 0.39), p = 0.96	0.10 (−0.29, 0.49), p = 0.62
TC (mmol/L) (mean, SD)	5.2 (1.3)	5.0 (1.1)	−0.24 (−0.50, −0.01), p = 0.063	−0.18 (−0.43, 0.08), p = 0.17
HDL-c (mmol/L) (mean, SD)	1.5 (0.4)	1.5 (0.4)	−0.03 (−0.12, 0.06), p = 0.49	−0.05 (−0.13, 0.04), p = 0.31
TG (mmol/L) (mean, SD)	1.4 (0.6)	1.4 (0.8)	−0.01 (−0.18, 0.16), p = 0.89	0.07 (−0.10, 0.23), p = 0.43
LDL-c (mmol/L) (mean, SD)	3.4 (1.1)	3.2 (1.0)	−0.21 (−0.45, 0.03), p = 0.085	−0.15 (−0.38, 0.09), p = 0.23
TC to HDL-c ratio (mean, SD)	3.5 (1.1)	3.3 (1.0)	−0.10 (−0.34, 0.14), p = 0.40	−0.03 (−0.26, 0.21), p = 0.82
TIgE (IU/ml) (geometric mean [95%CI])*	743.3 (613.5–900.5)	1080.3 (927.5–1258.3)	1.41 (1.09, 1.84), p = 0.010	1.39 (1.07, 1.82), p = 0.015
hs-CRP (mg/l) (geometric mean [95%CI])*	0.6 [0.4–0.7]	0.6 [0.5–0.7]	1.20 (0.77, 1.37), p = 0.88	1.21 (0.74, 1.55), p = 0.72
TNF (pg/ml) (geometric mean [95%CI])*	223.5 [168.2–297.0]	265.4 [226.5–310.6]	1.21 (0.89, 1.65), p = 0.22	1.18 (0.85, 1.62), p = 0.32
IL-10 (pg/ml) (geometric mean [95%CI])*	123.9 [95.3–161.1]	119.2 [104.3–136.2]	0.97 (0.74, 1.27), p = 0.81	0.96 (0.73, 1.27), p = 0.77

1. positive by microscopy.
2. positive by PCR.
Abbreviations: BMI = body mass index, WHR = waist to hips ratio, FBG = fasting blood glucose, TC = total cholesterol, TG = triglyceride, HDL-c = high density lipoprotein cholesterol, LDL-c = low density lipoprotein cholesterol, TIgE = total immunoglobulin E, hs-CRP = high sensitive C reactive protein, TNF = tumor necrosis factor, IL-10 = interleukin 10. *Adjusted mean difference for TIgE, hs-CRP and cytokines were anti-log transformed and represent ratio.
doi:10.1371/journal.pone.0054855.t002

mentioned have focused on the adaptive responses and include Tregs [6–9]. It should be noted that, in line with the current data, a recent study in Gabon found that *S. haematobium* infected children develop a more pro-inflammatory TLR-mediated response [30]. We did not observe substantial differences in STH species and their effects on CVD risk factors.

Although we showed a relationship between helminth infections and traditional CVD risk factors, we did not find an association between helminth infections and cIMT. The following explanations may account for the absence of a relationship between helminth infection status and cIMT. In our study, helminth infection was assessed at one time-point. Theoretically, it may be possible that helminth-negative subjects in our study have only recently become helminth negative. In this situation, the beneficial effects of helminths can be seen on some of the traditional CVD risk factors, but not atherosclerosis development, as the dewormed state may have been too short to affect cIMT. As the development of atherosclerosis is a chronic process, ideally, lifetime exposure to helminth infections should be assessed in relation to atherosclerosis development. Although this is not feasible, the association was also not significant when TIgE, reflecting current and prior helminth infection, was considered. Another explanation is that the population studied has a low cardiovascular risk profile. Although the association of CVD risk factors and IMT in the Flores population was similar to other studies [4,31–34], mean IMT in the study population was lower than comparable age groups in Europe [35], the USA [36], and Japan [37]. Indeed most other CVD risk parameters were well within the normal reference range. In this situation, the absolute levels of CVD risk factors may be below the threshold for accelerated atherosclerosis development.

In conclusion, in a large cross-sectional study in an area endemic for helminth infections, we found an association between STH infection and conventional risk factors for CVD. The effect of helminth infections on CVD was at least partially mediated by an effect on BMI. However, no direct association between helminth infections and IMT was found. Nevertheless we believe that this study shows that in the explanation of increase in CVD in non-western societies, changes in infectious pressure should be taken into account. Further studies are necessary to assess the causal relationships between helminth infections and atherosclerosis.

Acknowledgments

The authors thank the team from University of Indonesia, the staff from Puskesmas Nangapanda, Ende health authorities, the community field workers and most of all the study participants from Nangapanda, Flores, Indonesia. Mylab25 ultrasound system and a LA523 13–4 MHz transducer were kindly provided for the duration of the study by ESAOTE, S.p.A, Maastricht, The Netherlands, who also provided the technical training regarding the ultrasound system for the investigators (AEW and LJW).

Author Contributions

Reviewed the manuscript: ES OMD MY JWAS. Conceived and designed the experiments: AEW JTT FP ES TS MY JWAS. Performed the experiments: AEW LJW FH MAP MMMK JJV. Analyzed the data: AEW LM OMD MY JWAS. Wrote the paper: AEW.

References

1. World Heatlh Organization (2010) Prevention of cardiovascular disease: guideline for assessment and management of cardiovascular risk. Geneva: WHO.
2. Ramachandran A, Ma RC, Snehalatha C (2010) Diabetes in Asia. Lancet 375: 408–418.
3. Chan JC, Malik V, Jia W, Kadowaki T, Yajnik CS, et al. (2009) Diabetes in Asia: epidemiology, risk factors, and pathophysiology. JAMA 301: 2129–2140.
4. Berenson GS, Srinivasan SR, Bao W, Newman WP III, Tracy RE, et al. (1998) Association between multiple cardiovascular risk factors and atherosclerosis in children and young adults. The Bogalusa Heart Study. N Engl J Med 338: 1650–1656.
5. Wiria AE, Djuardi Y, Supali T, Sartono E, Yazdanbakhsh M (2012) Helminth infection in populations undergoing epidemiological transition: a friend or foe? Semin Immunopathol 34: 889–901.
6. Ricci ND, Fiuza JA, Bueno LL, Cancado GG, Gazzinelli-Guimaraes PH, et al. (2011) Induction of CD4(+)CD25(+)FOXP3(+) regulatory T cells during human hookworm infection modulates antigen-mediated lymphocyte proliferation. PLoS Negl Trop Dis 5: e1383.
7. Watanabe K, Mwinzi PN, Black CL, Muok EM, Karanja DM, et al. (2007) T regulatory cell levels decrease in people infected with Schistosoma mansoni on effective treatment. Am J Trop Med Hyg 77: 676–682.
8. Wammes LJ, Hamid F, Wiria AE, de GB, Sartono E, et al. (2010) Regulatory T cells in human geohelminth infection suppress immune responses to BCG and Plasmodium falciparum. Eur J Immunol 40: 437–42.
9. Wammes LJ, Hamid F, Wiria AE, Wibowo H, Sartono E, et al. (2012) Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics. PLoS Negl Trop Dis 6: e1655.
10. Coutinho HM, Acosta LP, McGarvey ST, Jarilla B, Jiz M, et al. (2006) Nutritional status improves after treatment of schistosoma japonicum-infected children and adolescents. J Nutr 136: 183–188.
11. Gupta M, Arora KL, Mithal S, Tandon BN (1977) Effect of periodic deworming on nutritional status of ascaris-infested preschool children receiving supplementary food. Lancet 2: 108–110.
12. Doenhoff MJ, Stanley RG, Griffiths K, Jackson CL (2002) An anti-atherogenic effect of Schistosoma mansoni infections in mice associated with a parasite-induced lowering of blood total cholesterol. Parasitology 125: 415–421.
13. Stanley RG, Jackson CL, Griffiths K, Doenhoff MJ (2009) Effects of Schistosoma mansoni worms and eggs on circulating cholesterol and liver lipids in mice. Atherosclerosis 207: 131–138.
14. Wu D, Molofsky AB, Liang HE, Ricardo-Gonzalez RR, Jouihan HA, et al. (2011) Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. Science 332: 243–247.
15. Mancia G, De BG, Dominiczak A, Cifkova R, Fagard R, et al. (2007) 2007 Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). J Hypertens 25: 1105–1187.
16. Bots ML, Hoes AW, Koudstaal PJ, Hofman A, Grobbee DE (1997) Common carotid intima-media thickness and risk of stroke and myocardial infarction: the Rotterdam Study. Circulation 96: 1432–1437.
17. Cooper PJ, Alexander N, Moncayo AL, Benitez SM, Chico ME, et al. (2008) Environmental determinants of total IgE among school children living in the rural Tropics: importance of geohelminth infections and effect of anthelmintic treatment. BMC Immunol 9: 33.
18. Hamid F, Wiria AE, Wammes LJ, Kaisar MM, Lell B, et al. (2011) A longitudinal study of allergy and intestinal helminth infections in semi urban and rural areas of Flores, Indonesia (ImmunoSPIN Study). BMC Infect Dis 11: 83.
19. Wiria AE, Prasetyani MA, Hamid F, Wammes LJ, Lell B, et al. (2010) Does treatment of intestinal helminth infections influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study). BMC Infect Dis 10: 77.
20. Barzi F, Woodward M, Czernichow S, Lee CM, Kang JH, et al. (2010) The discrimination of dyslipidaemia using anthropometric measures in ethnically diverse populations of the Asia-Pacific Region: The Obesity in Asia Collaboration. Obes Rev 11(2): 127–36.
21. Friedewald WT, Levy RI, Fredrickson DS (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 18: 499–502.
22. Salonen JT, Salonen R (1993) Ultrasound B-mode imaging in observational studies of atherosclerotic progression. Circulation 87: II56–II65.
23. Pasha SM, Wiria AE, Wammes LJ, Smit JW, Partono F, et al. (2011) Blood pressure class and carotid artery intima-media thickness in a population at the secondary epidemiological transition. J Hypertens 29: 2194–2200.
24. Vasunilashorn S, Crimmins EM, Kim JK, Winking J, Gurven M, et al. (2010) Blood lipids, infection, and inflammatory markers in the Tsimane of Bolivia. Am J Hum Biol 22: 731–740.
25. Wiedermann U, Stemberger H, Unfried E, Widhalm K, Kundi M, et al. (1991) Intestinal worm burden and serum cholesterol or lipid concentration in a Shipibo population (Peru). Zentralbl Bakteriol 275: 279–286.
26. de la Llera MM, McGillicuddy FC, Hinkle CC, Byrne M, Joshi MR, et al. (2012) Inflammation modulates human HDL composition and function in vivo. Atherosclerosis 222: 390–394.
27. Coutinho HM, McGarvey ST, Acosta LP, Manalo DL, Langdon GC, et al. (2005) Nutritional status and serum cytokine profiles in children, adolescents, and young adults with Schistosoma japonicum-associated hepatic fibrosis, in Leyte, Philippines. J Infect Dis 192: 528–536.
28. Coutinho HM, Leenstra T, Acosta LP, Su L, Jarilla B, et al. (2006) Pro-inflammatory cytokines and C-reactive protein are associated with undernutrition in the context of Schistosoma japonicum infection. Am J Trop Med Hyg 75: 720–726.
29. Mwatha JK, Kimani G, Kamau T, Mbugua GG, Ouma JH, et al. (1998) High levels of TNF, soluble TNF receptors, soluble ICAM-1, and IFN-gamma, but low levels of IL-5, are associated with hepatosplenic disease in human schistosomiasis mansoni. J Immunol 160: 1992–1999.
30. Meurs L, Labuda L, Amoah AS, Mbow M, Ngoa UA, et al. (2011) Enhanced pro-inflammatory cytokine responses following Toll-like-receptor ligation in Schistosoma haematobium-infected schoolchildren from rural Gabon. PLoS One 6: e24393.
31. Jatoi NA, Jerrard-Dunne P, Feely J, Mahmud A (2007) Impact of smoking and smoking cessation on arterial stiffness and aortic wave reflection in hypertension. Hypertension 49: 981–985.
32. Lakka TA, Salonen R, Kaplan GA, Salonen JT (1999) Blood pressure and the progression of carotid atherosclerosis in middle-aged men. Hypertension 34: 51–56.
33. Van Bortel LM, Spek JJ (1998) Influence of aging on arterial compliance. J Hum Hypertens 12: 583–586.
34. Kallio K, Jokinen E, Saarinen M, Hamalainen M, Volanen I, et al. (2010) Arterial intima-media thickness, endothelial function, and apolipoproteins in adolescents frequently exposed to tobacco smoke. Circ Cardiovasc Qual Outcomes 3: 196–203.
35. Touboul PJ, Labreuche J, Vicaut E, Belliard JP, Cohen S, et al. (2009) Country-based reference values and impact of cardiovascular risk factors on carotid intima-media thickness in a French population: the 'Paroi Arterielle et Risque Cardio-Vasculaire' (PARC) Study. Cerebrovasc Dis 27: 361–367.
36. Soliman EZ, Ding J, Hsu FC, Carr JJ, Polak JF, et al. (2010) Association between carotid intima-media thickness and pericardial fat in the Multi-Ethnic Study of Atherosclerosis (MESA). J Stroke Cerebrovasc Dis 19: 58–65.
37. Kitagawa K, Hougaku H, Yamagami H, Hashimoto H, Itoh T, et al. (2007) Carotid intima-media thickness and risk of cardiovascular events in high-risk patients. Results of the Osaka Follow-Up Study for Carotid Atherosclerosis 2 (OSACA2 Study). Cerebrovasc Dis 24: 35–42.

chapter 6

Infection with soil-transmitted helminths is associated with increased insulin sensitivity. Experiments of nature on immune modulation and metabolism

Abstract

Systemic inflammation has been propagated an important phenomenon in the pathogenesis of type-2 diabetes. Remarkably helminth infections shift the immune system to an anti-inflammatory profile. We therefore hypothesized that helminth infections, as an experiment of nature, lead to decreased insulin resistance, which has not been studied before in humans. We performed a cross-sectional study in Flores, Indonesia, an area endemic for soil-transmitted helminths to explore whether helminth infections are associated with increased insulin sensitivity. Stool samples from 646 participants aged 18-80 years were collected and screened for Trichuris trichiura by microscopy and for Ascaris lumbricoides, Necator americanus, Ancylostoma duodenale, and Strongyloides stercoralis by qPCR. We documented data on body mass index (BMI), waist-to-hip ratio, fasting blood glucose, insulin, high sensitive C-reactive protein level and E. coli lipopolysaccharide stimulated cytokines (TNF and IL-10). The HOMA-IR was calculated and the association between helminth infection status and insulin resistance was tested by linear regression adjusted for age, sex and BMI. Participants with any helminth infection had lower BMI (kg/m2) (mean difference -0.63, 95%CI [-1.22, 0.02], p=0.044), WHR (-0.01, [-0.02, -0.00], p=0.020) as well as insulin (pmol/L) (0.85, [0.74, 0.98], p=0.023) and HOMA-IR (0.83, [0.73, 0.95], p=0.0075) than uninfected subjects. After adjustment for BMI the association between helminth infection and insulin (pmol/L) (mean difference 0.89, 95%CI [0.78, 1.01], p=0.081) as well as HOMA-IR (0.88, [0.77, 0.99], p=0.036) remained. We conclude that helminth infections are associated with improved insulin sensitivity, which may support a direct association between systemic inflammation and type-2 diabetes.

Aprilianto Eddy Wiria[1,2], Firdaus Hamid[1,3], Linda J Wammes[1,2], Margaretta Prasetyani[1,2], Olaf M Dekkers[4,5], Linda May[2], Maria MM Kaisar[1,2], Jaco J Verweij[2*], Bruno Guigas[2], Felix Partono[1], Erliyani Sartono[2], Taniawati Supali[1†], Maria Yazdanbakhsh[2†], Johannes WA Smit[5,6†]

† These authors contributed equally

1. Department of Parasitology, Faculty of Medicine, University of Indonesia, 10430 Jakarta, Indonesia
2. Department of Parasitology, Leiden University Medical Center, 2333ZA Leiden, The Netherlands
3. Department of Microbiology, Faculty of Medicine, Hasanuddin University, 90245 Makassar, Indonesia
4. Department of Clinical Epidemiology, Leiden University Medical Center, 2333ZA Leiden, The Netherlands
5. Department of Endocrinology & General Internal Medicine, Leiden University Medical Center, 2333ZA Leiden, The Netherlands
6. Department of General Internal Medicine, Radboud University Medical Center, 6525GA Nijmegen, The Netherlands
*current address: Laboratory for Medical Microbiology and Immunology, St. Elisabeth Hospital, 5022GC Tilburg, The Netherlands

– Manuscript is submitted –

Introduction

The prevalence of type-2 diabetes is rising in urban areas in low-to-middle income countries (LMIC) (1). In many Asian countries, rapid socioeconomic development has led to a shift in infrastructure, technology and food supply that promotes over nutrition and sedentary lifestyles (2, 3). Indeed, the increased prevalence of type-2 diabetes has traditionally been explained by decreased physical activity or excess consumption of high-energy foods, which lead to a disturbed energy balance. However, there is now compelling evidence that inflammation plays a role in chronic non-communicable diseases, including type-2 diabetes (4). Indeed, in type-2 diabetes, elevated levels of inflammation-related markers such as interleukin 6 (IL-6), IL-8, tumor necrosis factor (TNF), and C-reactive protein (CRP) (4) have been reported. In this respect, it is interesting that some chronic infections, such as helminth infections, which are highly prevalent in rural areas of LMIC, have been shown to induce anti-inflammatory and immune regulatory effects (5). One particular modifier are the production of T helper (Th) 2 cells and regulatory cytokines (IL-4, IL-5, IL-10, and IL-13), that are capable of keeping Th1 and TNF responses in balance. Therefore it has been hypothesized that chronic helminth infections by inducing anti-inflammatory responses decrease systemic inflammation and might be beneficial to the prevention of inflammatory diseases, such as allergy (6), inflammatory bowel disease (7), and metabolic diseases (8).

In addition, in animal models, the response associated with helminth infections can forestall obesity and enhance glucose tolerance (9). Currently, there are no published human studies on the relationship between helminth infections and glucose metabolism.

In the present study, we aim to investigate the relationship between helminth infections and insulin resistance, as assessed by HOMA-IR (10) in an area endemic for soil-transmitted helminths on Flores Island, Indonesia.

Material and Methods

Study objectives

The primary objective of the study was to investigate the association between helminth infections and HOMA-IR. Chronic inflammation is a central feature in the pathophysiology of IR and type-2 diabetes. Therefore our hypothesis was that, since helminths might induce an immune evasion strategy by inducing anti-inflammatory responses, HOMA-IR is lower in subjects with helminth infections than in subjects without helminth infections.

Study population

The study area is Nangapanda on Flores Island in Indonesia, highly endemic for soil-transmitted helminths (6, 11). In this area, a large investigational project is being conducted on the relationship between helminth infections and the immune system (ImmunoSPIN study (6, 11)). For the current study, a cross sectional representative sample was included from all inhabitants aged 18 years and above. Data were collected between May-August 2009.

Study design

From 1841 inhabitants age 18 years and above in Nangapanda who participated in the ImmunoSPIN project, 683 were randomly selected to participate in the present cross-sectional

study and invited to provide data on BMI, WHR, and blood sampling for fasting glucose (FBG), insulin, High sensitive C-reactive protein (hs-CRP) and whole blood culture. 646 subjects of whom data on helminth infections, BMI and WHR ratio are available were included in the present analysis. In 584 of these subjects, laboratory measurements were performed.

The study was approved by the ethical committee of the Faculty of Medicine, University of Indonesia, ref: 194/PT02.FK/Etik/2006 with addendum ref: 96/PT02.FK/Etik/2010 and registered as clinical trial ref: ISRCTN83830814 and was filed by the Leiden University Medical Center Committee of Medical Ethics (CME). Because of the high rate of illiteracy amongst elderly participants, either written or verbal informed consent was obtained from each participant.

Clinical and laboratory assessment

Anthropometric measurements of body weight (SECA 761, SECA GMBH & Co. Kg., Hamburg, Germany), height (SECA 206, SECA GMBH & Co. Kg., Hamburg, Germany), waist and hip circumference (SECA 203, SECA GMBH & Co. Kg., Hamburg, Germany) were performed using the NHLBI practical guidelines (NHLBI web). Abnormal BMI is defined as ≥ 25 kg/m^2 and the Asian modified abnormal waist hip ratio (WHR) is >0.9 (men) and >0.8 (women) (12). Impaired fasting glucose (FBG) was defined as ≥5.6 mmol/L (13). A FBG ≥7.1 mmol/L of is indicative of diabetes mellitus (13). All participants were instructed to be fasting before venous sampling. FBG was analyzed using Breeze®2 glucose meter (Bayer Health Care LLC, Basel, Switzerland). Insulin was measured using MSD® 96-Well MULTI-ARRAY® Human insulin assay (Meso Scale Discovery, Gaithersburg, USA). HOMA-IR, a well-validated measure of IR was calculated to estimate insulin resistance (10). High sensitive C-reactive protein (Hs-CRP) level was measured using MSD® 96-Well MULTI-ARRAY® CRP Assay (Meso Scale Discovery, Gaithersburg, USA). Health questionnaires on smoking habits, medical history, and family history were collected.

Helminth status

Stool samples were collected and preserved in 4% formaldehyde for microscopy examination or frozen (-20°C) unpreserved for PCR detection. The formol-ether acetate concentration method was performed on the formalin preserved stool samples followed by microscopy examination for *Trichuris trichiura* infections (11). As described in detail before (11), DNA was isolated from approximately 100 mg unpreserved feces and a multiplex real-time PCR for the detection of *Ancylostoma duodenale*, *Necator americanus*, *Ascaris lumbricoides* and *Strongyloides stercoralis* was performed. The real-time PCR output from this system consisted of a cycle-threshold (CT) value, representing the amplification cycle in which the level of fluorescent signal exceeds the background fluorescence, and reflecting the parasite-specific DNA load in the sample tested. Negative and positive control samples were included in each run of the amplification. We defined a positive case for *T. trichiura* by the egg findings and for *A. duodenale*, *N. americanus*, *A. lumbricoides* and *S. stercoralis* by parasite-specific DNA amplification. Participants were also grouped by number of helminth species infections.

Whole blood stimulation and cytokine measurement, and haemoglobin count

The procedure of whole blood stimulation and cytokines measurement has been described previously (11). Briefly, heparinized blood was diluted 4x and stimulated within 6 hours after drawing.

Stimulations were performed with control medium or *E. coli* lipopolysaccharide (LPS, 1 ng/ml Sigma-Aldrich, Zwijndrecht, The Netherlands), incubated for 24 hours at 37^{0}C and 5% CO_2. The supernatants were frozen at -20^{0}C and transported to Jakarta where TNF and IL-10 supernatants were assessed by means of immunobead-based multiplex assays on a Liquichip 200® Workstation (Qiagen, Venlo, The Netherlands) using Liquichip analyzer software (Qiagen, Venlo, The Netherlands). Samples with TNF levels higher than 250 pg/ml in medium stimulation were excluded from further analyses (2 samples). Haemoglobin was determined using heparinized blood on a routine cell counter (Coulter® Ac-T™ diff Analyzer, Beckman Coulter Inc., Fullerton, CA, USA)

Statistical analysis

Participant characteristics were stratified for helminth uninfected and infected. Insulin, HOMA-IR, cytokines and CRP concentrations were normalized by log-transformation. Analyses were performed with these log transformed values but results were presented as geometric means after exponentiation of the values on a logarithmic scale. Linear regression was used to study the associations between infection with any helminth species as well as the number of helminth species and HOMA-IR and adjusted for age and sex. To assess whether a potential association between helminth infection and HOMA-IR is mediated through an effect on BMI or WHR, we adjusted for BMI or WHR. Furthermore, in the helminth infected group we tested the association of helminth intensity per species with HOMA-IR. Differences between infected and uninfected participants were reported as mean differences with 95% confidence intervals (95% CI). P values <0.05 were considered to be statistically significant. Statistical analysis was performed with SPSS statistics 17.0.2 (SPSS Inc., Chicago, Illinois, The USA).

Results

Characteristics of study participants

A total of 424 participants infected with at least one species of soil-transmitted helminths were compared to 222 uninfected participants (Table 1). There were slightly more males in the infected group (38%) than in the uninfected group (34%), whereas the mean age was similar (45.1 vs 44.7 years). The most prevalent soil-transmitted helminth species were *N. americanus* 334/646 (51.7%), *A. lumbricoides* 141/646 (21.8%) and *T. trichiura* 127/646 (19.7%). The proportion of participants infected with *A. duodenale* 24/646 (3.7%) and with *S. stercoralis* 4/646 (0.6%) was clearly lower. 261 participants were infected with one helminth species only, 124 with two helminth species and 39 with 3 or more helminth species. 322 of 584 (55.1%) participants had elevated FBG (≥5.6 mmol/L) of whom 27 (4.6%) with FBG ≥7-11 mmol/L and 10 (1.7%) ≥11 mmol/L. We found no significant differences in hemoglobin levels between participants infected with helminths and uninfected participants.

Association between helminth infection and glucose metabolism

Participants with any helminth infection had lower BMI (kg/m2) (mean difference -0.63, 95%CI [-1.22, -0.02]), WHR (-0.01, [-0.02, -0.00]), insulin (pmol/L) (0.85, [0.74, 0.98]) and HOMA-IR (0.83, [0.73, 0.95]) than uninfected participants (Table 1). After adjustment for BMI, the association between helminth infection and insulin (0.89, [0.78, 1.01]) as well as HOMA-IR (0.88,

Table 1. Characteristics of the study population

	Helminth u ninfected (n=222)	Helminth infected (n=424)	Mean difference adjusted for age and sex (95% confidence interval)	Mean difference adjusted for age, sex and BMI (95% confidence interval)
Age (year) (mean, Range)	44.4 (18.2-79.4)	45.2 (18.0-79.4)	-	-
Male (%)	33.5	37.8	-	-
Trichuris trichiura[1] (%)	0	30.7	-	-
Ascaris lumbricoides[2] (%)	0	33.1	-	-
Necator americanus[2] (%)	0	78.2	-	-
Ancylostoma duodenale[2] (%)	0	5.3	-	-
Strongyloides stercoralis[2] (%)	0	0.9	-	-
BMI (Kg/m2) (mean, SD)	23.2 (3.7)	22.5 (3.8)	-0.63 (-1.22, -0.02), p=0.044	-
WHR (mean, SD)	0.89 (0.07)	0.88 (0.06)	-0.01 (-0.02, -0.00), p=0.020	-
FBG (mmol/L) (mean, SD)	5.9 (1.5)	5.9 (1.6)	-0.05 (-0.31, 0.22), p=0.73	0.01 (-0.25, 0.27), p=0.93
Haemoglobin (g/dl) (mean, SD)	14.5 (2.5)	14.5 (2.7)	0.05 (-0.46, 0.56), p=0.84	0.07 (-0.44, 0.58), p=0.80
Insulin (pmol/L) (geometric mean [95%CI])	38.1 (34.1-42.5)	32.0 (29.5-34.8)	*0.85 (0.74, 0.98), p=0.023	*0.89 (0.78, 1.01), p=0.081
HOMA-IR (geometric mean [95%CI])	0.7 (0.7-0.8)	0.6 (0.6-0.7)	*0.83 (0.73, 0.95), p=0.0075	*0.88 (0.77, 0.99), p=0.036
Hs-CRP (mg/l) (geometric mean [95%CI])	0.5 (0.4-0.6)	0.5 (0.4-0.6)	*0.95 (0.75, 1.30), p=0.96	*1.02 (0.77, 1.36), p=0.88
TNF (pg/ml)† (geometric mean [95%CI])	222.5 (180.0-275.1)	281.2 (245.4-322.2)	*1.27 (0.99, 1.63), p=0.056	*1.26 (0.98, 1.61), p=0.073
IL-10 (pg/ml)† (geometric mean [95%CI])	132.3 (109.7-159.7)	127.9 (114.2-143.2)	*0.98 (0.79, 1.21), p=0.82	*0.96 (0.78, 1.19), p=0.74

1. positive by microscopy
2. positive by PCR

Abbreviations: BMI = body mass index, WHR = waist to hips ratio, FBG = fasting blood glucose, HOMA-IR = Homeostasis model assessment for insulin resistance, Hs-CRP = High sensitive C reactive protein, TNF = tumor necrosis factor, IL-10 = interleukin 10. †The cytokines were measured from stimulated whole blood for 24h with *E. coli* lipopolysaccharide (LPS). *Adjusted mean difference for insulin, HOMA-IR, hs-CRP and cytokines were anti-log transformed and represent ratio of the measurement between uninfected and infected group.

[0.77, 0.99]) remained (Figure 1). No clear associations were found between helminth infection and FBG, hs-CRP or TNF-LPS. Using WHR as a marker of central obesity gave similar results that there is negative association between helminth and insulin as well HOMA-IR (data not shown). Additional correction for smoking, medical history, and familial history did not change the association between helminth infection and HOMA-IR (data not shown).

When considering the relationship between the number of helminth species, we found that BMI, WHR, insulin and HOMA-IR were negatively associated with the number of helminth species (p=0.036, p=0.010, p<0.001, p<0.001, respectively). Adjustment for BMI attenuated the association between number of infections and insulin as well as HOMA-IR but both associations remained significant (p=0.0015 and p<0.001, respectively). The results were also similar after adjustment for WHR (p=0.0024 and p=0.0013, respectively). TNF-LPS was positively associated with increasing number of helminth species with the highest LPS-levels in participants with 3 or more infections. This association was not attenuated after adjustment for BMI or WHR (p=0.035 and p=0.032, respectively).

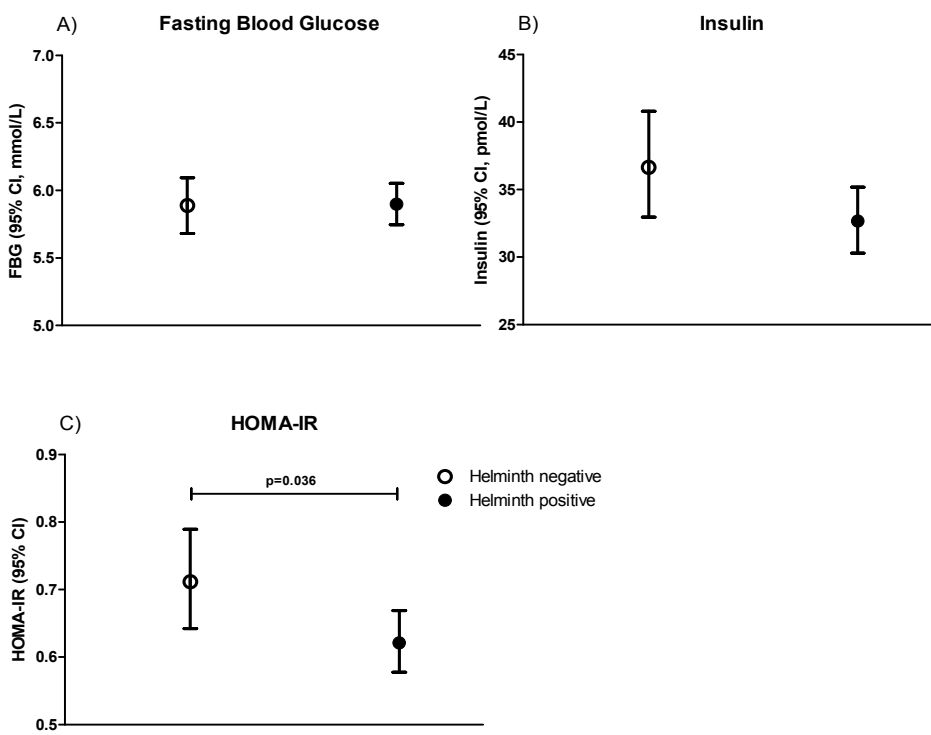


Figure 1. The association of soil-transmitted helminths infection and metabolic parameters. Relation of helminth infection with (A) fasting blood glucose (FBG), (B) insulin* and (C) HOMA-IR* with correction for age, sex, and BMI. 208 participants had no helminth infection and 376 were infected with at least one helminth species. Mean FBG was 5.9 mmol/L 95%CI (5.7, 6.1) and 5.90 (5.8, 6.1), insulin was 36.6 pmol/L (33.0, 40.8) and 32.7 (30.3, 35.2), and HOMA-IR was 0.7 (0.6, 0.8) and 0.6 (0.6, 0.7) for helminth-uninfected and infected group, respectively. *Adjusted mean for insulin and HOMA-IR were anti-log transformed and represents geometric means.

We also investigated whether there were differences in the association between different species of helminths with HOMA-IR. We found no association between *N. americanus*, *A. lumbricoides*, or *T. trichiura*, individually with HOMA-IR (prevalence of *S. stercoralis* and *A. duodenale* were too low to be considered).

Discussion

The objective of this study was to examine the association between helminth infections and insulin resistance in a population residing in an area highly endemic for soil-transmitted helminths. The hypothesis being tested is that helminth infections may have a direct beneficial effect on glucose metabolism, by influencing systemic inflammation.

The influence of helminth infections has been shown in animal models of type-1 diabetes (T1D) (14, 15) or type-2 diabetes (9) but has not been studied in humans. In our study, we found a negative association between HOMA-IR with helminth infections, which was independent of BMI or WHR.

While both helminth infected and uninfected participants in our study had relatively low HOMA-IR, we found that helminth infection was associated with even lower HOMA-IR. No clear association was found for each single helminth species in the absence of infection with other helminths.

Studies in experimental models have shown that injecting helminth antigens to young non-obese-diabetic mice prevented the onset of T1D. This inhibition of T1D development appeared to be due to the ability of helminth and its products to induce IL-10 production by dendritic cells, B cells, alternatively activated macrophages, as well as regulatory T cells (16). In the model of type-2 diabetes, mice with high fat diet that were infected with helminths, had eosinophilia, became less obese and less insulin resistant which seemed to be in conjunction with maintenance of alternative activated macrophages in adipose tissues (9). Ricardo-Gonzales *et al*, have also shown that the IL-4/STAT6 immune axis, which is a key pathway affected by helminths, promotes control on peripheral nutrient metabolism and insulin sensitivity (17).

Inflammation is known to be an important factor in the pathogenesis of type-2 diabetes (4) and increased CRP has been shown to be either a causal or a prediction marker of metabolic syndrome and IR (18-22). Individuals with Asian descent may exhibit the characteristics of inflammation while relatively lean (2, 23, 24), however, our participants who were lean, had also very low hs-CRP level (0.4-0.6 mg/L) and even lower than what has been reported in China (0.6-0.8 mg/L) (21, 22), South Asia (0.9-2.8 mg/L in rural and in urban 2.2-2.6 mg/L) (19, 20) or US population of various ethnicities (1.1-4.5 mg/L) (18, 24, 25).

We acknowledge the limitation of this cross-sectional study in nature. As the development of type-2 diabetes is a chronic process, ideally, lifetime exposure to helminth infections should be related to the disease development, which is however not feasible. The causal relationship between helminth infection and glucose metabolism could be studied in a placebo-controlled trial with anti-helminth treatment.

Conclusion

In a large cross-sectional study in an area endemic for helminth infections, we found an association between soil-transmitted helminths infection and decreased insulin resistance. We

believe that this experiment of nature supports the notion of a direct relationship between systemic inflammation and the pathogenesis of type-2 diabetes, although further studies are needed to assess the causal relationships.

Acknowledgments

The authors thank the team from University of Indonesia, the staff from Puskesmas Nangapanda, Ende health authorities, the community field workers and most of all the study participants from Nangapanda, Flores, Indonesia.

Transparency declaration

All authors declare that they have no conflict of interest.

This work was supported by the following grants: The Royal Netherlands Academy of Arts and Science (KNAW), Ref.KNAW-05-PP-35, European Commission contracts INCO-CT-2006-031714 and INCO-CT-2006-032436, and the Prof. Dr. P.C. Flu Foundation.

Authorship/Contribution

Conceived and designed the experiments: AEW, FP, ES, TS, MY and JWAS. Patient enrolment and performed the experiment: AEW, FH, LJW, and MAP. PCR detection on helminths: MMMK and JJV. Analyzed and interpreted the data: AEW LM OMD MY JWAS. Wrote the paper: AEW. Review of paper: ES, OMD, BG, MY and JWAS. All authors revised the report for important intellectual content and have seen and approved the final version. MY and TS had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Reference List

1. World Health Organization. Diabetes Programme. Available at: http://www.who.int/diabetes/en/. Accessed 30 November 2012.
2. Chan JC, Malik V, Jia W, et al. Diabetes in Asia: epidemiology, risk factors, and pathophysiology. JAMA 2009; 301:2129-2140.
3. Ramachandran A, Ma RC, Snehalatha C. Diabetes in Asia. Lancet 2010; 375: 408-418.
4. Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. J Clin Invest 2006; 116: 1793-1801.
5. Wiria AE, Djuardi Y, Supali T, Sartono E, Yazdanbakhsh M. Helminth infection in populations undergoing epidemiological transition: a friend or foe? Semin Immunopathol 2012; 34: 889-901.
6. Hamid F, Wiria AE, Wammes LJ, et al. A longitudinal study of allergy and intestinal helminth infections in semi urban and rural areas of Flores, Indonesia (ImmunoSPIN Study). BMC Infect Dis 2011; 11: 83.
7. Summers RW, Elliott DE, Urban JF, Jr., Thompson RA, Weinstock JV. Trichuris suis therapy for active ulcerative colitis: a randomized controlled trial. Gastroenterology 2005; 128: 825-832.
8. Chawla A, Nguyen KD, Goh YP. Macrophage-mediated inflammation in metabolic disease. Nat Rev Immunol 2011; 11: 738-749.
9. Wu D, Molofsky AB, Liang HE, et al. Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. Science 2011; 332: 243-247.
10. Wallace TM, Levy JC, Matthews DR. Use and abuse of HOMA modeling. Diabetes Care 2004; 27: 1487-1495.
11. Wiria AE, Prasetyani MA, Hamid F, et al. Does treatment of intestinal helminth infections influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study). BMC Infect Dis 2010; 10: 77.
12. Barzi F, Woodward M, Czernichow S, et al. The discrimination of dyslipidaemia using anthropometric measures in ethnically diverse populations of the Asia-Pacific Region: the Obesity in Asia Collaboration. Obes Rev 2010; 11: 127-136.
13. IDF Clinical Guidelines Task Force. Global guideline for Type 2 diabetes. Brussels: International Diabetes Federation, 2005. Available at: http://www.idf.org/webdata/docs/IDF%20GGT2D.pdf. Accessed 30 November 2012.
14. Liu Q, Sundar K, Mishra PK, et al. Helminth infection can reduce insulitis and type 1 diabetes through. Infect Immun 2009; 77: 5347-5358.
15. Saunders KA, Raine T, Cooke A, Lawrence CE. Inhibition of autoimmune type 1 diabetes by gastrointestinal helminth infection. Infect Immun 2007; 75: 397-407.
16. Cooke A. Review series on helminths, immune modulation and the hygiene hypothesis: how might infection modulate the onset of type 1 diabetes? Immunology 2009; 126: 12-17.
17. Ricardo-Gonzalez RR, Red EA, Odegaard JI, et al. IL-4/STAT6 immune axis regulates peripheral nutrient metabolism and insulin sensitivity. Proc Natl Acad Sci U S A 2010; 107: 22617-22622.
18. Coe CL, Love GD, Karasawa M, et al. Population differences in proinflammatory biology: Japanese have healthier profiles than Americans. Brain Behav Immun 2011; 25: 494-502.
19. Mahajan A, Jaiswal A, Tabassum R, et al. Elevated levels of C-reactive protein as a risk factor for metabolic syndrome in Indians. Atherosclerosis 2012; 220: 275-281.
20. Indulekha K, Surendar J, Mohan V. High sensitivity C-reactive protein, tumor necrosis factor-alpha, interleukin-6, and vascular cell adhesion molecule-1 levels in Asian Indians with metabolic syndrome and insulin resistance (CURES-105). J Diabetes Sci Technol 2011; 5: 982-988.
21. Ye X, Yu Z, Li H, Franco OH, Liu Y, Lin X. Distributions of C-reactive protein and its association with metabolic syndrome in middle-aged and older Chinese people. J Am Coll Cardiol 2007; 49: 1798-1805.
22. Yang T, Chu CH, Hsieh PC, et al. C-reactive protein concentration as a significant correlate for metabolic syndrome: a Chinese population-based study. Endocrine 2012; 10.1007/s12020-012-9743-7 [doi]
23. Pandit K, Goswami S, Ghosh S, Mukhopadhyay P, Chowdhury S. Metabolic syndrome in South Asians. Indian J Endocrinol Metab 2012; 16: 44-55.
24. Shah T, Newcombe P, Smeeth L, et al. Ancestry as a determinant of mean population C-reactive protein values: implications for cardiovascular risk prediction. Circ Cardiovasc Genet 2010; 3: 436-444.
25. Ford ES. Body mass index, diabetes, and C-reactive protein among U.S. adults. Diabetes Care 1999; 22: 1971-1977.


WILL
WORK
FOR

chapter 7

Summarizing Discussion

To de-worm or to re-worm:
the impact of helminth infections
on co-infections and on health
outcomes on Flores island, Indonesia

Abstract

Many countries are currently at various stages of an "epidemiological transition" in which infectious disease is replaced by cardiovascular disease (CVD) as the major cause of death. Evidence suggests that a sedentary lifestyle, with low physical activity and excessive caloric intake, are risk factors for metabolic syndrome and CVD. In addition to life style, low grade chronic inflammation seems to also be associated with CVD. In fact, the so called inflammatory diseases such as allergies, autoimmunities, inflammatory bowel diseases are all on the rise in affluent countries and in urban centers of low-to-middle income countries (LMIC). It is interesting, that certain infections, such as bacterial or protozoan, lead to inflammation, which when uncontrolled can lead to death, while others, such as helminth infections are generally believed to lead to anti-inflammatory responses. As helminth infections can induce host immune hyporesponsiveness, we hypothesized that this would affect co-infections, such as malaria, and inflammatory diseases, such as allergies, and that deworming would modulate these effects. The variability of the geographical and disease landscapes in Indonesia provided an opportunity to investigate whether helminth infections and a deworming program could have an impact on co-infections and other health outcomes. This thesis describes the results of a longitudinal study on a household-based cluster-randomized double-blinded placebo-controlled anthelminthic trial on malarial parasitemia, allergy and immune responsiveness, with particular focus on malarial parasitemia. In addition and as a starting point for future studies, a cross-sectional study in a subset population on the association between helminth infection and risk factors for CVD and insulin resistance is also reported.

Thesis outline

The previous chapters of this thesis described the findings of our study on the Island of Flores, Indonesia, where we explored the hypothesis that helminth infections, by inducing immune hyporesponsiveness, have an impact on co-infections (lead to higher malarial parasitemia and lower malaria disease) and on inflammatory diseases (suppress allergic reactions and allergic inflammation). We investigated whether periodic anthelminthic treatment, using single dose albendazole, can alleviate helminth-induced immune hyporesponsiveness. In **Chapter 1**, we reviewed and discussed current knowledge of major helminth infections in the shaping of host immune responses, consequences for other infections, and the outlook related to the increasing prevalence of allergy, autoimmune disease, type-2 diabetes (T2D) and CVD. In the context of malaria, while a host requires adequate pro-inflammatory responses as protection against malarial parasites (1), the inability to control inflammatory responses can be associated with pathology (2;3). The question can be posed as to whether deworming in areas where malaria is co-endemic can improve the clearance of malarial parasites but increase risk of unwanted clinical outcomes (**Chapter 2)**. Using a household-clustered randomized double-blinded placebo-controlled anthelminthic trial, we investigated the effects of reducing helminth infections on the prevalence of malarial parasitemia in albendazole-treated group compared to a placebo-treated group (**Chapter 3**). We also asked whether we can alleviate helminth-induced immune hyporesponsiveness in the albendazole treatment group (**Chapter 4**). The household-clustered treatment randomization was aimed at reducing possible infection through other household members. As inflammation is a known risk factor for T2D and CVD (4;5), we explored the possibility that helminth-induced hyporesponsiveness might have a beneficial effect on preventing the development of T2D and CVD (6). We therefore investigated the association between helminth infection and risk factors for CVD and carotid intima media thickness (cIMT) (**Chapter 5**), as well as between the parasite and metabolic parameters and insulin resistance (IR) (**Chapter 6**).

Introduction

The term "epidemiological transition" is used to classify a population undergoing a transition from a state where infectious diseases (communicable diseases) as the major cause of death to a state in which CVD (non-communicable diseases) is the main cause of death (7). This transition is at different stages in different regions of the world. In industrial/developed countries (also known as high income countries [HIC]), infectious diseases are relatively well controlled compared to developing or low-to-middle income countries (LMIC), especially when compared to rural areas of LMIC. Causes of death in HIC are mainly due to degenerative diseases or to metabolic syndrome (MetS) related non-communicable diseases such as T2D and CVD (8). The results of a global disease burden assessment were reported recently and in some HIC regions, although still showing a high incidence, CVD is now better controlled (9). However, while the LMIC, which include the majority of the world's population, are still struggling with infectious diseases, in their urban areas, they are also facing the emergence of health problems similar to those of HIC. Indonesia is one of the emerging world economies and covers a large geographic area that includes a constellation of islands between the Asian and the Australian

continental plates, forming a crossroad between the Pacific and Indian Oceans. In terms of lifestyle and disease landscape, the urban areas in Indonesia are reasonably comparable to developed countries and deworming programs in these areas have been reported (10). The recent improvements in infrastructure and control of infectious diseases in rural and semi-urban areas may represent a paradigm for the transition to a sedentary lifestyle coupled to a decline in infections (also including reduced helminth infections). A better understanding of the dynamic changes between communicable and non-communicable diseases could help guide a healthier transition.

Helminth parasites generally cause chronic infections in their host, which results from their ability to influence the immune system of the host leading to immune hyporesponsiveness. This ensures the long term survival of the parasites in the human host while possibly benefiting the host by preventing immune pathological reactions (11). As it can be assumed that both the host and the helminth evolve within this relationship, a sudden deworming process might disrupt this relationship. It is important to note that helminth induced immune hyporesponsiveness may also affect host responses to bystander antigens (12). In rural LMIC, helminth infections are commonly co-endemic with malaria, tuberculosis (TB) or HIV/AIDS (13) and therefore the question what the consequence of deworming will be on co-endemic infections needs to be answered. Helminths are also thought to be associated with a lower incidence of allergy (14) and autoimmune disease (15) again raising the question whether these diseases patterns would change in the absence of helminth infections. The prevalence of T2D and CVD in rural LMIC, while increasing, are lower than urban areas of LMIC or in HIC (9). The evidence of the possible association between helminth infections and T2D and CVD in experimental models is gradually becoming available but in humans this has remained largely unexplored (6).

The large geographic area of Indonesia and the widespread population, with a declining helminth burden in some places, provides an opportunity to investigate whether helminth infections and deworming have an impact on co-infections such as malaria and on other health outcomes such as allergy, T2D and CVD. Specifically, here, we discuss our investigation of the relationship between soil-transmitted helminths (STH) and malaria, allergy, IR (a marker for T2D) and atherosclerosis (a marker for CVD).

Albendazole treatment reduced but did not eliminate helminth infections

In **Chapters 3** and **4**, we showed that a two-year course of intensive albendazole treatment reduced but did not entirely eliminate helminth infections. Other longitudinal, three to four monthly interval deworming trials, using various drugs against STH have also reported difficulties in eliminating helminths (16-18). These poor results could be related to highly contaminated environment and reinfection but also to the limited efficacy of available anthelminthic drugs or the possible emergence of drug resistance (19). Altogether, in terms of egg load, these earlier studies (16-18) reported significant decreases that were similar to our own findings. Using a sensitive PCR diagnostic method, we have also shown that evaluation of deworming by microscopy can fail to identify submicroscopic infections. This represents a possible source of continuing transmission when treatment is discontinued. Given that global campaigns are underway to control and eliminate STH, our findings and those of others suggest an urgent need for new approaches in helminth elimination programmes.

The impact of deworming on malarial outcomes

Despite a significantly reduced load of helminth infections following deworming, these parasites remained, in part due to persisting *Trichuris* worms, which appear not to be affected by albendazole used as a single dose at three monthly intervals. In addition, compared to our preliminary findings during the piloting phase of the program, the prevalence of malarial parasitemia in our study population dropped at the beginning of the study and gradually declined during the study period, which led to the low level of malarial prevalence in our co-infection study. This situation affected the study power and the statistical analyses, especially at the end of the study. Nevertheless, we found that compared to placebo, the prevalence of malarial parasitemia was transiently increased in the albendazole group, when there was sufficient transmission. Findings regarding clinical symptoms were not significant (**Chapter 3**). Malaria-related clinical symptoms were also not paralleled by the malarial parasitemia findings during the study, suggesting that most malaria in the population is asymptomatic. This might be related to the previously high rates of malaria transmission in the area, which may have led to a certain degree of immunity to malaria in this population.

Regarding the recent fall in the prevalence of malarial parasitemia, a model has shown that the loss of protective immunity is gradual (22). Our malaria-infected subjects were found to be relatively asymptomatic, but malarial parasitemia prevalence was higher in the younger age group. Therefore, while both immunity to disease severity and parasitemia might be well established in the older age group, in younger children an earlier malaria exposure may have only resulted in immunity to clinical disease. The low malaria transmission may have played a role in our study, resulting in new infections that were insufficient to provoke clinical symptoms. This was indeed shown in a study in five communities in Kenya and Gambia with differing levels of malaria transmission, in which higher malaria transmission was related to greater severity of malaria (23).

The increased risk of malarial parasitemia was not expected. However, a recent review by Nacher suggested that *Ascaris* infection is associated with protection against malaria disease or parasitemia (20), perhaps indicating that the increased risk for malarial parasitemia in our study participants may have been caused by a reduced level of *Ascaris* infection. However, as our study was not designed to stratify the analyses based on helminth species at baseline, the power of the study was insufficient to address this specific question. The same problem precluded the investigation of the effects of deworming on different malaria species.

Another question is why the effect of albendazole treatment was primarily confined to individuals >15 years of age. A possible explanation is that the lower *Ascaris* infections in the older age group compared with the younger, may mean better reduction of infection and therefore more profound effect on malarial parasitemia in those older than 15 years of age. Similar findings were reported from a small study in Madagascar where higher malarial parasitemia was seen in older age group, the group in which *Ascaris* egg loads were strongly decreased following anthelminthic treatment (16;21).

Deworming to improve immune responsiveness

Through investigations in children infected with STH, we showed that depletion of regulatory T cells (Treg) improved responses to in vitro malaria antigen stimulation (18). Indeed, we found that in vitro immune responses were enhanced after albendazole treatment and significant

increase in malaria-specific and mitogen-induced tumor necrosis factor (TNF) and interferon (IFN)-γ cytokine production was seen (**Chapter 4**). However, our hypothesis that alleviation of immune suppression by helminth infections would stimulate a better clearance of malarial parasites (and therefore result in lower prevalence of this parasite in our study population), in parallel with an increase in clinical outcomes, was not clearly resolved. As mentioned previously, albendazole treatment had neither a beneficial nor a detrimental impact in terms of clinical outcomes (**Chapter 3**). Despite the possibly already established immunity to clinical malaria, one can speculate that the increased pro-inflammatory responses to the malarial parasite observed during the trial may not be sufficient to cause clinical symptoms. This could be due to the maintenance of a certain level of anti-inflammatory responses, because of incomplete deworming that resulted in a sufficient immune suppression to prevent the development of clinical symptoms.

Two years of deworming has a minimal impact on allergy outcomes

In brief, we also found no significant impact of albendazole treatment on allergy outcomes. Although not statistically significant, the trend for increased skin prick test (SPT) reactivity was in line with our hypothesis. The risk of SPT reactivity increased incrementally with longer treatment and raises the question of whether even longer deworming periods are needed to produce more pronounced effects on allergic outcomes, as seen in a study where 15-17 years of ivermectin treatment for onchocerciasis in Ecuador resulted in increased prevalence of SPT reactivity (24). The question of whether this is related to differences in the prevalence and type of helminth infections remains unanswered. As with investigations of helminth-malaria co-infection outcomes, further research is needed before sufficient information is available on whether helminths may provide protection or actually increase the risk for the development of allergies as suggested by some investigations (25;26).

Helminth infection is negatively associated with risk factors for T2D and CVD

We have shown in cross sectional manner, in **Chapter 5,** that STH infection was associated with lower risk factors for CVD. Individuals with helminth infections had a significantly lower body mass index (BMI) or waist-to-hip ratio, and lower lipid levels, compared to uninfected individuals. However, a direct association between helminth infection and CVD markers, such as cIMT as a marker of atherosclerosis (**Chapter 5**), was not clear cut. In addition, no significant difference in fasting blood glucose (FBG) was found between infected and uninfected individuals. Indeed, the cIMT of our study participants was relatively low compared to individuals of the same age living in HIC (27;28). It is possible that cIMT level and FBG are still within the normal range, and therefore could not be affected by helminth infections.

In **Chapter 6** we have shown that helminth infection was associated with improved insulin sensitivity, despite this, there was no significant difference in FBG level as already alluded to. The improvement in insulin sensitivity seemed to be related, in part, to lower levels of insulin. This lower insulin level might be due to less insulin being needed to maintain normal blood glucose in individuals with helminth infections.

In line with our study, where we showed helminth infections were associated with lower lipids, in apoE-/- mice, factors released from *Schistosoma mansoni* eggs appeared to have

lipid-lowering effects (29). Indeed, it was recently shown in an experimental hookworm model that infection with *Nippostrongylus brasiliensis* could prevent obesity, as well as reduce lipid levels and improve insulin sensitivity (30). The authors showed that the protective effect was associated with eosinophilia induced by helminth infection and was related to interleukin (IL)-4 mediated alternatively activated macrophages in adipose tissue. Moreover, Bhargava et al. also demonstrated that lacto-N-fucopentose III, an immunomodulatory glycan that can be found in human milk and in parasitic helminths (in *S. mansoni* soluble egg antigen), could improve insulin sensitivity by enhancing white adipose tissue insulin signalling through induction of IL-10 production (31). Whether helminth infections lead to similar changes in humans still requires further investigation (Fig. 1).

C-reactive protein (CRP) is commonly measured as a marker of inflammation (32). Measuring high sensitivity (hs)-CRP in our study participants, we found a very low level of hs-CRP despite the probable high infection pressure in the community. As blood samples were analyzed in a laboratory facility in the Netherlands that carries out routine measurements using a gold standard assay, differences in laboratory protocols cannot account for the low hs-CRP level. Although studies in Philippines (33) and Ghana (34) show also low level of CRP in rural areas where high infection rates are expected, in the Tsimane Indians in Bolivia, high levels of CRP were measured (35). These variable findings need to be further investigated. Interestingly, total IgE (TIgE) levels have been reported to be inversely correlated with growth and the hs-CRP level in LMIC (36). The level of TIgE is thought to reflect the length of helminth exposure during the host lifetime (37). The inverse association is suggested to be related to a concept of trade off in the use of limited energy available to the host defence (36;37). Blackwell and colleagues have put forward the following explanation: when facing high burden of different infections, there would be a trade-off between defence against helminths (with production of TIgE) and defence against other infections, such as bacterial or protozoan, that are associated with elevated CRP (36). This argument is not entirely satisfactory, as helminth infections are altogether considered less life threatening than bacterial or protozoan infections. Alternative explanations such as the ability of helminth infections (high IgE) to suppress inflammation (low CRP) needs to be tested.

Is there a similarity in the association of helminths and CVD to that of helminths and allergy?

It might be interesting to test whether helminths or their products can be used as a treatment for CVD, as extensively highlighted in the allergy field. Around two decades ago, an elevated TIgE was shown to be associated with risk for CVD (myocardial infarction, stroke or peripheral arterial disease) in studies conducted in affluent countries (38-40). A recent study by Wang et al. (41) showed that IgE was most elevated in patients with myocardial infarction, followed by unstable angina pectoris, and least elevated in stable angina pectoris. This suggests that different types of CVD are associated with specific differences in the level of TIgE. A number of these authors have also reported increased levels of IgE and FcεR1 in the atherosclerotic lesions of patients. To demonstrate the role of IgE and FcεR1 in atherosclerotic lesions, an apoE-/- mice model deficient for the FcεR1α receptor (apoE−/−, Fcer1a −/−) has been used and it has been demonstrated that these mice have lower occurrence of atherosclerotic lesions. Interestingly, it was also mentioned that the reduced levels of atherosclerotic lesions in apoE−/−, Fcer1a −/−

A. Animal models without the present of helminths or human living in non-helminth endemic areas.

B. Animal models with the present of helminths or human living in helminth endemic areas.

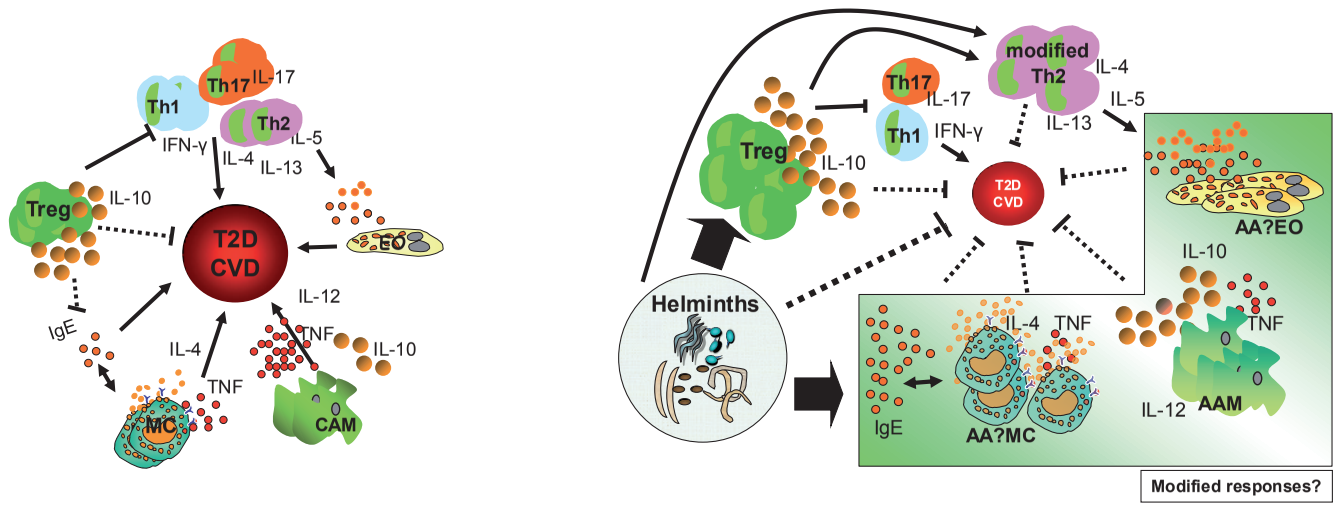


Figure 1. A possible schematic role of inflammatory cells network on type-2 diabetes and cardiovascular diseases with or without involvement of helminth infections. Various cells of the immune system seem to be involved in cardiovascular disease (CVD) or type-2 diabetes (T2D). In high income countries or in experimental models, inflammatory conditions created by the activity of inflammatory T cells such as T helper (Th) 1, Th17 and even Th2 and their cytokines, has been shown to increase the risk of T2D and CVD. Moreover, classically activated macrophages (CAM), mast cells (MC) and eosinophils (EO) have been shown to take part in the pathogenesis of the disease in target organs. However, the activity of regulatory T cells (Treg) and/or anti-inflammatory cytokines such as interleukin (IL) 10, might be able to prevent T2D and CVD (A). The situation seems to be different in rural areas of low-to middle income countries where helminth infections are highly prevalent. The same applies to animal models where the effect of helminth infections has been studied on atherosclerosis or diabetes. Helminths lead to the expansion of Treg, IL-10 and Th2 cells. During helminth infections, inflammatory cells might be modified: modified Th2, alternatively activated macrophages (AAM) have anti-inflammatory properties while eosinophils and mast cells seem no longer to behave as pro-inflammatory immune cells. Thus in presence of helminth infections, the immune system is in an anti-inflammatory mode which is considered to beneficial against development of T2D and CVD (B). The solid lines represent associations based on data available, while dotted lines represent theoretical associations that are yet to be tested.

mice did not affect total cholesterol (TC) or LDL levels, although they were associated with increased serum triglyceride (TG) and HDL levels (41). Altogether, these results seem to be in contrast to our findings: while in western countries IgE levels were positively associated with FBG, in our study in Indonesia, a high level of TIgE was associated with lower FBG. Moreover, in contrast to studies of atherosclerosis mentioned above, we noted a negative association between TIgE, which in our studies is related to helminth exposure, and risk factors for CVD that included TC, LDL and HDL.

The question arises whether findings in CVD may have some analogy with studies of allergic disorders. In areas where helminths are not endemic, allergy is associated with high levels of TIgE, while in helminth endemic populations, high levels of TIgE are associated with helminths, but inversely correlate with allergy (14). It is tempting to speculate that the possible protective effect of living in areas where helminths are highly endemic against development of CVD is based on similar mechanisms to the protection against the development of allergy, namely, the presence of strong anti-inflammatory and modified responses (Fig. 1).

Conclusions and future perspectives

We have shown in this thesis that helminth infections were associated with hyporesponsiveness. Yet we observed no consistent beneficial or detrimental effect regarding malaria or allergy after two years of community based anthelminthic therapy, which reduced but did not eliminate helminths. We also showed that helminth infections were negatively associated with risk factors for CVD, as well as being possibly beneficial for insulin sensitivity. The role of helminths in T2D and CVD needs to be followed up.

Altogether long-term, well-powered, placebo controlled anthelminthic trial are needed with better anthelminthics to investigate the causal relationship between helminth infections and malaria and allergy. A number of issues need to be considered for such future studies:

1. more adequate anthelminthic drugs and/or schedules
2. longer deworming period
3. areas where the endemicity of malaria is high
4. use of molecular methods to determine *Trichuris* worm burden

Only then will it be possible to bridge the gap between the findings in animal models that show a beneficial effect of helminths on a number of diseases and the situation in humans.

References

1. McCall MB, Sauerwein RW. Interferon-gamma-central mediator of protective immune responses against the pre-erythrocytic and blood stage of malaria. J Leukoc Biol 2010;88:1131-43.
2. Andrade BB, Reis-Filho A, Souza-Neto SM et al. Severe Plasmodium vivax malaria exhibits marked inflammatory imbalance. Malar J 2010;9:13.
3. Lyke KE, Burges R, Cissoko Y et al. Serum levels of the proinflammatory cytokines interleukin-1 beta (IL-1beta), IL-6, IL-8, IL-10, tumor necrosis factor alpha, and IL-12(p70) in Malian children with severe Plasmodium falciparum malaria and matched uncomplicated malaria or healthy controls. Infect Immun 2004;72:5630-7.
4. Donath MY, Shoelson SE. Type 2 diabetes as an inflammatory disease. Nat Rev Immunol 2011;11:98-107.
5. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005;352:1685-95.
6. Wiria AE, Djuardi Y, Supali T, Sartono E, Yazdanbakhsh M. Helminth infection in populations undergoing epidemiological transition: a friend or foe? Semin Immunopathol 2012;34:889-901.
7. McKeown RE. The Epidemiologic Transition: Changing Patterns of Mortality and Population Dynamics. Am J Lifestyle Med 2009;3:19S-26S.
8. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med 2006;3:e442.
9. Lim SS, Vos T, Flaxman AD et al. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 2013;380:2224-60.
10. Margono SS. Review on the control of soil-transmitted helminthiases in Indonesia: the role of parasitologists. In: Hayashi S, ed. Collected Papers on the Control of Soil-transmitted Helminthiases. 2001:169-72.
11. McSorley HJ, Maizels RM. Helminth infections and host immune regulation. Clin Microbiol Rev 2012;25:585-608.
12. Maizels RM, Yazdanbakhsh M. Immune regulation by helminth parasites: cellular and molecular mechanisms. Nat Rev Immunol 2003;3:733-44.
13. Elliott A, Yazdanbakhsh M. Troubles never come alone. Curr Opin HIV AIDS 2012;7:211-3.
14. Yazdanbakhsh M, Kremsner PG, van RR. Allergy, parasites, and the hygiene hypothesis. Science 2002;296:490-4.
15. Elliott DE, Urban JF JR, Argo CK, Weinstock JV. Does the failure to acquire helminthic parasites predispose to Crohn's disease? FASEB J 2000;14:1848-55.
16. Brutus L, Watier L, Briand V, Hanitrasoamampionona V, Razanatsoarilala H, Cot M. Parasitic co-infections: does *Ascaris* lumbricoides protect against Plasmodium falciparum infection? Am J Trop Med Hyg 2006;75:194-8.
17. Kirwan P, Jackson AL, Asaolu SO et al. Impact of repeated four-monthly anthelmintic treatment on Plasmodium infection in preschool children: a double-blind placebo-controlled randomized trial. BMC Infect Dis 2010;10:277.
18. Wright VJ, Ame SM, Haji HS et al. Early exposure of infants to GI nematodes induces Th2 dominant immune responses which are unaffected by periodic anthelminthic treatment. PLoS Negl Trop Dis 2009;3:e433.
19. Keiser J, Utzinger J. Efficacy of current drugs against soil-transmitted helminth infections: systematic review and meta-analysis. JAMA 2008;299:1937-48.
20. Nacher M. Interactions between worms and malaria: good worms or bad worms? Malar J 2011;10:259.
21. Brutus L, Watier L, Hanitrasoamampionona V, Razanatsoarilala H, Cot M. Confirmation of the protective effect of *Ascaris* lumbricoides on Plasmodium falciparum infection: results of a randomized trial in Madagascar. Am J Trop Med Hyg 2007;77:1091-5.
22. Ghani AC, Sutherland CJ, Riley EM et al. Loss of population levels of immunity to malaria as a result of exposure-reducing interventions: consequences for interpretation of disease trends. PLoS One 2009;4:e4383.

23. Snow RW, Omumbo JA, Lowe B et al. Relation between severe malaria morbidity in children and level of Plasmodium falciparum transmission in Africa. Lancet 1997;349:1650-4.
24. Endara P, Vaca M, Chico ME et al. Long-term periodic anthelmintic treatments are associated with increased allergen skin reactivity. Clin Exp Allergy 2010;40:1669-77.
25. Flohr C, Tuyen LN, Quinnell RJ et al. Reduced helminth burden increases allergen skin sensitization but not clinical allergy: a randomized, double-blind, placebo-controlled trial in Vietnam. Clin Exp Allergy 2010;40:131-42.
26. Moncayo AL, Vaca M, Oviedo G et al. Effects of geohelminth infection and age on the associations between allergen-specific IgE, skin test reactivity and wheeze: a case-control study. Clin Exp Allergy 2013;43:60-72.
27. Soliman EZ, Ding J, Hsu FC, Carr JJ, Polak JF, Goff DC, Jr. Association between carotid intima-media thickness and pericardial fat in the Multi-Ethnic Study of Atherosclerosis (MESA). J Stroke Cerebrovasc Dis 2010;19:58-65.
28. Touboul PJ, Labreuche J, Vicaut E et al. Country-based reference values and impact of cardiovascular risk factors on carotid intima-media thickness in a French population: the 'Paroi Arterielle et Risque Cardio-Vasculaire' (PARC) Study. Cerebrovasc Dis 2009;27:361-7.
29. Stanley RG, Jackson CL, Griffiths K, Doenhoff MJ. Effects of Schistosoma mansoni worms and eggs on circulating cholesterol and liver lipids in mice. Atherosclerosis 2009;207:131-8.
30. Wu D, Molofsky AB, Liang HE et al. Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. Science 2011;332:243-7.
31. Bhargava P, Li C, Stanya KJ et al. Immunomodulatory glycan LNFPIII alleviates hepatosteatosis and insulin resistance through direct and indirect control of metabolic pathways. Nat Med 2012;18:1665-72.
32. Black S, Kushner I, Samols D. C-reactive Protein. J Biol Chem 2004;279:48487-90.
33. McDade TW, Rutherford J, Adair L, Kuzawa CW. Early origins of inflammation: microbial exposures in infancy predict lower levels of C-reactive protein in adulthood. Proc Biol Sci 2010;277:1129-37.
34. Koopman JJ, van BD, Jukema JW, Westendorp RG. Risk of cardiovascular disease in a traditional African population with a high infectious load: a population-based study. PLoS One 2012;7:e46855.
35. Gurven M, Kaplan H, Winking J et al. Inflammation and infection do not promote arterial aging and cardiovascular disease risk factors among lean horticulturalists. PLoS One 2009;4:e6590.
36. Blackwell AD, Snodgrass JJ, Madimenos FC, Sugiyama LS. Life history, immune function, and intestinal helminths: Trade-offs among immunoglobulin E, C-reactive protein, and growth in an Amazonian population. Am J Hum Biol 2010;22:836-48.
37. Blackwell AD, Gurven MD, Sugiyama LS et al. Evidence for a peak shift in a humoral response to helminths: age profiles of IgE in the Shuar of Ecuador, the Tsimane of Bolivia, and the U.S. NHANES. PLoS Negl Trop Dis 2011;5:e1218.
38. Criqui MH, Lee ER, Hamburger RN, Klauber MR, Coughlin SS. IgE and cardiovascular disease. Results from a population-based study. Am J Med 1987;82:964-8.
39. Kovanen PT, Manttari M, Palosuo T, Manninen V, Aho K. Prediction of myocardial infarction in dyslipidemic men by elevated levels of immunoglobulin classes A, E, and G, but not M. Arch Intern Med 1998;158:1434-9.
40. Langer RD, Criqui MH, Feigelson HS, McCann TJ, Hamburger RN. IgE predicts future nonfatal myocardial infarction in men. J Clin Epidemiol 1996;49:203-9.
41. Wang J, Cheng X, Xiang MX et al. IgE stimulates human and mouse arterial cell apoptosis and cytokine expression and promotes atherogenesis in Apoe-/- mice. J Clin Invest 2011;121:3564-77.

Summary

In this thesis we reported our investigations of the relationship between soil-transmitted helminths (STH) and a number of outcomes, in particular malaria, insulin resistance (a marker for type-2 diabetes (T2D)) and atherosclerosis (a marker for cardiovascular diseases (CVD)) on Flores island, Indonesia.

Chapter 1

Here we discussed how the major helminth infections that affect a large proportion of the population in the developing world can have impact on the immune system and the consequences of this for other infections which are co-endemic in the same areas. Furthermore, we addressed the issue of decreasing helminth infections in many parts of the world within the context of increasing inflammatory, metabolic, and cardiovascular diseases. We assembled from the available literature the evidence from experimental models and epidemiologic studies that helminth parasites appear to be able to induce immune hyporesponsiveness, which results in the host's inability to eliminate the parasites and affects the host responses to bystander antigens. In the context of malaria, while a host requires adequate pro-inflammatory responses to protect itself against malaria parasites, the inability to control overt inflammatory responses can be associated with pathology. Therefore, helminth infections might be associated with more susceptibility to malaria infection but less severe pathology. Moreover, as inflammation is a known risk factor for T2D and CVD, we addressed the possibility that helminth-induced hyporesponsiveness might have a beneficial effect on preventing the development of T2D and CVD.

Chapter 2

Here we described in detail our study protocol and our study population in the context of helminth and malaria co-infection. In order to investigate the effect of helminths on malaria infection and disease outcome, as well as on immunological parameters, the area of Nangapanda on Flores Island, Indonesia, where malaria and helminth parasites are co-endemic, was selected for a longitudinal study. A household-clustered, double-blind randomized trial, incorporating repeated treatment with albendazole (400 mg) or placebo at three monthly intervals was performed to elucidate the impact of helminth infections on malaria longitudinally. We collected information on household characteristics, anthropometry, the presence of intestinal helminths and *Plasmodium spp* infections, and the incidence of malaria episodes. Detailed methods of PCR detection of studied parasites, which were used in our trial were described, in addition to the whole blood procedures for immunological analysis of the trial.

Chapters 3 and 4

These chapters reported the outcome of the 2 years duration randomized trial. **Chapter 3** reported results of malarial parasitemia and allergy outcome, while **Chapter 4** reported the immunological outcomes. Helminth infections in our albendazole treated group decreased significantly (**Chapter 3 and 4**). However, this intensive deworming could not eliminate helminth infections. This was perhaps due to persisting helminths such as *Trichuris,* which appear not to be affected by albendazole used as a single dose at three monthly intervals as well as to the strong contribution of contaminated environment to the transmission of the helminths studied.

We found a transient increased risk of malarial parasitemia (mainly in >15 years of age) in albendazole treatment group (**Chapter 3**). However, it is still unclear what the mechanism behind the high malarial parasitemia in the treatment group is. We also showed that there did not seem to be any effect on clinical symptoms. The consistently decreasing malarial parasitemia prevalence in our study population during the study period, led to very low extent of malaria helminth co-infection than expected based on our findings during the piloting phase prior to the start of our deworming trial. This might have had an effect not only on the power of the study but also on the immunological status regarding anti malaria responses. Studies in areas with high prevalence of clinical malaria are needed to establish whether helminth infections affect this outcome.

As shown in **Chapter 4**, we found that *in vitro* immune responses were improved after albendazole treatment and significant increases in malaria-specific and mitogen-induced tumor necrosis factor and interferon γ cytokine production were observed. However, these increased pro-inflammatory responses to the malaria parasite observed during the trial may not have been sufficient to cause clinical symptoms; no changes in clinical symptoms observed in **Chapter 3**. This could also be due to the maintenance of a certain level of anti-inflammatory responses, because of incomplete deworming, which would still be able to prevent the development of clinical symptoms.

Chapter 5

There is now compelling evidence that inflammation plays a role in chronic non-communicable diseases, including CVD and T2D. In this respect, it is interesting that some chronic infections, such as helminth infections, which are highly prevalent in rural areas of low-to middle income countries, have been shown to induce anti-inflammatory and immune regulatory effects as discussed in **Chapter 1**. Here we showed in a cross-sectional manner that STH infection was associated with lower risk factors for CVD that include lower body mass index (BMI) or waist-to-hip ratio (WHR), and lower lipid levels, compared to uninfected individuals. We also noted a negative association between total IgE (TIgE), which related to helminth exposure, and risk factors for CVD that included total cholesterol, low density lipoprotein and high density lipoprotein. In this study, the effect of helminth infections on CVD risk factors was at least partially mediated by an effect on BMI/WHR. However, a direct association between helminth infection and CVD markers, such as to carotid intima media thickness (cIMT) as a marker of atherosclerosis, was not seen. Indeed, the cIMT of the study participants was relatively low compared to individuals of the same age living in high income countries (HIC) and still below what is considered pathological. Further studies are necessary to assess the causal relationship between helminth infections and CVD.

Chapter 6

With respect to T2D, it has been hypothesized that chronic helminth infections by inducing anti-inflammatory responses decrease systemic inflammation and may help to prevent metabolic diseases. Here, we have shown that helminth infection was associated with improved insulin sensitivity even though there was no significant effect on fasting blood glucose (FBG) level. The improved insulin sensitivity seemed to be related, in part, to lower levels of insulin meaning that less insulin was needed to maintain normal blood glucose in individuals with helminth infections.

Chapter 7

In this chapter I discussed questions that remained or emerged from our findings. The first one is why with our intensive deworming treatment, helminth infections remained. Secondly, why we found increased risk of malarial parasitemia (mainly in >15 years of age) in the albendazole treatment group. Moreover, I questioned whether the association between helminths and CVD or T2D is similar to that seen between helminths and allergy.

First, I propose that the poor result of our intensive deworming trial could be related to highly contaminated environment and reinfection as well as to the limited efficacy of albendazole or possible emergence of drug resistance. Given that global campaigns are underway to control and eliminate STH; our finding suggests an urgent need for new approaches to helminth elimination programs. Furthermore, well-designed population studies that include adequate anthelminthic treatment regiment are needed to confirm our propositions. Diagnostic methods that are able to reveal submicroscopic infection such as *Trichuris* infection or other intestinal infections are also needed.

For the second question, I thought that the increased risk for malarial parasitemia in our study participants may have been caused by a reduced level of *Ascaris* infection. It is possible that *Ascaris* infection in the older age groups which was lower than in the younger age group prior to treatment was more effectively cleared. Therefore, the level of *Ascaris* infection might have been sufficiently high to keep malarial parasitemia at a low level in the younger age group, while the low infection after treatment in older age group was not able to do so. Whether this association between helminth and malaria is immune mediated or competition for resources is currently unknown.

In non-helminth endemic populations, allergy is associated with high levels of TIgE, while in helminth endemic populations, high levels of TIgE are associated with helminths, which are inversely correlated with allergy. Interestingly, in HIC, elevated TIgE has been shown to be associated with risk for CVD (myocardial infarction, stroke or peripheral arterial disease). In contrast, we found that TIgE levels were related to helminth exposure and we noted a negative association between TIgE and risk factors for CVD as well as association between TIgE and lower FBG. One can hypothesize that during helminth infections, the immune system may be somehow modified, that it no longer predisposes to the development of CVD. Also, chronic exposure to infections, in addition to the reduction in energy intake and association with poor nutritional status, could play an important role in the establishment of effective regulatory networks that, to a certain degree, are beneficial in preventing CVD and T2D. A possible approach will be a long-term, well-powered, placebo controlled anthelminthic trials to investigate whether alleviation of helminthic pressure is inversely correlated with anti-inflammation, lipid levels and insulin sensitivity, and therefore leads to an accelerated development of T2D and CVD.

Samenvatting

In dit proefschrift beschrijven we ons onderzoek naar de relaties tussen worminfecties en een aantal andere kenmerken, in het bijzonder malaria infectie, insulineresistentie (een marker voor type-2 diabetes (T2D)), en atherosclerose (een marker voor hart- en vaatziekten (HVZ)) op het eiland Flores in Indonesië.

Hoofdstuk 1

In hoofdstuk 1 wordt beschreven dat parasitaire wormen, waarmee een groot deel van de bevolking van ontwikkelingslanden is geïnfecteerd, het afweersysteem kunnen beïnvloeden en wat gevolgen kan hebben voor andere infecties die in dezelfde gebieden ook voorkomen. Daarnaast beschrijven we dat in ontwikkelde gebieden in verschillende delen van de wereld er een toename van ontstekingsziekten, metabole ziekten en hart- en vaatziekten is vastgesteld, wat mogelijk een gevolg is van het verdwijnen van worminfecties. Er is een aantal bewijzen uit muismodellen en uit humaan epidemiologisch onderzoek dat aantoont dat wormparasieten het afweersysteem van de gastheer kunnen onderdrukken. Deze onderdrukking resulteert in het onvermogen van de gastheer om de parasieten te elimineren en beïnvloedt tevens reacties van de gastheer tegen andere infecties en ziekteprocessen. Bij een malaria infectie heeft de gastheer voldoende afweerreacties nodig om zichzelf te beschermen tegen malariaparasieten, maar als deze reacties niet gecontroleerd worden kan dit leiden tot ongewenste bijverschijnselen, zoals te veel ontsteking en schade aan de weefsels. Daarom zouden worminfecties geassocieerd kunnen zijn met de vatbaarheid voor malaria infectie, maar tegelijkertijd ook met de beperking van de pathologie. Aangezien ontstekingsreacties een bekende risicofactor zijn voor T2D en HVZ, bespreken we ook de mogelijkheid dat de onderdrukking van afweerreacties door wormen een remmend effect kan hebben op de ontwikkeling van T2D en HVZ.

Hoofdstuk 2

Hier zijn de details van ons onderzoeksprotocol en de studiepopulatie op Flores beschreven. Voor ons onderzoek naar het effect van wormen op malaria infectie, de ziekteverschijnselen, en de daarmee samenhangende immunologische parameters is het dorp Nangapanda geselecteerd, waar malaria en worminfecties beide voorkomen. Er is een grote, longitudinale studie uitgevoerd, waarbij per huishouden de inwoners elke drie maanden behandeld werden met albendazol (een medicijn tegen wormen) of placebo. Hiermee wilden we bekijken wat voor effect worminfecties hebben op malaria over een periode van twee jaar. We hebben veel informatie verzameld over de huizen, het huishouden, de lichaamsbouw, de aanwezigheid van worm- en malaria infecties, en het voorkomen van klachten door malaria. Verder worden ook de in ons onderzoek gebruikte methoden voor immunologische analyses beschreven, naast details van de PCR methode om parasieten te detecteren.

Hoofdstukken 3 and 4

In deze hoofdstukken hebben we de uitkomsten van dit twee jaar durende onderzoek beschreven. **Hoofdstuk 3** behandelt de resultaten op het gebied van malaria en allergie, terwijl **Hoofdstuk 4** over de immunologische analyse gaat. Worminfecties zijn aanzienlijk gedaald in de groep die door ons met albendazol behandeld is (**Hoofdstukken 3 en 4**). Echter, niet alle worminfecties werden

geëlimineerd; vooral de zweepworm (*Trichuris*) bleef volhardend aanwezig bij veel mensen. Dit zou kunnen komen door de hoge graad van besmetting in de omgeving en door de beperkte gevoeligheid van *Trichuris* voor behandeling met een enkele dosis albendazol per 3 maanden. We hebben gevonden dat de aanwezigheid van malaria infectie in het bloed kortstondig toenam in de albendazol behandeling groep (**Hoofdstuk 3**). Het is echter niet duidelijk welk mechanisme hierachter zit. We hebben ook aangetoond dat er geen effect was van wormbehandeling op de ziekteverschijnselen van malaria. Door het voortdurend afnemen van malaria infecties in onze studiepopulatie tijdens de onderzoeksperiode, was er een veel lager aantal co-infecties van malaria en wormen dan wij verwachtten op basis van onze bevindingen in de testfase van het onderzoek. Er zijn studies nodig in regio's met veel malaria om vast te kunnen stellen of worminfecties een effect hebben op malaria infecties of op klinische symptomen van malaria.

Zoals beschreven in **Hoofdstuk 4**, hebben we gevonden dat afweerreacties zijn verbeterd na de behandeling met albendazol. Er was een aanzienlijke toename van afweer tegen malaria, en er werden grotere hoeveelheden van de ontstekingseiwitten tumor necrosis factor en interferon-γ aangemaakt. Samen met de resultaten in **Hoofdstuk 3** moeten we echter vaststellen dat deze toename van reacties tegen malariaparasieten tijdens de onderzoeksperiode geen veranderingen in de klinische symptomen veroorzaakt. Dit zou veroorzaakt kunnen worden door persisterende 'anti-inflammatoire' reacties omdat er nog steeds wormen aanwezig zijn, waardoor de ontwikkeling van klinische symptomen onderdrukt worden.

Hoofdstuk 5

Tegenwoordig is er duidelijk bewijs dat ontsteking een rol speelt bij het ontstaan van chronische ziekten, zoals HVZ en T2D. Daarom is het interessant dat sommige chronische infecties, in het bijzonder worminfecties, die veel voorkomen op het platteland van lage- tot middeninkomenslanden, ontstekings- en afweerreacties kunnen remmen, zoals besproken in **Hoofdstuk 1**. In **Hoofdstuk 5** hebben we aangetoond dat mensen met worminfecties een lagere body mass index (BMI), een kleinere heupomvang, en lagere waarden van cholesterol en andere vetten hebben vergeleken met mensen zonder infecties. Daardoor lopen zij een kleiner risico op HVZ. We hebben ook een negatief verband gezien tussen totaal IgE (TIgE), een afweerstof die een verband heeft met blootstelling aan wormen, met HVZ risicofactoren zoals cholesterol en een aantal lipoproteinen (HDL en LDL). Echter, een direct verband tussen worminfectie en aderverkalking, gemeten aan de hand van de dikte van de vaatwand in de halsslagader (intima media thickness (IMT)) werd niet gezien. Dit komt misschien doordat in de bevolking van Nangapanda het IMT niveau over het algemeen erg laag was vergeleken met mensen van dezelfde leeftijd in hoge inkomenslanden. Verdere studies zijn nodig om het oorzakelijk verband tussen worminfecties en HVZ aan te tonen.

Hoofdstuk 6

Met betrekking tot T2D, wordt er verondersteld dat chronische worminfecties algemene systemische ontstekingen onderdrukken, waardoor metabole ziekten zoals T2D voorkomen zouden kunnen worden. We hebben hier beschreven dat worminfecties inderdaad geassocieerd zijn met verhoogde gevoeligheid voor insuline, ofschoon er geen aanzienlijk effect op de bloedsuikerwaarde is. Dit betekent dat er minder insuline nodig is om een normaal bloedsuiker

te behouden in mensen met worminfecties. Dit zou kunnen betekenen dat mensen die worminfecties hebben of hebben gehad minder snel diabetes krijgen.

Hoofdstuk 7

In dit hoofdstuk bediscussieer ik een aantal kwesties die uit onze bevindingen naar voren zijn gekomen. Ten eerste: waarom zijn worminfecties gebleven ondanks onze intensieve wormbehandeling? Ten tweede: waarom hebben we een verhoogd risico op malaria infectie (vooral in de groep 15 jaar en ouder) in de groep met albendazol behandeling gevonden? Daarnaast heb ik me afgevraagd of het verband tussen wormen en HVZ of T2D vergelijkbaar is met het verband dat tussen wormen en allergie is gezien.

Met betrekking tot de eerste vraag, denk ik dat het slechte resultaat van onze intensieve ontworming gerelateerd zou kunnen zijn aan de hoge besmettingsgraad van de omgeving en het veel vóórkomen van hernieuwde infecties. Ook zou dit verklaard kunnen worden door de beperkte effectiviteit van de albendazol of de mogelijkheid dat er resistentie is ontstaan tegen de geneesmiddelen. Aangezien er uitgebreide campagnes aan de gang zijn om worminfecties te controleren en te elimineren, geeft onze bevinding aan dat het noodzakelijk is om nieuwe benaderingen te vinden voor worm-eliminatie programma's. Bovendien zijn er goed opgezette bevolkingsstudies nodig waarin procedures voor wormbehandeling worden getest om onze voorlopige conclusies te bevestigen. Ook zijn er diagnostische methoden nodig om de lichte *Trichuris* infecties of andere intestinale infecties aan te kunnen tonen.

Betreffende de tweede vraag denk ik dat de verhoging van het risico op malaria infectie in onze studie-deelnemers veroorzaakt zou kunnen zijn door het verlaagde niveau van *Ascaris* infectie. Het is mogelijk dat *Ascaris* infecties in de oudere mensen voor de behandeling minder zwaar zijn geweest dan in de jongere groep, en dat de infecties in de oudere groep daadwerkelijk zijn verdwenen. De *Ascaris* infectie in de jongere leeftijdsgroep kan echter voldoende zijn geweest om malaria infectie onderdrukt te houden. Of dit verband tussen worm- en malaria infecties te maken heeft met het afweersysteem of met concurrentie om voedingsmiddelen is op dit moment onbekend.

In gebieden waar geen worminfecties voorkomen is allergie geassocieerd met hoge TIgE niveaus, terwijl in gebieden met hoge TIgE niveaus vanwege worminfecties, die worminfecties juist omgekeerd zijn gecorreleerd met allergie. Het is boeiend dat er in hoge inkomenslanden is aangetoond dat verhoogd TIgE een hoger risico op HVZ geeft. In tegenstelling hebben wij gevonden dat TIgE niveaus hoger zijn bij blootstelling aan wormen, maar dat dit ook een lager risico geeft op HVZ, bijvoorbeeld met een lagere bloedsuikerspiegel. Men zou zich voor kunnen stellen dat het afweersysteem tijdens worminfecties op één of andere manier wordt veranderd waardoor dit niet meer tot HVZ kan leiden. Bovendien kan chronische blootstelling aan worminfecties vermindering van de energie-inname veroorzaken en door de slechte voedingstoestand die dan ontstaat een belangrijke rol spelen bij het opzetten van een effectief regulatoir netwerk, gunstig om HVZ en T2D te voorkomen. Een mogelijke benadering om dit verder te onderzoeken is een lange-termijn, robuust, placebo-gecontroleerd onderzoek met wormbehandeling, om na te gaan of wormen een verband hebben met minder ontsteking, lagere hoeveelheden vetten en suikers en gevoeligheid voor insuline. In dat geval zou wormbehandeling kunnen leiden tot versnelde ontwikkeling van T2D en HVZ.

List of Abbreviations

AAM	Alternatively activated macrophages
ACT	Artemisinin-combination therapy
Ag	Antigen
BMI	Body mass index
Breg	Regulatory B cells
CAM	Classically activated macrophages
CBC	Complete blood count
cIMT	Carotid Intima Media Thickness
CT	Cycle-treshold
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CRP	C-reactive protein
CVD	Cardiovascular diseases
Dreg	Regulatory D cells
DM2	Type-2 diabetes
FBG	Fasting blood glucose
HC	Hip circumference
HDL	High density lipoprotein
HIC	High income countries
HIV	Human immunodeficiency virus
HOMA-IR	Homeostasis model assessment for insulin resistance
IBD	Inflammatory bowel diseases
IFN-γ	Interferon γ
IgE	Immunoglobulin E
IL	Interleukin
iRBC(PfRBC)	Infected red blood cells (*Plasmodium falciparum* infected red blood cells)
ITN	Insecticide-treated nets
LDL	Low density lipoprotein
LLIN	long-lasting insecticide-treated nets
LMIC	Low-to-middle income countries
LPS	Lipopolysaccharide
miRNA	MicroRNA
metS	Metabolic syndrome
MS	Multiple sclerosis
PRDX	Peroxiredoxin
PHA	Phytohaemagglutinin
RCT	Randomized control trial
SPT	Skin prick test
STH	Soil-transmitted helminths
T2D	Type-2 diabetes
TB	Tuberculosis
TC	Total cholesterol

TG	Triglyceride
Th	T-helper
TGF-β	Transforming growth factor β
TIgE	Total IgE
TNF/TNF-α	Tumor necrosis factor/ Tumor necrosis factor- α
Treg	Regulatory T cells
TST	Tuberculin skin test
uRBC	Uninfected red blood cells
WC	Waist circumference
WHR	Waist-to-hip ratio

Curriculum Vitae

Aprilianto Eddy Wiria was born in Jakarta, Indonesia on the 29th of April 1980. After completing his secondary education at Canisius College High School in Jakarta, in 1998, he entered the Faculty of Medicine at the University of Indonesia (FMUI), Jakarta in the same year and obtained his medical degree in January 2006. Thereafter he worked at the Department of Parasitology FMUI where he became appointed as a lecturer in 2007.

During his time at medical school, he was active in the organization of Media Aesculapius, a Bimonthly National Health Newspaper and in the Asian Medical Students' Association (AMSA). In 2003 he was the chair of the 24th AMSA Conference and later was the head of AMSA (OC) in 2003/4. Currently, he is active in developing the AMSA Alumni Club.

In 2006 he was selected as a PhD candidate for a collaborative project between FMUI and Leiden University Medical Center funded by the Royal Netherlands Academy of Arts and Sciences (KNAW). The project was to undertake a longitudinal randomized anthelminthic-control trial (www.ImmunoSPIN.org) coordinated by Prof. Dr. Maria Yazdanbakhsh and Dr Taniawati Supali. Toward the end of his PhD he also worked under the supervision of Prof. Dr. Jan Smit on metabolic syndrome and cardiovascular diseases. The results of the ImmunoSPIN project and the outcome of the research on the effect of helminth infection on insulin sensitivity and on atherosclerosis are described in this thesis.

As part of his PhD he established the Flores field research site, collaborated with the local public health authority and oversaw activities of the community workers on the research project. After his PhD he will be involved in projects investigating non communicable diseases. Overall he is driven to link clinical care to research and would like to pursue a career as a research-clinician.

Publication List

1. **Wiria AE**, Lianawati A. Looking for new protection against malaria by studying the interconnection between malaria and thalasemia. *Majalah Kedokteran Indonesia*. 2001;51:57-62.
2. **Wiria AE**, Tjokronegoro A, Sungkar S, Siagian M. The Controversy on Autism: Do Vaccine induce austism? *Majalah Kedokteran Indonesia*.2003;53:222-227.
3. Wammes LJ, Hamid F, **Wiria AE**, de Gier B, Sartono E, Maizels RM, Luty AJ, Fillié Y, Brice GT, Supali T, Smits HH, Yazdanbakhsh M. Regulatory T cells in human geohelminth infection suppress immune responses to BCG and *Plasmodium falciparum*. European Journal of Immunology. 2010;40:437-442.
4. **Wiria AE**, Prasetyani MA, Hamid F, Wammes LJ, Lell B, Ariawan I, Uh HW, Wibowo H, Djuardi Y, Wahyuni S, Sutanto I, May L, Luty AJ, Verweij JJ, Sartono E, Yazdanbakhsh M, Supali T. Does treatment of intestinal helminth infections influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study). BMC Infectious Disease. 2010;10:77.
5. Supali T, Verweij JJ, **Wiria AE**, Djuardi Y, Hamid F, Kaisar MM, Wammes LJ, van Lieshout L, Luty AJ, Sartono E, Yazdanbakhsh M. Polyparasitism and its impact on the immune system International Journal of Parasitology. 2010;40:1171-1176.
6. Hamid F, **Wiria AE**, Wammes LJ, Kaisar MM, Lell B, Ariawan I, Uh HW, Wibowo H, Djuardi Y, Wahyuni S, Schot R, Verweij JJ, van Ree R, May L, Sartono E, Yazdanbakhsh M, Supali T. A longitudinal study of allergy and intestinal helminth infections in semi urban and rural areas of Flores, Indonesia (ImmunoSPIN Study). BMC Infectious Disease. 2011;11:83.
7. Pasha SM*, **Wiria AE***, Wammes LJ, Smit JW, Partono F, Supali T, Yazdanbakhsh M, Tamsma JT. Blood pressures class and carotid intima media thickness in a population at secondary epidemiological transition. Journal of Hypertension 2011:29:2194-2200. *the authors contributed equally.
8. Wammes LJ, Hamid F, **Wiria AE**, Wibowo H, Sartono E, Maizels RM, Smits HH, Supali T, Yazdanbakhsh M. Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics. PLoS Neglected Tropical Disease. 2012;6:e1655.
9. **Wiria AE**, Djuardi Y, Supali T, Sartono E, Yazdanbakhsh M. Helminth infection in the population at transition: an old friend or foe? Seminars in Immunopathology. 2012;34:889-901
10. **Wiria AE**, WammesLJ, Hamid F, Dekkers OM, Prasetyani MA, May L, Kaisar MM, Verweij JJ, Tamsma JT, Partono F, Sartono E, Supali T, Yazdanbakhsh M, Smit JWA. Relationship between Carotid Intima Media Thickness and Helminth Infections on Flores Island, Indonesia. PLoS One. 2013;8:e54855
11. Wammes LJ, **Wiria AE**, et al. Asymptomatic plasmodial infection is associated with increased TNFRII-expressing Tregs and suppressed type 2 immune responses. Journal of Infectious Disease. J Infect Dis. 2013;207:1590-1599
12. **Wiria AE***, Hamid F*, Wammes LJ*, Kaisar MM, May L, Prasetyani MA, Wahyuni S, Djuardi Y, Ariawan I, Wibowo H, Lell B, Sauerwein R, Brice GT, Sutanto I, Lieshout L, de Craen AJM, van Ree R, Verweij JJ, Tsonaka R, Houwing-Duistermaat JJ, Luty AJF, Sartono E, Supali T, Yazdanbakhsh M. The effect of three-monthly albendazole treatment on malarial

parasitemia and allergy: a household-based cluster-randomized, double-blind, placebo-controlled trial. *the authors contributed equally. PLoS One. 2013;8:e57899

13. Hamid F, **Wiria AE**, Wammes LJ, Kaisar MM, Djuardi Y, Versteeg S, Wahyuni S, Van Ree R, Sartono E, Supali T, Yazdanbakhsh M. Risk factors associated with the development of atopic sensitization in Indonesia. PLoS One. *In press*
14. **Wiria AE**, Hamid F, Wammes LJ, Prasetyani MA, Dekkers OM, May L, Kaisar MM, Verweij JJ, Guigas B, Partono F, Sartono E, Supali T*, Yazdanbakhsh M*, Smit JWA*. Infection with soil-transmitted helminths is associated with increased insulin sensitivity. Experiments of nature on immune modulation and metabolism. *Submitted*. *these authors contributed equally
15. Wammes LJ*, Hamid F*, **Wiria AE***, May L, Kaisar MM, Prasetyani MA, Djuardi Y, Ariawan I, Wibowo H, Kruize YCM, Suryani H, Verweij JJ, Tsonaka R, Houwing-Duistermaat JJ, Luty AJF, Sartono E, Supali T, Yazdanbakhsh M. Three-monthly albendazole treatment alleviates geohelminth-induced immune hyporesponsiveness; results of a double blind placebo-controlled household-randomized trial. *the authors contributed equally. *Submitted*.
16. Kaisar MM, Supali T, **Wiria AE**, Hamid F, Wammes LJ, Sartono S, Luty AJF, Brienen EAT, Yazdanbakhsh M, Lieshout L, Verweij JJ. Epidemiology of Plasmodium infections in Flores Island, Indonesia using real-time PCR. *Submitted*.
17. Sartono E, **Wiria AE**, Wammes LJ, Hamid F, Kaisar MM, Djuardi Y, Kruize YCM, Lieshout L, Mathias E, Luty AJF, Verweij JJ, Supali T, Yazdanbakhsh M. Soil-transmitted helminth and Plasmodium coinfection in Flores island, Indonesia: its impact on anemia, parasitemia and cytokine responses. *Submitted*.

Acknowledgements

I have got my PhD. Yeayyy!!!

To thank everyone that enabled me to finish this PhD, I would need to write another book.

I feel myself as a modulated immune cell. I was on my way to prepare for a specialist training and thereafter to try and get into research, but the modulatory forces took over.

The first modulation was towards the end of my medical study when I met Prof Saleha whom I have known for a long time via Prof Arjatmo. She offered me to join Parasitology at FMUI.

I was introduced to Dr Taniawati Supali who later introduced me to Prof Maria Yazdanbakhsh. Sometime ago I would have said I felt trapped, but now, I would say thanks to both of you for all the 'troubles' that have thought me science in an academic and non-academic way.

From the beginning until the very end of my PhD, Dr Erliyani Sartono helped me accumulate scientific knowledge. Also thanks to Yvonne Kruize who was always there for me for all scientific and non-scientific issues related to my work. A list of others who have been important for my scientific forming: Dr Adrian Luty, Dr Jaco Verweij, Dr Lisette van Lieshout, Prof Jeanine Houwing-Duistermaat, Dr Roula Tsonaka, Dr Haewon Uh, Prof Inge Sutanto, Dr Sitti Wahyuni, Dr Iwan Irawan, Prof Jan Wouter Jukema, Dr Bruno Guigas, thank you all for your expertise. The thesis would not have been possible without input and enthusiasm from you all. Mas Agus dan Mas Awal, thanks for helping with the mapping. Caroline Remmerswaal, Jantien Guldemond and Corrie Verbree, thank you so much for helping me with all the paper work.

Then I met Dr Partono. A couple of week's interaction with you opened my eyes and I learnt that to answer a difficult question you have to find the simplest answer.

Thanks to Dr Jouke Tamsma who made me enjoy the topics surrounding vascular medicine, especially over intima media thickness, and also to Dr. Sharif Pasha who companied me in this process. To Dr. Bertrand Lell who introduced me to database management. To Dr. Linda May who thought me to perform data analyses systematically. Also to Dr. Olaf Dekker who has helped me with communicating the results of my data analyses to the clinicians.

Thanks to Prof Jan Smit who has shown me how one can be a physician and a scientist at the same time as well as a nice person. You were a strong modulator towards becoming a clinical researcher, a track I have chosen now.

Humans are social beings and cannot function well alone, their peers and interactions with them are important. Therefore I would like to give big hugs to all my friends. Mba Eva Suarthana (friend and supporter). My first housemate (landlord :D)) Faber, and colleagues (Weng Weng, Ira, Reza kum, Bene, and more) and Luiz (which was later also a good companion in Leiden and everywhere in the Netherlands) as well as all members of the International Student Chaplaincy in Delft who made me feel welcome. Also friends in Leiden: Tita, Ichwan, Icha, Ari (later also became my landlord ☺), Pak Insa, Natashia, Iqbal were all good companions. LUMC friends: Bart, Benedicta, Akim, Jemmy, Astrid, Ellen, Sunny, Alwin who made my first time in the Netherlands so unforgettable. Of course the support from Dr Heri Wibowo, Dr Yenny Djuardi, Pak Sudirman, Pak Suwarto, Heni, Mba Erlinda, Maria Kaisar, Ani, Tryana, Sofi were also important as co-workers as well as friends.

Sometimes I felt exhausted. Therefore gathering, hangouts, eating times, traveling, or just some random emails, chats and facebook comments would make my day. For that in addition to friends mentioned above, thanks to Iwan, Tri L, Dwi, Riri, Ella, Linda&Carl, Avi, Donna, Dola, Bedil, Pokpok, Cindy, Nuril, Mba Nuning, Mba Dinar, Weta, Abi, Nindi, Mas Amiq, group of Encompass program Gesit, Uji, Intan, Ratih, Mas Wahid. Our times were always joyful. Also for T5 inhabitants before I migrated: Lucien, Anouk (both have helped me with my Dutch), Abena, Lucja (both also have helped me with my English), Kit Yeng, Regina, Simone, and Ulysse; and also other members of the Dept of Parasitology at LUMC: Hermelijn, Arifa the new mommy, Ron (thank you for the Netherlandse vertaling) and Nicole whose birthdays are shared in the best month of the year with me, and Honorine. Not to forget Ge Wangge, Rizal, Wulan and Widi (pretend to be) in the group of Rabbit 95, and Hendy (???☺).

Special thanks also to alumni Canisius College in Europe, especially the one in The Netherlands.

Now I have to go to Ende where the project was conducted. A migration to a new place needs a suitable environment to be optimal. Unforgettable hospitality was given by Ko Acang dan Mama Kapus sekeluarga, Ko Acing dan dr Helda sekeluarga, keluarga besar Bintang Timur, keluarga besar Gemilang, para guru, tokoh-tokoh agama dan masyarakat, juga seluruh warga Nangapanda which support the study as well as myself. Terutama juga untuk Sensi, Ical, Ima, Markus dan para kader yang telah bekerja keras dan menemani berlangsungnya program penelitian ini.

Firdaus and Linda, my paranymphs, my partners in crime. Thanks a bunch for your companionship, cooperation and friendship. This will not be an end, but beginning of our new chapter in life.

Many thanks to my brothers (Arief, Budi, Chandra) who have helped me and have done many things for me in Jakarta while I was most of the time away and my sister (Augustine) who repeatedly worried about my future plans that implies she cares. Also to Tika who also gave me support and understanding.

I would like to send my gratitude to Stichting van Helten that helped me to finance my Dutch Courses which also form the basis for my future development. My big thanks also for Enna, mijn docent Nederlands die mij bij de stichting van Helten introduceerde. Indeed to learn new things adequate resources are needed.

I believe there are still lots of people that I haven't mentioned; I also would say big thanks to you all.

This thesis is for my parents Lambang dan Mariati who always encouraged me to reach my dream whatever it takes. Their love is always my greatest resource.