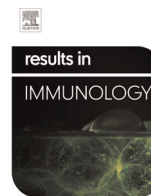




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Effective anthelmintic therapy of residents living in endemic area of high prevalence for Hookworm and *Schistosoma mansoni* infections enhances the levels of allergy risk factor anti-Der p1 IgE

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ABSTRACT

In this work were investigated the relationship between Hookworm/*Schistosoma mansoni* infections and allergy related risk factors in two endemic areas with distinct prevalence of infections and co-infection. The intensity of infections, eosinophilia, allergy risk factors, infections status and anti-Der p1 IgE levels before and 2 years (population 1) and 3 years (population 2) after anthelmintic treatment, were evaluated. It was observed that the population with lower prevalence and intensity of infection (population 2) had lower eosinophils counts ($> 600/\text{mm}^3$) and higher animal contact than the population with higher parasites intensity (population 1). After anthelmintic treatment the intensity of *S. mansoni* single infection decreased, but no changes were observed in Hookworm and co-infected individuals. The anthelmintic treatment also enhanced anti-Der p1 IgE optical density in ELISA on the subgroups that became negative for helminth infection regardless of their previous infection condition in population 1. Facing that, we evaluated the anti-Der p1 IgE reactivity index, and the ratio (after/before treatment) was significantly higher in patients co-infected before treatment. On the other hand, no association between anti-Der p1 IgE reactivity index and the intensity of infections were observed. In conclusion, effective anthelmintic therapy of subjects from endemic areas with high prevalence of Hookworm and *S. mansoni* infections enhances anti-Der p1 IgE levels.

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1. Introduction

The relationship between environmental factors and allergic disorders was first published by Strachan [1]. The author proposed the Hygiene Hypothesis, attributing the progressive growth of numbers of allergic individuals in the last decades to the reduction of infectious agents exposure during childhood. Immunologically, infections can modify the immune responses and consequently inhibit an exacerbated inflammatory condition. This phenomenon can be explained by a perturbation of the immune responses caused mostly by pathogens in which helminths play an important role [2,3]. The effects of helminth infections upon the immune responses to other health conditions are an area of great interest, mainly because helminth infections are able induce immune

regulation through stimulation of regulatory T cells and IL-10 production [4,5]. Previous studies [6,7] have shown that *Schistosoma mansoni* infection induces IL-10 production which leads to a decrease in allergic symptoms.

In this context, additional studies of the effect of helminth infections on allergic disorders are needed to better determine whether these infections reduce allergic symptoms and if this is dependant on the exposure conditions and sensitization. Moreover, it is important to determine whether treatment of individuals from endemic areas with anthelmintic drugs increase allergic occurrence. Under this scope, our study aimed to investigate the relationship between prevalence and intensity of *S. mansoni* (SCH) and hookworm (HW) infection and IgE responses to *Dermatophagoides pteronyssinus* antigen (Der p1) before and after treatment as well as risk factors to allergy such as eosinophil

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count, allergy episodes, animal contact and smoking in two populations of medium and high *S. mansoni* transmission.

2. Population, material and methods

2.1. Study area and population

The study was carried out in Caju (population-1) with 413 individuals and São Pedro do Jequitinhonha (population-2) with 314 individuals, 20 km distant from each other and both in the Municipality of Jequitinhonha. These are poor rural endemic areas for *S. mansoni* and hookworm infections. The population in Caju is of 632 inhabitants that live by subsistence agriculture. São Pedro do Jequitinhonha, which population is 2265 inhabitants, has similar economic condition but is closer to a larger non-rural community and is bordered by Jequitinhonha river. This region is characterized by poor sanitation and social economic conditions and the residents are constantly exposed to the risk of helminth infections [8]. The entire population was screened for infection and due to the fact that prevalence of infection was above 50%, all inhabitants were treated. Follow up treatment was performed at 1 month post-therapy and yearly for the period of 5 years. Informed consent was obtained for all volunteers, including children. For adolescents, consent was obtained from the volunteer and from its parents. The samples used were from two distinct collection times in each locality: before treatment (2004/Caju and 2007/São Pedro do Jequitinhonha) and after treatment (2006/Caju and 2010/São Pedro do Jequitinhonha). All participants were registered and assigned unique household identification (HHID) and personal identification numbers (PID). This study was approved by the National Committee of Ethics in Research of Brazil (CONEP/ 268/08).

2.2. Parasitological survey and eosinophils count

Parasitological survey was conducted using the Kato-Katz test. All individuals living in the studied villages received three containers with identification numbers for stool sample collection. The recipients were collected on three consecutive days. On the day of sample collection, all volunteers were submitted to a questionnaire to collect additional information on socio-economic status and activities as previously described by our group [8]. For parasite egg detection and quantification, two slides were prepared for each sample, totalling six slides per participant.

Parasite burdens, as determined by eggs per gram of faeces (epg), were calculated from the media of absolute egg number multiplied by 24 and divided by the number of slides (6). The *S. mansoni* infected adults were treated by single dose of Praziquantel (50–60 mg/kg) while children were treated single dose of Oxamniquine (20 mg/kg). Hookworm infected individuals were treated by single dose of Albendazole (400 mg). All treatments were given under medical supervision and according to Brazilian Ministry of Health regulations.

The CBC (complete blood count) was performed using the automated haematology system Advia 60 (Bayer Health Care, USA). Eosinophilia was defined as eosinophils count over 600/mm³.

2.3. Serum samples and ELISA

Serum was obtained by collection of blood in vacutainer tubes and one additional tube containing EDTA was collected for haematological analysis (Vacutainer, BD, EUA). Tubes were refrigerated and sent to the Laboratório de Imunologia Celular e Molecular from Centro de Pesquisas René Rachou, where samples

were aliquoted in microtubes and stored at -70°C until use. Serum IgE reactivity against *D. pteronyssinus* crude extract (Derp1, LG 5449, Cosmo Bio Co. Ltd., Japan) antigen was tested using an Enzyme Linked Immunosorbent Assay (ELISA). Briefly, each well was coated with 100 μL of mite Der p1 antigen at the final concentration of 1 $\mu\text{g}/\text{mL}$ in phosphate buffer saline pH 7.2 (PBS 1 \times). The ELISA plates were incubated for 16 h at 4°C , washed twice with PBS Tween 20 (Biorad, USA) 0.05% and blocked with 200 μL of PBS with 3% bovine serum albumin (BSA) for 1 h at 37°C . The serum samples were diluted 1:50 in PBS 1 \times with BSA at 3% and were added to the wells and incubated for 1 h at 37°C . The plates were washed five times and incubated for 1 h at 37°C , washed five times again and incubated with 100 μL of peroxidase labelled monoclonal antibody anti-human IgE (KPL, USA) for 1 h at 37°C . The assays were developed by addition of the substrate (H_2O_2 , Sigma-Aldrich Co., USA) and the chromogen (O-phenylenediamine, Sigma-Aldrich Co., USA). The optical density was determined using an automatic ELISA microplate reader at 492 nm (SpectrMax 340 PC reader, Molecular Devices, Sunnyvale, CA, USA) running Softmax Pro software (Molecular Devices). A serum pool obtained from urban area subjects and free of parasitic infection was used as control sample.

2.4. Questionnaire used to obtain information about respiratory disease

The interviews were held at the subject's homes in 2011 (Caju) and 2008 (São Pedro de Jequitinhonha). Children under 13 years old had the questionnaire answered by their tutors. Data were collected by a portable computer PDA-Personal Digital Assistant-Dell-Axim X 50 (Dell Inc., Texas, EUA). The frequency of allergic disorders was estimated by the International Study of Asthma and Allergies in Childhood questionnaire. This questionnaire was created to develop an epidemiological study of asthma and allergy distributions worldwide and is divided in three phases with questions of behavioural and environmental aspects. Questions of the first phase, related to previous symptoms and confirmed diagnosis of allergic disease, were used to estimate allergic disorders distribution on these two localities. Furthermore, secondary questions were asked to characterize the occurrence of risk factors as contact with animals and smoking habit.

2.5. Statistical analysis

For statistical analysis were used Microsoft Excel 2010 SP1 (Microsoft Corporation, Washington, EUA) and Graph Pad Prism 5.0.3 (San Diego, CA, EUA). Chi-square, Mann-Whitney and Tukey's post-tests were used for multiple comparisons tests to investigate differences between frequencies. In all cases, differences were considered significant when $p < 0.05$.

3. Theory

Long-term anthelmintic treatment can modify the allergen specific immune response in *S. mansoni* and hookworm infected individuals.

4. Results

4.1. Prevalence of Hookworm and *S. mansoni* infections pre and post-treatment

Our data demonstrated that the prevalence of HW infection as well as HW+SCH co-infection were higher in population 1 when

compared to population 2 (Fig. 1A). No significant differences were observed on the age ranges as well as gender distribution between the two localities (Fig. 1B). Population 1 also had higher intensity of infection (Fig. 1C) in all age ranges as compared to population 2 (Fig. 1D). Moreover, population 1 also presented greater frequency of individuals displaying high intensity *S. mansoni* infection (Sm > 100 epg) (Fig. 1E).

4.2. Analysis of anti-Der p1 antibody responses of individuals living in endemic areas with distinct prevalences for Hookworm and *S. mansoni* infections

To determine the effectiveness of the ELISA technique to measure the serum levels of Anti-Der p1 IgE, we first established the optical density cut-off point of the assay. To determine this point, the median of anti-Der p1 IgE optical density values from both populations together before treatment was evaluated in order to separate the individuals into “Low and High anti-Der p1 IgE producers” (Fig. 2A). The cut-off used to evaluate the data were of reading on O.D. of 0.420.

Although population 2 showed a lower frequency of individuals with eosinophilia (EOS > 600/mm³), it had increased frequency of individuals reporting animal contact, and > 50% of “High anti-Der p1 IgE producers”(Fig. 2B).

4.3. Effect of treatment on the frequency on high anti-Der p1 IgE responder

Although the albendazole/praziquantel combined treatment was unable to completely eradicate the helminthic infections in

both studied populations, the frequency of negative (NEG) individuals in these localities increased (Fig. 3A). It is interesting to note that combined anthelmintic therapy reduced the intensity of *S. mansoni* (SCH) infection in single infected patients in the population 1. However, it did not reduce the intensity of infection in those individuals that remained HW infected or HW+SCH co-infected after treatment (Fig. 3B). No significant differences in the intensity of infection were observed in the population 2 (Fig. 3B).

With the objective of identifying the effect of albendazole/praziquantel anthelmintic therapy on the levels of anti-Der p1 IgE antibody response, we categorized the studied populations into two subgroups, referred as “TREATED-NEG” and “TREATED-POS” since these individuals cleared the infection or remained infected (or re-infected) 2 years after treatment. The re-infected and infected participants were treated over again until the infection clearance. The posterior data about repeatedly treatments were not use on this study.

Our data demonstrated that the frequency of “High anti-Der p1 IgE producers” increased selectively in the TREATED-NEG subgroup of population 1, with no significant changes in the TREATED-POS subgroup of population 1 neither in both subgroups of population 2 (Fig. 4A).

Detailed analysis of TREATED-NEG and TREATED-POS subgroups based on their pre-treatment (Before-NEG, Before-HW, Before-SCH and Before-HW+SCH) and post-treatment status (After-NEG, After-HW, After-SCH and After-HW+SCH), further demonstrated that, regardless of their pre-treatment condition, the increased frequency of “High anti-Der p1 IgE producers” were evenly distributed amongst the TREATED-NEG patients of population 1 (Fig. 4B).

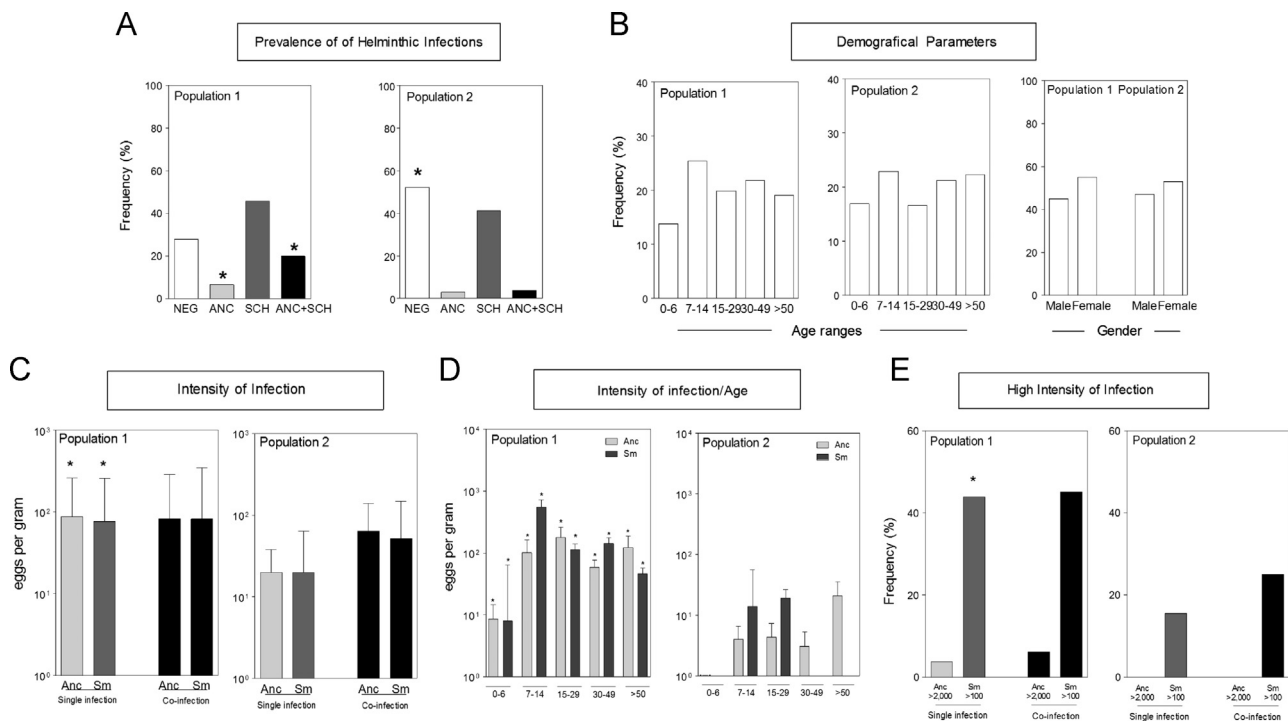


Fig. 1. Prevalence of helminthic infections, demographical parameters and intensity of infection related to age and parasite burden in two municipalities of Minas Gerais state Brazil. The infected individuals were identified by Kato Katz examinations against hookworm and *S. mansoni* and the prevalence was estimated in populations 1 and 2 following the examinations results: negative in kato-Katz test (NEG/ white bar), positive for hookworm in kato-Katz test (HW/ light grey bar), positive for *S. mansoni* (SCH/ dark grey bar) in Kato-Katz test and positive for co-infection with hookworm and *S. mansoni* (HW + SCH/ black bar). (A) The frequency of gender and age were also evaluated. (B) The intensity of infection was expressed as egg per grams in hookworm (Anc) and *S. mansoni* (Sm) infections. (C) Its distributions amongst different age ranges. (D) and the frequency of highly infected individuals was determined by ≥ 2000 epg for hookworm (Anc) and ≥ 400 epg for *S. mansoni* (Sm). (E) The median of each population 1 group was compared with their respective on the population 2. The error bars represent the interquartile range statistical analyses were performed by Fisher's exact and by Mann-Whitney tests. In both cases significance were considered at $p < 0.05$ and highlighted by *.

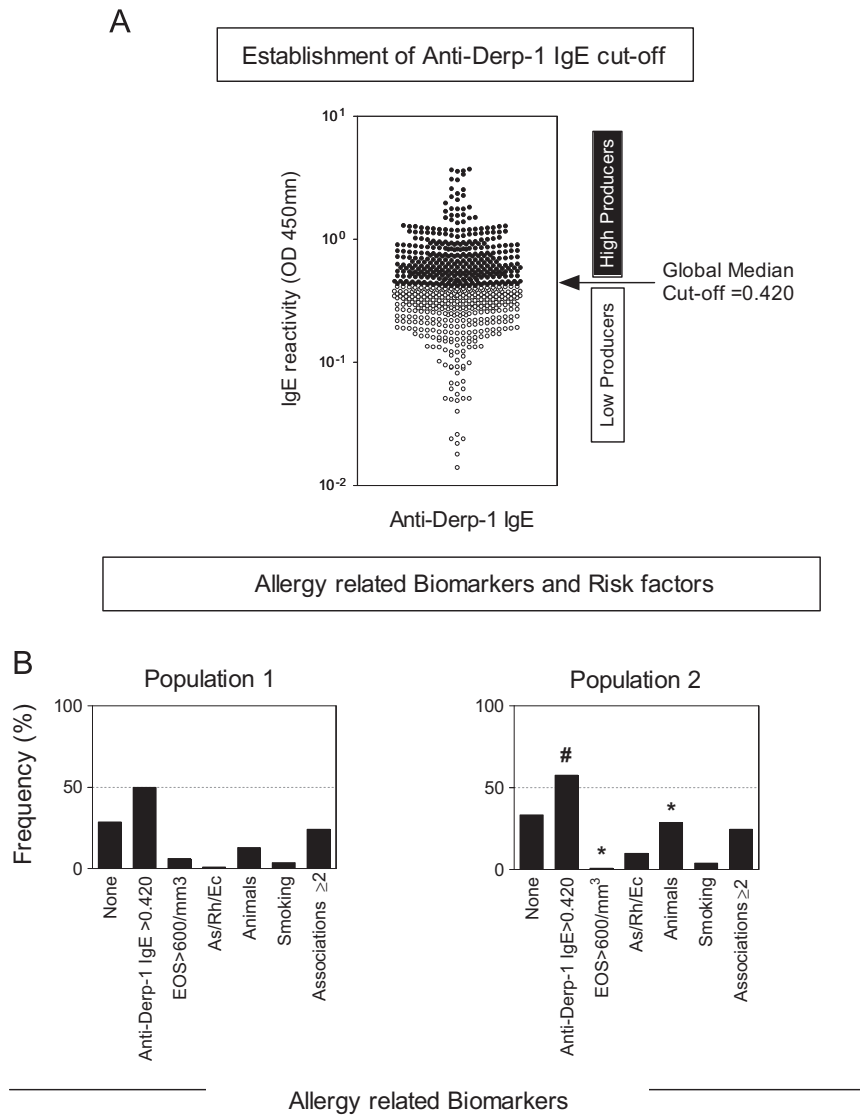


Fig. 2. Analyses of allergy related biomarkers and risk factors in two endemic areas for helminthic infection in Minas Gerais, Brazil. The global median value of to anti-Der p1 IgE reactivity was used as the cut-off to segregate “low” and “High producers”. The anti-Der p1 values correspond to the absorbance before treatment (A). Five allergy related biomarkers and risk factors, including anti-Der p1, peripheral blood eosinophil count (EOS > 600/mm³), asthma, rhinitis and eczema previous episodes (As/Rh/Ec), animals contact and smoking addiction were used to assess the likelihood of allergic disease, according to Isaac questionnaire. Individuals presenting more than one allergy risk factor were also related (association ≥ 2). Each parameter of the population 1 was compared with the equivalent in population 2(B). Statistical analyses were performed by Fisher’s exact and by Mann–Whitney tests. In both cases significance were considered at $p < 0.05$ and highlighted by *. Although there was not a statistical significance on the frequency of higher anti-Der p1 producers, it was slightly higher on population 2 (#).

4.4. Analysis of post-treatment anti-Der p1 Ige Index and its relationship with helminthic infection/co-infection status and infection intensity

In order to further investigate whether the changes in the anti-Der p1 IgE observed in the TREATED-NEG subgroup of population 1 could be related to the patient pre-treatment condition, we calculated for each patient the anti-Der p1 Ige Index as the ratio between the optical densities observed after/before treatment. Interestingly, our data demonstrated that the anti-Der p1 Ige Index was significantly higher in the subgroup of patients with co-infection status before treatment (Before-HW+SCH) (Fig. 5A). Additional analysis demonstrated that the increase on the anti-Der p1 Ige Index was not associated with either the intensity of Hookworm or the *S. mansoni* egg counts (Fig. 5B).

5. Discussion

Although several authors have evaluated changes in anti-Der p1 responses following anthelmintic treatment [9,10], in this study we performed comparisons of anti-Der p1 IgE response before and after treatment in all age range and in both genders. The populations evaluated had similar age and gender parameters and also allergy risk factors were equally distributed. Treatment against *S. mansoni* and hookworm was effective in both localities as indicated by negative individuals’ stool egg counts. We also observed that re-infection frequently occurs.

Furthermore, we demonstrated that anti-Der p1 IgE levels increased after treatment on population with higher intensity of infection. Worms are known as master pieces on immune regulation and are commonly associated with allergy downregulation.

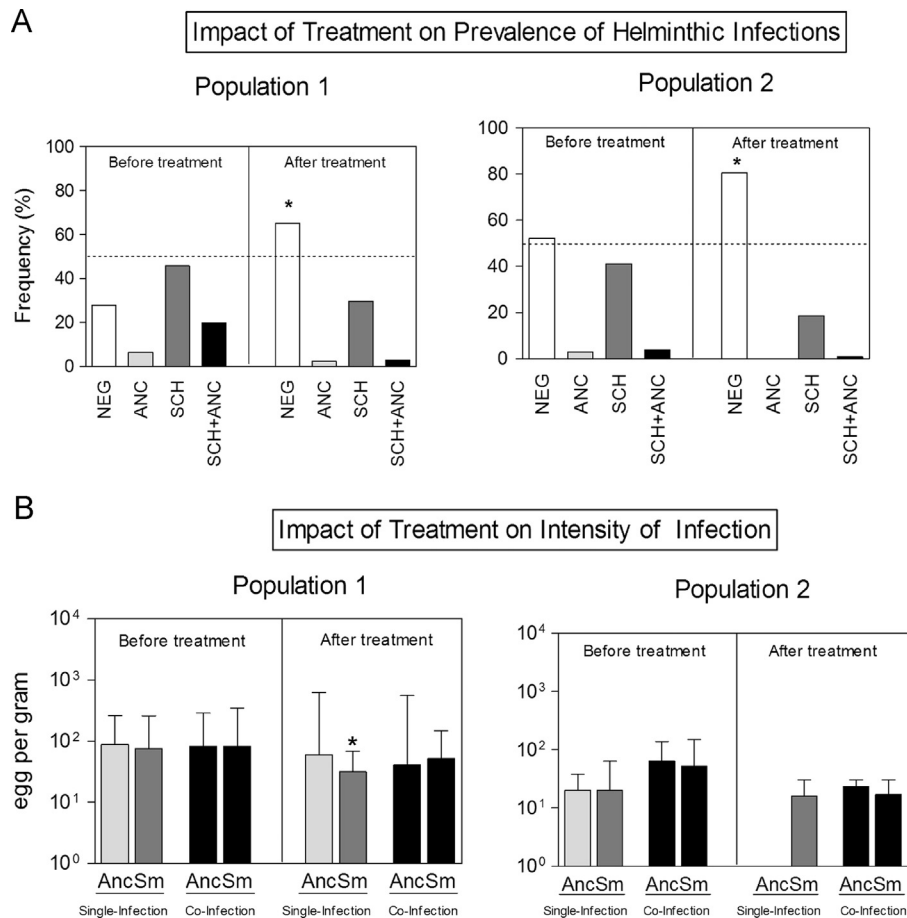


Fig. 3. Impact of treatment on prevalence and intensity of infection in two endemic areas for helminthic infection in Minas Gerais, Brazil. The prevalence of infection before and after treatment was determined for each one of these conditions: individuals with negative kato-Katz test (NEG) were represented by white bar; hookworm positive individuals on kato-Katz test (HW) were represented by light grey bar; positive individuals for *S. mansoni* on Kato-Katz test (SCH) were represented by dark grey bar and co-infected individuals determined by Kato-Katz test (HW + SCH) were represented by black bar (A). The intensity of hookworm (Anc) and *S. mansoni* (Sm) infections were also evaluated before and 2 years after treatment and the absolute number obtained on Kato-Katz test was also used. The medians of each group of the populations were compared before and after treatment with their respective groups. The error bars represent the interquartile range. Statistical analyses were performed by Fisher's exact and by Mann–Whitney tests. In both cases significance were considered at $p < 0.05$ and highlighted by *.

Van den Biggelaar and colleagues [10], showed an association between *S. mansoni* infection and IL-10 production on patients serum. IL-10, combined or not with TGF- β , secreted by antigen presenting cells and regulatory T cells may directly interfere with allergic effectors mechanisms by inhibiting mast cell degranulation or Th2 immune response [11,12]. This mechanism was corroborated in animal models demonstrating that allergic immune response was suppressed by helminthes and this event was related to IL-10 increase production and regulatory CD4⁺Foxp3⁺CD45⁺ T cells [13].

Moreover, it has also been previously shown that PBMC of patients from endemic areas produce lower levels of IL-5 and IL-4 when exposed to *D. pteronyssinus* antigen (Der p1) than individuals with no helminth infection, resulting in lower levels of anti-Der p1 IgE [14]. On the other hand, worm antigens exposure stimulated by anthelmintic therapy promotes an increase of IL-5 and IL-4 productions [15,16]. At the same time, IL-10 production is suppressed, what may lead to IgE production including IgE anti-Der p1 [17,18]. *In vitro* studies demonstrate that Praziquantel can alter worm tegument, liberating or exposing inner worm antigens can also stimulate IL-6, TNF- α and eotaxin production culminating in antibodies isotypes changes over weeks or months [12,19–22]. Our data also support these findings as we showed that anti-Der p1 IgE levels increase after treatment. We propose that this

might be related to decreased IL-10 resulting in increased IL-4 production.

Concerning geohelminths, it has been demonstrated that Albendazole treatment was strongly associated with recurrent wheeze [21]. Moreover, it was observed that Albendole treatment during pregnancy enhances infantile eczema [23] and also increases in anti-Der p1 IgE [24,25] In this study we demonstrated that post-treatment response of individuals with high intensity of infection of geohelminths also have high levels of anti-Der p1 IgE but the opposite was observed in the population with individuals with low intensity of infection.

Rujeni and colleagues [26], found a negative correlation between IgE anti-Der p1 and the intensity of *S. mansoni* infection in high transmission areas. The authors also did not observed relationship between these two variables in low transmission areas. In our study, the anti-Der p1 IgE levels ratio analysis after and before treatment demonstrated that co-infected individuals had higher levels of anti-Der p1 IgE after treatment than the other groups of infected individuals evaluated. Although the increased anti-Der p1 IgE index of co-infected/cured individuals was observed, we did not observe a relationship between the intensity of infection and anti-Der p1 IgE. This event might be explained by the presence of different worms antigens released after treatment of co-infected individuals exacerbated the IgE productions, probably by stimulating IL-4 production [27].

Impact of treatment on Anti-Derp-1 IgE

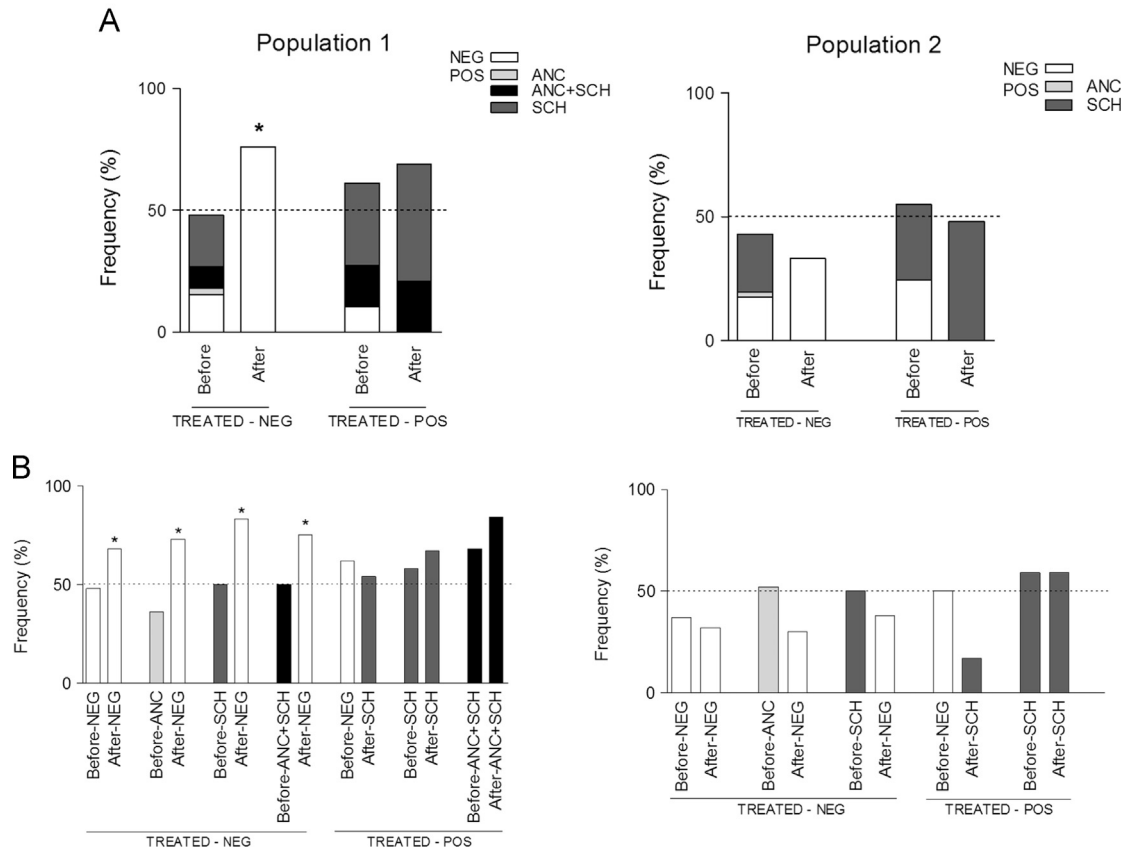


Fig. 4. Impact of treatment on Anti- Derp 1 IgE in two endemic areas for helminthic infection in Minas Gerais, Brazil. The frequency of “High anti-Der p1 IgE producers” and the the status before and after treatment (NEG/ white; HW/ light grey; SCH/ dark grey and HW + SCH/ black) were considered for population 1 (A) and population 2 (B). Statistical analyses were performed by Fisher’s exact and by Mann–Whitney tests. Each group of the populations was compared before and after treatment with their respective groups. In both cases significance were considered at $p < 0.05$ and highlighted by *.

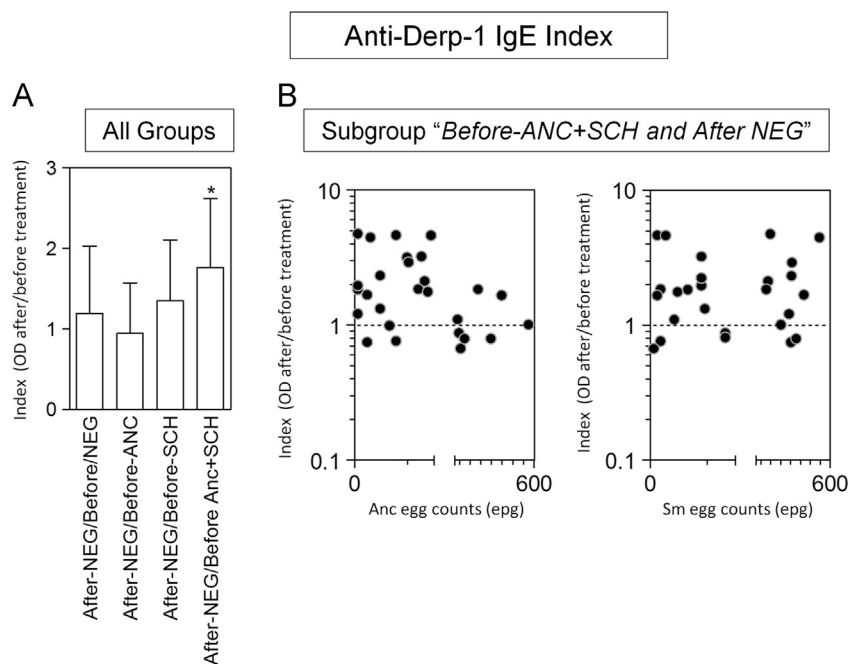


Fig. 5. Anti- Derp 1 IgE index. The index of anti- Der p1 IgE was defined as ration of anti-Der p1 IgE optical density value before treatment and anti- Der p1 IgE optical density value after treatment. The subgroups evaluated were those that presented negative results on Kato-Katz examinations. The groups were compared between them (A). The anti- Der p1 IgE index of co-infected subgroup (before HW + SCH and after NEG) was correlated with intensity of infection (B). Statistical analyses were performed by Fisher’s exact and by Mann–Whitney tests. In both cases significance were considered at $p < 0.05$ and highlighted by *.

It is important to underscore that data interpretation should be taken with caution, considering some particularities of experimental design and data collection approaches used in our study. In fact, the population 1 measurements were performed 2 years after treatment whereas the population 2 was assessed 3 years after treatment. Moreover, the post-treatment samples were taken years rather than weeks after treatment and therefore the infection level does not reflect efficacy of treatment but rather rates of re-infection in two areas with different rates of transmission. In addition, the questionnaires were administered 7 and 2 years after the beginning of the study in population 1 and 2, respectively.

6. Conclusions

The determination whether helminth infections confer protection or are risk factor to allergic disorders and the effect of anthelmintic treatment on allergic responses is still debatable. Although we could not find an association between helminth intensity of infection and allergy related risk factors it was demonstrated that effective chemotherapy of subjects from endemic areas with high prevalence of infection enhances the levels of anti-Der p1 IgE, that is a risk factor to allergic disorders development.

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