Age-Stratified Profiles of Serum IL-6, IL-10, and TNF-α Cytokines among Kenyan Children with *Schistosoma haematobium*, *Plasmodium falciparum*, and Other Chronic Parasitic Co-infections

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Abstract. In a study of children having polyparasitic infections in a *Schistosoma haematobium*–endemic area, we examined the hypothesis that *S. haematobium*–positive children, compared with *S. haematobium*–negative children (anti-soluble worm antigen preparation [SWAP] negative and egg negative) have increased systemic production of pro-inflammatory cytokines (interleukin [IL]-6, tumor necrosis factor [TNF]-α) and decreased down-regulatory IL-10. A total of 804 children, 2–19 years of age, were surveyed between July and December 2009 and tested for *S. haematobium*, *Plasmodium falciparum*, filariasis, and soil-transmitted helminth infections. Plasma levels of IL-6, TNF-α, and IL-10 were compared for *S. haematobium*–positive and *S. haematobium*–negative children, adjusting for malaria, filaria, and hookworm co-infections, and for nutritional status, age group, sex, and geographic location. IL-10 was significantly elevated among children infected with *S. haematobium*, showing bimodal peaks in 7–8 and 13–14 years age groups. IL-10 was also higher among children who were acutely malnourished, whereas IL-10 levels were lower in the presence of *S. haematobium*–filaria co-infection. After adjustment for co-factors, IL-6 was significantly elevated among children of 5–6 years and among those with *P. falciparum* infection. Lower levels of IL-6 were found in malaria–hookworm co-infection. High levels of TNF-α were found in children aged 11–12 years regardless of infection status. In addition, village of residence was a strong predictor of IL-6 and IL-10 plasma levels. In adolescent children infected with *S. haematobium*, there is an associated elevation in circulating IL-10 that may reduce the risk of later morbidity. Although we did not find a direct link between *S. haematobium* infection and circulating pro-inflammatory IL-6 and TNF-α levels, future T-cell stimulation studies may provide more conclusive linkages between infection and cytokine responses in settings that are endemic for multiple parasites and multiple co-infections.

INTRODUCTION

Data are scarce regarding the immune responses of children affected by chronic schistosomiasis and other overlapping parasitic infections, including malaria, filaria, and soil-transmitted helminth infections, experienced either sequentially or as polyparasitic disease. With an estimated 230 million people affected by schistosomiasis, and almost half of them being children,[1] there remains a significant knowledge gap regarding the timing and mechanisms for the initiation of *Schistosoma*-associated morbidity. As a result, schistosomiasis treatment campaigns may not be targeting an appropriate age range to prevent advanced disease due to end-organ fibrosis.[2]

Laboratory and clinical studies suggest that adaptive host immune responses can modulate manifestations of schistosomiasis morbidity.[3] However, production of pro-inflammatory cytokines is an essential part of chronic *Schistosoma* infection, and have been associated with malnutrition and anemia of inflammation, for example, by IL-6 stimulation of hepcidin production, a liver hormone responsible for sequestration of iron.[4–6] In *S. japonicum*–endemic settings, end-organ damage, manifesting as liver fibrosis, has been associated with elevated pro-inflammatory cytokine responses highlighting the correlation between inflammation and fibrosis.[5,7] Less is known about the link between *S. haematobium*–related cytokine response and infection-associated morbidity.[8]

The exact timing of the organ damage is largely unknown. Therefore, by analyzing the age profile of circulating pro-inflammatory (IL-6 and TNF-α) and immunomodulatory (IL-10) cytokines in the presence and absence of active *S. haematobium* infection, we aimed to identify the onset of inflammation and adaptive downregulation in chronically exposed children. To do this, we used a refined definition of *S. haematobium* infection that included either anti-parasite antibody positivity or egg patent infection. Our second objective was to correlate these cytokine levels with the presence or absence of schistosomiasis-associated morbidities such as anemia and undernutrition, and to explore interactions related to concurrent co-infections.

METHODS

Children aged 2–19 years (*N* = 804) were surveyed between July and December 2009 from two *S. haematobium*–endemic villages in Matuga and Msambweni sub-counties (in former Coast Province), Kenya (Figure 1) as detailed elsewhere.[9] The selected villages included low *S. haematobium* prevalence Vuga, and high prevalence Milalani (Table 1). Briefly, data collection included demography, urine filtration for *S. haematobium* eggs (one urine),[10] and Kato-Katz[11] stool examination for soil-transmitted helminths (one stool). Blood collection was performed by finger prick for the rapid antigen detection of *P. falciparum* (ICT Diagnostics, Sydney, Australia) and *Wuchereria bancrofti* filarial infection (Binax, Portland, ME).[9] After centrifugation, plasma samples were kept frozen at −80°C.

Hemoglobin was determined (Hemocue, Ångelholm, Sweden), and anemia and severe anemia were categorized according to World Health Organization (WHO) criteria for age and sex, and scored as present or absent for each child.[12] Reference population Z scores were calculated for each subject's height-for-age (HAZ) and body mass index-for-age

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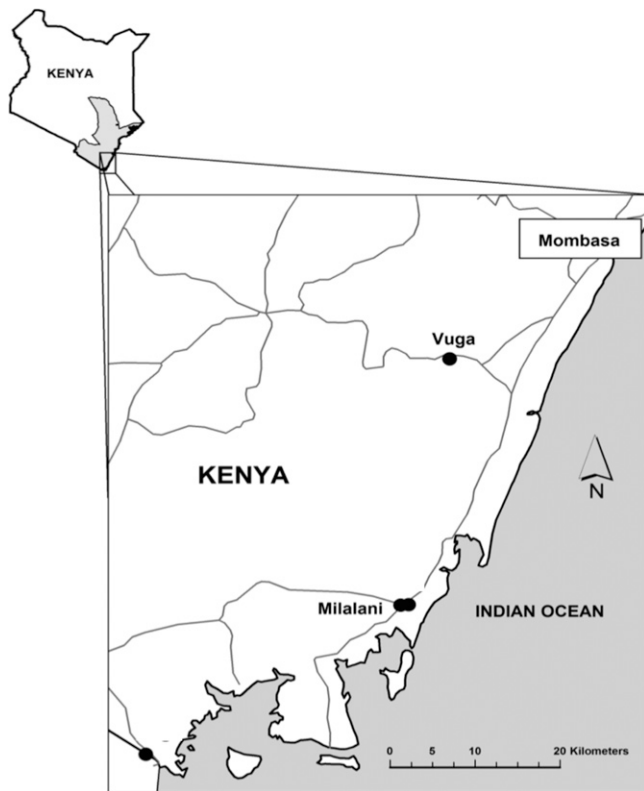
Figure 1. Map of the study villages in Kwale County, Kenya.

(BAZ) using international standards for comparison, taken from the WHO's Anthro program for ages 0–5 years and WHO Anthro-plus program for ages 5–19 years (WHO, Geneva, Switzerland).[13] According to WHO standards, stunting was categorized as an observed HAZ that was two or more standard deviations (SDs) below average (HAZ score < –2). Children were categorized as clinically wasted if their BAZ was more than 2 SDs below average for their age (BAZ score < –2).[13]

Ethical clearance was obtained from the Institutional Review Board at Case Western Reserve University and the Ethical Review Committee at the Kenya Medical Research Institute (KEMRI). Informed consent was obtained from each child's parent or guardian and verbal assent was obtained from children above 7 years of age. All infections detected during this study were treated in accordance with the national guidelines of Kenyan Ministry of Medical Services. Ultimately, 790 children provided full clinical and laboratory data (see Figure 2 for flow chart of enrollment), and these subjects with complete data were included in the data analysis presented in this article.

Anti-*Schistosoma* IgG4 antibody detection assay. To increase sensitivity for the detection of early, possibly egg-negative *S. haematobium* infection among younger children, we performed serologic testing for circulating anti-parasite IgG4 in all study subjects. Soluble worm antigen preparation (SWAP) (crude extract) was provided courtesy of Christopher King, Center for Global Health and Diseases, Cleveland, OH. Ninety-six-well plates were coated with 50 µL of 10 µg/mL SWAP diluted in enzyme-linked immunosorbent assay (ELISA) coating buffer and allowed to incubate at 4°C overnight. The plates were then blocked with ELISA blocking buffer and washed with ELISA wash buffer as described previously.[14] Serum samples prepared in ELISA diluent buffer were then allowed to incubate on the antigen-coated plate at 4°C overnight.[14] The plates were washed six times and incubated with 50 µL of a 1:2,000 dilution of mouse anti-human IgG4 (Jackson ImmunoResearch, West Grove, PA) for 2 hours. Plates were then washed and incubated with 50 µL of 1:1,000 dilution of goat anti-mouse, alkaline phosphatase–conjugated

TABLE 1
Population characteristics and cytokine distribution by village

	Milalani (N = 191)	Vuga (N = 613)
Demography		
Mean age ± SD in years (range)	12.4 ± 3.2 (5–19)	11.0 ± 3.7 (2–18)*
Female sex, n (%)	96 (50)	313 (51)
Morbidity		
Mean hemoglobin ± SD (range)	11.8 ± 1.9 (4.6–16.4)	11.8 ± 1.4 (6.1–15.9)
Anemia, n (%)	90 (47)	251 (41)
Stunting (HAZ < –2)	61 (32%)	263 (43%)*
Wasting (BAZ < –2)	21 (11%)	188 (31%)*
Parasitic infections		
Schistosoma haematobium		
Mean egg count	127 (0–1,000)	54 (0–1,000)*
Any intensity positive, n (%)	130 (68)	155 (25)*
Light (< 50 eggs/10 mL urine)	62 (32%)	80 (13%)
Heavy (> 50 eggs/10 mL urine)	68 (36%)	75 (12%)
Anti-SWAP IgG4‡		
Positive, n (%)	147 (77)	253 (41)*
Malaria (*P. falciparum*), n (%)	33 (17)	77 (13)
Filaria (*W. bancrofti*), n (%)	21 (11)	97 (16)
Hookworms, n (%)	43 (22)	64 (10)*
Cytokines		
Median IL-6 pg/mL (range)	204 (2–165,586)	298 (4–9,033)†
Median IL-10 pg/mL (range)	180 (1–2,473)	353 (2–424,996)†
Median TNF-α pg/mL (range)	102 (7–769)	104 (2–7,916)

SD = standard deviation; HAZ = age-adjusted height Z-score; BAZ, age-adjusted body-mass index Z-score; IL = interleukin; TNF = tumor necrosis factor.
* $P < 0.01$, significant difference between villages: χ^2 test for categorical variables, Student's t test for continuous variables.
† $P < 0.001$ for significant difference between villages, nonparametric testing by Mann–Whitney U test.
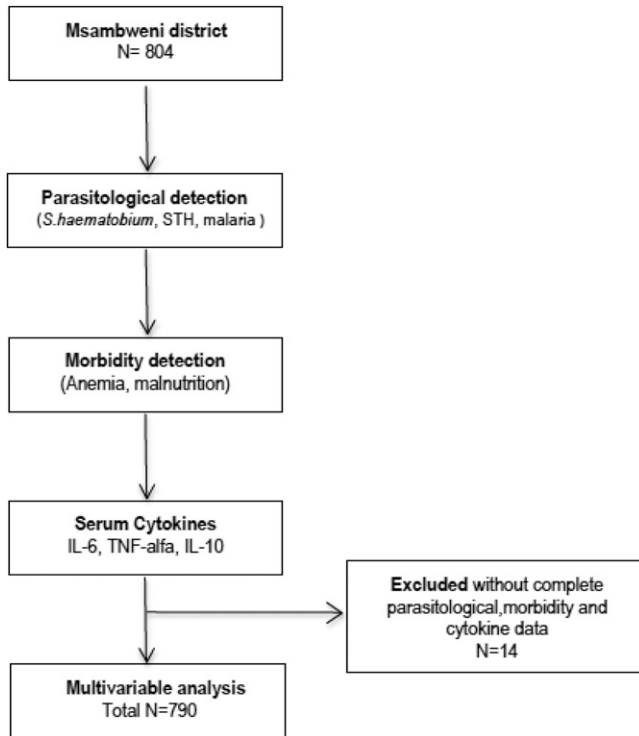‡ Antibody against *Schistosoma* adult Soluble Worm Antigen.

FIGURE 2.   Flow diagram with the design of the study.

antibody (Jackson ImmunoResearch) for 1 hour. The plates were washed and incubated with alkaline phosphatase substrate in buffer (Sigma Aldrich, St. Louis, MO) for 15 minutes. After 15 minutes, the reaction was stopped with 50 µL of 5% EDTA. Bound IgG4 was then measured by the determination of optical density at 415 nm. The cutoff for a positive test was set at 2 SDs above the mean optical density of 20 negative control sera obtained from an area in northern Kenya endemic for multiple parasites, but not endemic for schistosomiasis or filariasis (courtesy Christopher King Laboratory, CWRU, Cleveland, OH).

Circulating cytokine detection and quantification assays. Ninety-six-well Immulon IV ELISA plates were coated with 50 µL of 2.5 µg/mL primary antibody against IL-10, IL-6, and TNF-α (BD Diagnostics, San Jose, CA) diluted in ELISA coating buffer and allowed to incubate at 4°C overnight. After further washing and blocking of the plates, subject samples with standards and blanks were added in a 1:2 dilution and allowed to incubate at 4°C overnight. The plates were then washed and respective detection (biotinylated) antibodies were added, and the plates were incubated at room temperature for 45 minutes. The plates were rewashed and incubated in the presence of streptavidin alkaline phosphate for 30 minutes. The plates were then washed and incubated with alkaline phosphatase substrate buffer (Sigma Aldrich) for 1 hour. After 15 minutes, the reaction was stopped with 50 µL of 5% EDTA. Bound cytokines (IL-10, IL-6, and TNF-α) were then measured by the determination of optical density at 415 nm. There was a standard ELISA curve for each plate from the positive control standards, and the individual subject plasma levels (in pg/mL) were then interpolated from that curve.

Data entry and statistical analysis. All data were entered in Microsoft Excel (Redmond, King County, WA) and analyzed using the R statistical package version 2.14.1 (The R Foundation for Statistical Computing, Vienna, Austria) and SPSS v.21 software (IBM Corp., Armonk, NY). Arithmetic and geometric mean (GMW) were calculated for *S. haematobium* infection intensity. For percentages, 95% confidence intervals (CI) were calculated by applying the exact method. *S. haematobium* mean egg count, hookworm egg count, antibody values, and cytokine values were found to have a non–Gaussian distribution and were compared using non-parametric statistics, or were log transformed for multivariable linear modeling. Categorical variables were created for malaria, hookworm, anemia, *S. haematobium* intensity of infection, wasting, and stunting. A case definition of schistosomiasis was created with aggregated variables *S. haematobium* egg and/or anti-SWAP positive. Analysis of variance (ANOVA) or χ^2 testing was performed to assess the significance of differences detected among the study villages. Initial exploratory analysis included bivariate correlations between IL-6, IL-10, TNF-α levels, and the different covariates of interest: infection status, age, sex, village anemia, wasting, stunting, and other cytokines. Concurrent cytokine level predictors were also included in multivariable linear regression models.

RESULTS

Location-related differences. A total of 804 children were surveyed from two villages (see Figure 1 and Table 1). Milalani children were slightly older (mean age 12.4 versus 11.0, $P < 0.01$), but the male and female composition of the two community survey samples was not significantly different (Table 1). In high-prevalence Milalani ($N = 191$), 68% of the children had egg-patent *S. haematobium* infection in contrast to 25% of children in Vuga ($N = 613$). *Schistosoma* seroprevalence was again markedly higher in Milalani (77%) in contrast to Vuga (41%). More high-intensity *S. haematobium* infections (based on standardized urine filtration egg counts) were present in Milalani and the mean egg count per subject was higher in Milalani (Table 1). Other parasitic infections including malaria, hookworm, and filariasis were found in both villages (Table 1). Whereas levels of detected malaria and filarial infection were not significantly different between the two villages, hookworm was more common in Milalani (22% prevalence versus 10% in Vuga, $P < 0.01$) (Table 1).

In terms of clinical morbidity, the prevalence of anemia was not significantly different between the two villages: 47% in Milalani versus 41% in Vuga. However, a significantly greater proportion of children were malnourished in Vuga, with 43% of them being stunted and 31% wasted, compared with 32% and 11%, respectively, in Milalani.

For the cytokine outcomes measured, we observed overall higher levels of pro-inflammatory IL-6 and down-regulatory IL-10 among children from Vuga village as compared with Milalani. Levels of TNF-α did not vary significantly between locations.

Cytokine age-related distribution. The age-related distribution of the cytokines analyzed by infection status is shown in Figure 3. After controlling for possible confounders (sex, malaria, anemia, schistosomiasis, hookworm, wasting, stunting, and other cytokines), age remained a significant independent predictor of IL-6 response, with children aged 5–6 years having higher levels of IL-6 (see Table 2). IL-10 levels were significantly lower in children aged 11–12 years and 17–19 years as
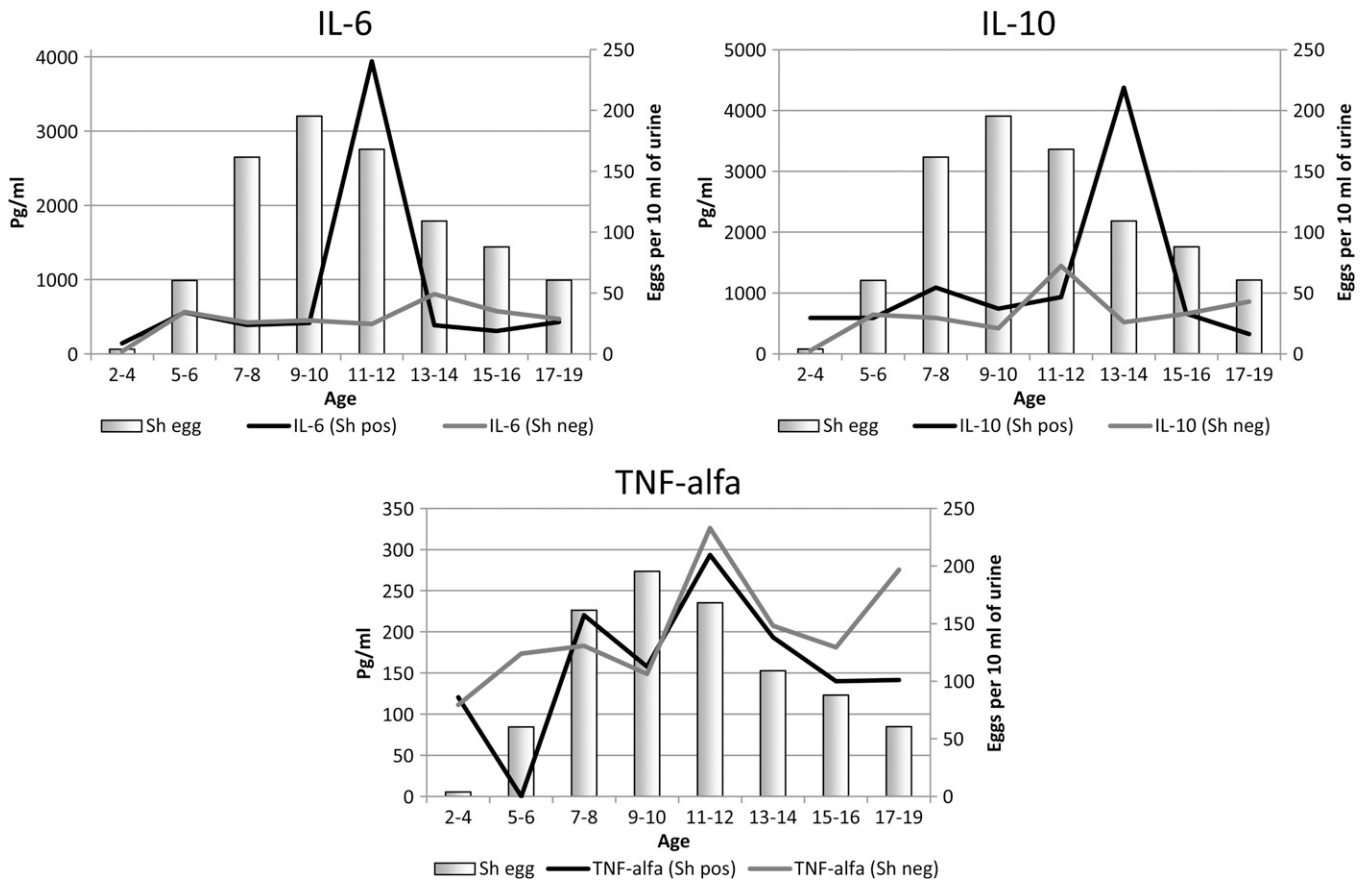
FIGURE 3. Age-related changes in cytokine levels according to *S. haematobium* infection status (Anti-SWAP positive and/or egg positive), contrasted with age-group-specific intensity of infection.

compared with 13–14 years when the model was adjusted for possible confounders (see Table 3). TNF-α levels were significantly higher in 11- to 12-year-old children (see Table 4).

Cytokine levels and infection. Overall, median IL-6 levels were higher ($P = 0.008$, Mann–Whitney U test), and IL-10 levels were also higher ($P = 0.003$), among children infected with *S. haematobium* (antibody positive and/or egg positive)

when compared with those not infected by *S. haematobium*. However, after adjustment for co-factors, the *S. haematobium* effect on IL-6 was no longer significant (Table 2), whereas the *S. haematobium* effect on IL-10 remained significant (Table 3). Nevertheless, we observed that IL-6 was particularly elevated among *S. haematobium*–infected children aged 11–12 years

TABLE 2

Multivariable log-linear modeling for the association of IL-6 with potential explanatory variables

Variables	Multivariable model for $\log_{10}$-IL6 level		
	B coefficient	95% CI	P value
IL-10*	−0.018	(−0.079, 0.051)	0.673
TNF-α*	0.262	(0.201, 0.391)	< 0.0001
Sex (male)	0.028	(−0.040, 0.094)	0.424
Village (Vuga)	0.131	(0.067, 0.240)	0.001
5–6 years old	0.091	(0.007, 0.269)	0.040
Wasted†	−0.013	(−0.113, 0.084)	0.771
Stunted‡	0.021	(−0.060, 0.102)	0.609
Malaria	0.112	(0.015,0.210)	0.024
S. haematobium§	0.052	(−0.024,0.129)	0.180
Hookworm	0.029	(−0.070,0.127)	0.567
Malaria–hookworm	−0.076	(−0.503, −0.011)	0.040

Final models adjusted for sex, age category, location, wasting, stunting, anemia, individual infections (*S. haematobium*, malaria, hookworm), and any significant interactions for infection by two or more parasites.
*$\log_{10}$ of IL-10 and TNF-α.
†Wasting: Body mass index-for-age Z score (BAZ) < −2 SD.
‡Stunting: Height-for-age Z score (HAZ) < −2 SD.
§Positive infection status is defined by *S. haematobium* egg +/antibody +, egg −/antibody +, or egg +/antibody −.

TABLE 3

Multivariable log-linear modeling for the association of IL-10 with potential explanatory variables

Variables	Multivariable model for $\log_{10}$-IL-10 level		
	B coefficient	95% CI	P value
TNF-α*	0.584	(0.773, 0.947)	< 0.0001
IL-6*	−0.013	(−0.094, 0.061)	0.673
Sex (male)	0.011	(−0.059, 0.087)	0.713
Village (Vuga)	0.123	(0.092, 0.281)	< 0.0001
11–12 years	−0.104	(−0.308, −0.057)	0.004
17–19 years old	−0.121	(−0.468, −0.146)	< 0.0001
Wasted†	0.107	(0.001, 0.214)	0.048
Stunted‡	−0.035	(−0.123, 0.054)	0.434
Malaria	−0.083	(−0.189, 0.022)	0.121
S. haematobium§	0.207	(−0.288, −0.126)	< 0.0001
Hookworm	0.001	(−0.105, 0.107)	0.985
Schisto–Filaria	−0.069	(−0.350, −0.033)	0.018

Final models adjusted for sex, age category, location, wasting, stunting, anemia, individual infections (*S. haematobium*, malaria, hookworm), and significant interactions for infection by two or more parasites.
*$\log_{10}$ of IL-6 and TNF-α.
†Wasting: Body mass index-for-age Z score (BAZ) < −2 SD.
‡Stunting: Height-for-age Z score (HAZ) < −2 SD.
§Positive infection status is defined by *S. haematobium* egg +/antibody +, egg −/antibody +, or egg +/antibody −.

TABLE 4

Multivariable log-linear modeling for the association of TNF-α with potential explanatory variables

Variables	Multivariable model for log$_{10}$-TNF-α level		
	B coefficient	95% CI	P value
IL-10*	0.561	(0.343, 0.420)	< 0.0001
IL-6*	0.176	(0.106, 0.207)	< 0.0001
Sex (male)	−0.038	(−0.081, 0.016)	0.149
Village (Vuga)	−0.024	(−0.088, 0.039)	0.126
11–12 years old	0.135	(0.078, 0.244)	< 0.0001
Wasted†	−0.063	(−0.134, 0.009)	0.087
Stunted‡	0.020	(−0.041, 0.077)	0.545
Malaria	0.026	(−0.038, 0.105)	0.356
S. haematobium§	0.056	(−0.006, 0.105)	0.080
Hookworm	−0.025	(−0.103, 0.040)	0.389

Final models adjusted for sex, age category, location, wasting, stunting, anemia, individual infections (*S. haematobium*, malaria, hookworm), and significant interactions for infection by two or more parasites.
*log$_{10}$ of IL-10 and IL-6.
†Wasting: Body mass index-for-age Z score (BAZ) < −2 SD.
‡Stunting: Height-for-age Z score (HAZ) < −2 SD.
§Positive infection status is defined by *S. haematobium* egg +/antibody +, egg −/antibody +, or egg +/antibody −.

(compared with *S. haematobium*–negative children of the same and other age groups), and IL-10 was highest among *S. haematobium*–infected children in the 13- to 14-year age group (compared with *S. haematobium*–uninfected children in that and other age groups) (see Figure 2). We also noted that 11- to 12-year-old children had higher TNF-α levels regardless of *S. haematobium*–infection status.

After adjusting for age, sex, location, and other variables, children affected by malaria were younger (5–6 years old) and had significantly higher IL-6 levels (Table 2) although those co-infected with malaria and hookworm infection had relatively lower IL-6 levels, suggesting a significant immunomodulation between these two infections. Although IL-10 levels were significantly higher in *S. haematobium*–infected children, they were lower among those who had *S. haematobium*–filarial co-infection (Table 3). There was overall a positive correlation between IL-6 and TNF-α levels, and TNF-α and IL-10 levels, respectively.

Cytokine levels and morbidity outcomes. Morbidity status measured as stunting (chronic malnutrition), wasting (acute malnutrition), or anemia was not associated with significant differences in measured circulating cytokines in our cross-sectional survey. The exception was IL-10 levels that were significantly elevated among wasted children.

DISCUSSION

Our results show an overall association between down-regulatory IL-10 cytokine and *S. haematobium* infection, but with relatively lower levels in early puberty (11–13 years old) and late adolescence (17–19 years old). Contrary to what was expected, circulating levels of pro-inflammatory cytokines (IL-6 and TNF-α) were not associated with urogenital schistosomiasis, even though granulomatous inflammation with T-cell activation is a hallmark of *S. haematobium* infection.[3,15] Children with malaria had a significant higher pro-inflammatory IL-6 levels, particularly in the younger ones (5–7 years old), although lower levels were seen in children co-infected with both malaria and hookworm. Striking inter-village differences were found for cytokine levels and morbidity outcomes, suggesting a clustering effect possibly related to geographical

transmission patterns and nutritional and/or socioeconomic disparities.[16,17] This highlights the multi-dimensional approaches needed to understand schistosomiasis infection and disease in the context of other endemic parasitic infections,[18] and the social determinants of health that remain so far unexplored in this field. The unforeseen lack of association between schistosomiasis-associated morbidities (anemia, wasting, and stunting) and pro-inflammatory cytokines previously seen in other endemic settings[5,18,19] might be explained by the intrinsic plasma level variability of cytokine levels when compared with laboratory responses seen in antigen T-cell-stimulating assays performed in other studies.[18–20] Co-infections could have also played an important role. For example, interactions across the immune responses to co-infecting parasites have been suggested in studies in Mali, where immune responses to acute malaria were blunted in children co-infected with urogenital schistosomiasis whose baseline production of IL-6, IL-4, IL-10, and interferon (INF)-γ was initially high.[21,22] In subsequent acute malaria infection, among 4- to 8-year-olds, the production of IL-6 was blunted in *S. haematobium*–positive children compared with those who were *S. haematobium* negative, suggesting a relative protective effect of *S. haematobium* co-infection on the outcomes of acute *P. falciparum* malaria.[22] Age- or experience-related factors likely modify this interaction. A smaller study in Senegal found increased production of anti-fibrogenic INFγ in *S. haematobium*–infected children who had *P. falciparum* malaria.[23] Increased levels of INFγ, seen in acute schistosomiasis[24] have also been associated with protection against periportal fibrosis.[25] The interesting inverse relationship between IL-6 and malaria–hookworm co-infection found in this study could potentially have the same immune mechanism as *Plasmodium–Schistosoma*, as hookworm and *Schistosoma* share some common host-regulatory pathways.[26]

In intestinal schistosomiasis, IL-10 is believed to have anti-inflammatory immunomodulatory effects as its greater presence is associated with reduced risk of periportal fibrosis from either *S. mansoni* or *S. japonicum* infection.[7,27] Therefore, our findings of high IL-10 in children with urogenital schistosomiasis in mid-puberty suggest a possible acquired, age-related protective effect against further morbidity following the 13- to 14-year-old age mark.[28] Protection against *S. japonicum* re-infection has also been described in association with sex hormone production during late puberty,[29] and there may well be a cytokine–hormone interplay contributing to this effect. In the same geographical location as our study, a positive correlation has been found in adolescent males between structural urinary tract pathology and the simultaneous presence of low levels of IL-10 and high levels of TNF-α in response to *S. haematobium* antigens.[18] Elevated pro-inflammatory TNF-α is associated with hepatic fibrosis in a *S. mansoni*–endemic area in Uganda and *S. japonicum*–endemic settings in the Philippines and China.[6,20,27] Parasite-induced inflammation is thought to be part of the causal pathway of under-nutrition and anemia.[5,6,9] Our results, however, did not show any association between IL-6 or TNF-α and the wasting, stunting, or anemia in this group of children.

There are several limitations in our study. First, current serum cytokine levels taken in cross section may not accurately reflect the ongoing in vivo effects of infection related to different antigenic stimulation. Single time point cytokine detection in the circulation, rather than in the local tissue microenvironments, is an inherent limitation to this kind of

study. The study was a single cross section, and therefore no post-treatment changes could be established to support parasite-specific associations. The effect of treatment on cytokine profile and its relationship to anemia and nutritional morbidity reversal would be an important question to address given that hepatic vascular remodeling has been shown to occur after treatment in studies in animal models of schistosomiasis.[20,30,31] Exploring other cytokines not included in this study (i.e., IL-4, IL-5, and IL-13) and important in understanding the immune response to multiple parasitic infections should be integrated in future research efforts.[20]

In summary, we have shown age-specific variation in serum cytokine profiles among children with *S. haematobium* and other chronic parasitic infections. The high levels of IL-10 in young children with schistosomiasis could potentially protect them against developing inflammation-related morbidity. Further pairing of morbidity assessment studies with more refined T-cell stimulation assays will help to strengthen assessments for the appropriate timing of anti-schistosomal treatment for prevention or remission of infection-related organ fibrosis.

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