

Multidisciplinary biomarkers aggrieve morbidity in schistosomiasis

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Abstract. Background: Biomarkers by definition are measurable molecules that mark the evidence of certain pathological processes. Collaboration of various biomarkers influences morbidity of schistosomiasis in Egypt. Objectives: To identify the biomarkers: CRP, IgE, hemoglobin, ferritin, vitamin D, and platelets in terms of relationship with active and chronic schistosomiasis; demographic data, and their interinfluence. Study design: A cross-sectional study. Methods: Parasitological analysis of stool and urine samples, Indirect Hemagglutination Test, Enzyme linked Immunoassay, Hematology Analyzer, and Statistical Package SPSS (Statistical Package for the Social Sciences) version 25. Results: Out of 400 participants, 25% suffered of schistosomiasis: active *S. mansoni* infections in 7 cases (1.75%), *S. haematobium* infections in 6 cases (1.5%), and chronic schistosomiasis infections in 20 cases (5%). C-reactive protein (CRP) likewise IgE levels were higher in active *S. mansoni* and *S. haematobium* infections when compared with chronic schistosomiasis. IgE levels appeared to affect infection intensity in *S. haematobium*. Inversely, hemoglobin (Hb) values were low in active schistosomiasis and upgraded in chronic infection (* $p < 0.05$). Ferritin levels varied in active *Schistosoma* infection and normalized during chronicity. Vitamin D was reduced in active and chronic schistosomiasis. Platelet counts were within normal ranges throughout the study groups. Distribution of ferritin, vit D, and platelets was statistically insignificant among *Schistosoma* infected population. Age affected only hemoglobin, CRP, and IgE biomarkers. CRP and IgE were in direct relationship together and inversely proportional with hemoglobin (* $P < 0.05$). Conclusion: Anemia increased proportionally with biomarkers of inflammatory stress (CRP and IgE) in early infections. Meanwhile, Hb and ferritin (iron stores) improved during chronicity. Hypovitaminosis-D associated the entire course of schistosomiasis while platelet counts were not affected.

INTRODUCTION

Human schistosomiasis affects at least 230 million people worldwide (Barakat 2013 and Youssef and Uga, 2014). Both types of *Schistosoma* (intestinal and urinary) are endemic in Egypt (Youssef and Uga, 2014). Adult schistosome worms colonize human blood vessels for years and exert multiple immune evasion mechanisms while producing hundreds of eggs daily (Confalonieri *et al.*, 1988 and McDonald *et al.*, 2010). Eggs

are either excreted with intensive blood loss, iron deficiency anemia, easy fatigue, stunt growth, impaired cognition and low productivity or become trapped in nearby tissues (Confalonieri *et al.*, 1988) to elucidate immune-mediated granulomatous response that will be terminated by fibrosis and lifelong complications (Jones *et al.*, 2007 and Dienz and Rincon, 2008).

Course of schistosomiasis is thought to be affected by various factors that might promote or hinder inflammatory course of the

disease. C-reactive protein (CRP), the acute phase reactant produced by the liver as quick response in acute inflammations. It is considered to be a more sensitive indicator of stress than erythrocyte sedimentation rate (ESR) (Osei-Bimpong *et al.*, 2007). Secretion of IgE from B cells occurs under the umbrella of type-2 cytokines (de Jesus *et al.*, 2004). Another study conducted by Pinot de Moira *et al.* (2013) demonstrated that the collaboration of high IgE serum levels and Praziquantel not only decline infection intensity but also hinders reinfection in early disease.

Hemoglobin is essential for oxygen carriage to tissues for ATP production. However, several factors can influence hemoglobin concentration including nutritional deficiencies, co-infection of other parasites, age, sex, weight and increased physiological iron requirements in some conditions e.g. pregnant women (Coutinho *et al.*, 2005). Ferritin is the iron stores and was considered as an inflammatory biomarker (Ruddell *et al.*, 2009) as it is a member of large protein family that masters the cellular defense in cases of stress and inflammation (Ruddell *et al.*, 2009 and Elizabeth *et al.*, 2008). High ferritin had been suggested to increase aggressiveness of active schistosomiasis (McDonald *et al.*, 2010). Iron appears to be absorbed across the tegument of the parasite to be stored intracellular especially in the vitelline glands of female schistosomes. Based on ultra-structural studies, egg yolk ferritin is a fundamental requirement for miracidia to develop. These findings emphasize the importance of the patient's iron stores (ferritin) in schistosome fertility and egg formation (Clemens and Basch, 1989).

Vitamin D has immunomodulatory and anti-inflammatory effects (Bonventre, 2013). Besides vitamin D plays an impact role in calcium homeostasis (Kruita and Pieter Zanenb, 2016). Several factors may affect its normal physiological levels including deprivation of the patients from exposures to the sun light besides the cyclic patterns of the ultraviolet B energy over the 12 months, poor dietary intake and the physiological

variations in patients with possible higher tissue requirements for vit D e.g. childhood and pregnancy. Genetic role in previous studies had been explained to be minor (Malloy and Feldman, 2010).

Previous experimental studies showed the direct cytotoxic effect of platelets on larval stages of *Schistosoma* parasites especially after being interacted with IgE via specific receptor or activated by CRP and cytokines (Bout *et al.*, 1986 and Da'dara and Skelly, 2014). In addition in murine model isolated eggs induced platelet aggregation that subsequently promoted extravasation and excretion of the schistosome eggs (Joseph *et al.*, 1986 and Ngaiza and Doenhoff, 1990).

This study aimed to evaluate levels of various biomarkers including IgE, C-reactive protein (CRP), hemoglobin (Hb), ferritin, vitamin D, and platelets up on morbidity of Egyptian patients suffer of active and chronic schistosomiasis, relationship of demographic data including age and sex to these biomarkers and interactions of these variable biomarkers together.

METHODOLOGY

Ethical Statement

Ethical approval was received from the ethical committee of Cairo University School of Medicine. In the present study all procedures concerning human participants were in accordance with the ethical standards settled by the National Research Committee and the 1964 Helsinki Declaration and its successive amendments and comparable ethical standards. Aims and procedures of the study were explained and all participants or parents' of the children above 5 years assigned informed consent before being enrolled into the study. Patients of any age who suffered of cancers were not included, as well as pregnant women and under five years old children.

After sample collection, *Schistosoma* – infected patients were offered the anti-helminthic drug “Praziquantel” with the standard dose of 40 mg/kg body weight.

Population characterization and study area

A cross-sectional study was performed in a convenience sample composed of (400) patients presented to Abu El-Reesh El-Mounira Pediatric Hospital and The Internal Medicine Hospital Cairo University out-patient clinics between November 2017 and August 2018.

Inclusion criteria were in accordance with the following measures: (1) all participants had to be residents in endemic areas in Egypt for most of their lives (defined by history taking via a questionnaire), (2) should be available to provide the team of the study with at least three urine and two stool samples on successive days to permit for proper parasitological diagnosis, (3) should provide the study with blood samples to obtain serum for the biochemical analysis, (4) should not receive any anthelmintic therapy prior sample collection in this study, (5) conditions that may alter the biochemical rational should all be avoided including repetitive defrosting of the samples.

Sample collection

Stool and mid-day urine samples were collected in sterile cups and were sent to the Medical Parasitology Department Cairo University. Serum samples obtained from 10 mL venous blood from each patient were stored at -20°C freezer to be subsequently sent to the Clinical Pathology Department Cairo University.

Parasitological examination

Stool samples were qualitatively evaluated for *Schistosoma mansoni* eggs by direct microscopic examination of fresh fecal samples and concentration by sedimentation using formalin-Ethyl-Acetate concentration technique (Confalonieri *et al.*, 1988 and Rabello, 1992). Quantitative study by 41.7 mg Kato template was performed for positive fecal samples to estimate the parasite worm burden (Rabello, 1992). The obtained material was then covered by cellophane slip soaked in glycerin and 3% Malachite Green that allowed dehydration. The prepared samples were then left to settle 30-60 minutes before being examined under a light micro-

scope and number of eggs per gram was calculated (Confalonieri, 1985 and Cringoli *et al.*, 2010). In an attempt to enhance validity of the readings microscopic examination was conducted by two parasitologists in 4-6 smears for each sample. Urine samples were physically examined and filtered using nucleopore filter membrane to detect *Schistosoma haematobium* eggs. Number of eggs per 10ml urine was calculated and recorded for each patient (Hai-Yong, 2013 and Richard *et al.*, 2014).

Immunoassays and biomarkers assessment

Anti-Schistosoma anti body titer was measured in the collected serum samples by the Commercial Indirect Hemagglutination Test in accordance with manufacturing instructions (FUMOUCHE DIAGNOSTICS). Serial dilutions for patients' serum were performed and serological titer greater than 1:60 was considered positive for schistosomiasis.

IgE was measured by ELISA kit from Abcam (Cat. #AB108650). CRP is an inflammatory biomarker (Osei-Bimpong *et al.*, 2007) and was assayed by ELISA kit from Sigma-Aldrich (Cat. # RAB0096). Hemoglobin and platelet count was performed by using a Sero Baker 9000 hematology analyzer (Sero Baker Diagnostics, Allentown, PA). Vitamin D serum level was evaluated in all patients by 25 (OH) Vitamin D ELISA (solid phase enzyme-linked immunoassay kit) from Sigma-Aldrich (Cat. #SE120139). The procedure was performed based on the competitive binding principal according to the manufacturer instruction. Ferritin was measured by an ELISA kit from Abcam (Cat. # AB200018).

Statistical analyses

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 25. For the statistical analyses, samples of schistosomiasis infection were classified into active *Schistosoma mansoni*; active *Schistosoma haematobium* and chronic schistosomiasis. Intensity of active schistosomiasis infection was estimated in agreement with the World

Health Organization and similar methodology enrolled: by Cringoli *et al.* (2010), Reilly *et al.* (2012), and Richard *et al.* (2014). Regarding *S. mansoni* infections eggs per gram (epg) were calculated and infection was in turn classified into light (1-99 epg); moderate (100-399 epg) and heavy (≥ 400 epg) while *S. haematobium* was evaluated as light worm burden (≤ 50 eggs/10ml) and high worm burden (≥ 50 eggs/10ml).

Hemoglobin status was described using previously published ranges (13.5 to 17.5 g/dl for men and 12.0 to 15.5 g/dl for women). Normal CRP level ranges below 3mg/L (Danesh *et al.*, 2004). In IgE, the usually accepted upper limit range from 100 to 200 I.U./ml in children (Csorba *et al.*, 1976) and from 150 up to 300 I.U./ml in adults (Laurent *et al.*, 1985).

To evaluate the vitamin D levels, the cut off was established according to the Endocrine Society Clinical Practice Guideline: deficiency ≤ 20 ng/ mL; insufficiency 21-29 ng/mL and sufficiency ≥ 30 ng/mL (Bonventre, 2013). Ferritin was assessed according to The World Health Organization normal reference ranges (15.0–150.0 $\mu\text{g/L}$ in females and 15.0–200.0 $\mu\text{g/L}$ in male) (Coutinho *et al.*, 2005). Referral to The American Society of Hematology normal platelet count ranges from 150,000 to 450,000 platelets / μl of blood.

In order to evaluate interactions between infection status and various biomarkers data was summarized using mean, standard deviation, median, minimum and maximum of the quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and Mann-Whitney tests (Chan, 2003a). For comparing categorical data, Chi squared test was

performed. Exact test was used instead when the expected frequency is less than 5 (Chan, 2003b). Correlations between quantitative variables were done using Spearman correlation coefficient (Chan, 2003c). P-values less than 0.05 were considered as statistically significant.

RESULTS

Characterization of schistosome infection

In 400 patients schistosomiasis infection was prevalent in 25% (n=33) of the study population. Samples for each schistosomiasis infections are n=7 (1.75%) for active *S. mansoni*, n=6 (1.5%) for active *S. haematobium*, and n=20 (5%) for chronic schistosomiasis. Infection intensity in active *S. mansoni* and *S. haematobium* was described in Table 1.

Infection status followed the typical schistosome age-infection pattern, chronicity raised with age to a peak in middle age with a mean value 29.7 (* $p < 0.001$), (Figure 1-a).

Population of the present study had higher mean value of C-reactive protein (CRP) in *S. mansoni* (16.5 mg/L) and *S. haematobium* (22.67 mg/L) when compared with chronic schistosomiasis (9.75 mg/L) (* $p < 0.001$) Figure 1-b. Similarly, IgE levels had elevated mean values 471.71 I.U./ml and 813.33 I.U./ml in active *S. mansoni* and *S. haematobium* successively while in chronic stages it was 188.45 I.U./ml (* $p < 0.001$), (Figure 1-c). Hemoglobin (Hb) values were low in active infection with mean value 9 g/dl in *S. mansoni* and 9.5 g/dl in *S. haematobium*. In contrary, Hb levels were raised in chronic schistosomiasis with a mean value 12.75 g/dl (* $p < 0.001$), (Figure 1-d).

Table 1. Distribution of infection intensity in active *S. mansoni* and *S. haematobium*

Active <i>S. mansoni</i> (n=7)			Active <i>S. haematobium</i> (n=6)	
Light worm burden	Moderate worm burden	High worm burden	Light worm burden	High worm burden
(1-99 epg)	(100-399 epg)	(≥ 400 epg)	(≤ 50 eggs/10 ml)	(≥ 50 eggs/10 ml)
4 (57.14%)	2 (28.57%)	1 (14.29%)	4 (66.67%)	2 (33.33%)

Variable ferritin levels were detected in active *Schistosoma* infection but went up to normal and high values in chronic schistosomiasis. When considering the percentiles of Vit D, results mainly went

down towards insufficiency and deficiency in active and chronic schistosomiasis. Platelet results were shifted mainly towards normal, Table (2).

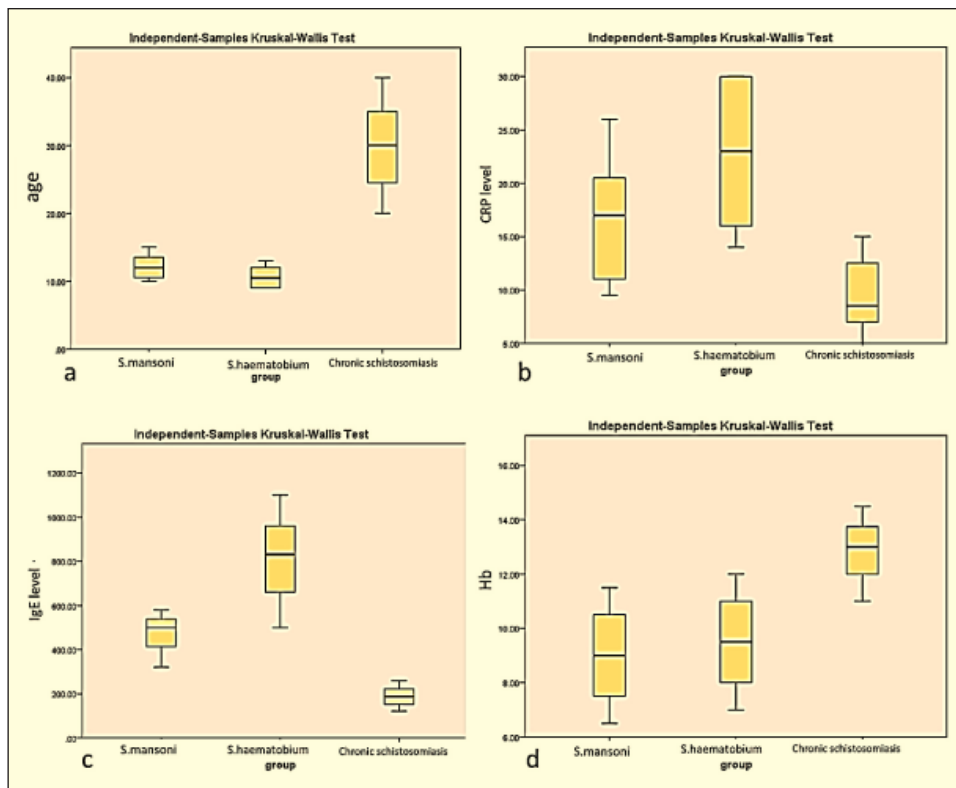


Figure 1. Stock columns represent mean, standard deviation, median, minimum and maximum value of the quantitative data of various biomarkers in each sample of schistosomiasis infections. (a) Age of infected population, (b) C-reactive protein (CRP), (c) immunoglobulin-E level (IgE), and (d) hemoglobin level (Hb) (* $p < 0.005$).

Table 2. Distribution of vitamin D, ferritin and platelets is shown versus the three samples of *Schistosoma* infections. However, statistical analyses showed that after allowing for the biomarkers (ferritin; vit D and platelets) there was no statistical significance in the three samples of schistosomiasis infection (* $p > 0.05$).

Schistosoma sample		<i>S. mansoni</i>		<i>S. haematobium</i>		Chronic schistosomiasis	
Biomarker	Value	Count	%	Count	%	Count	%
Ferritin	Low	2	28.6%	2	33.3%	0	0%
	Borderline	2	28.6%	2	33.3%	0	0%
	Normal	1	14.3%	0	0%	14	70.0%
	High	2	28.6%	2	33.3%	6	30.0%
Vit D titer	Normal	1	14.3%	0	0%	0	0%
	Insufficiency	3	42.9%	4	66.7%	6	30%
	Deficiency	3	42.9%	2	33.3%	14	70%
Platelets	Normal	5	71.4%	5	83.3%	13	65.0%
	Low	2	28.6%	1	16.7%	7	35.0%

Distribution of age-biomarkers profiles

The statistical analyses showed that age affected only levels of hemoglobin, IgE and CRP in a statistically significant pattern ($*P < 0.001$) as shown in Figure 2. Nevertheless, other biomarkers were not significantly affected ($*P > 0.05$). Yet, results

concerning other demographic variables e.g. sex, were statistically insignificant.

Interactions of various biomarkers

Statistics demonstrated the significant direct relationship between CRP and IgE ($*P < 0.001$) (Figure 3-a). Whereas both IgE and CRP were

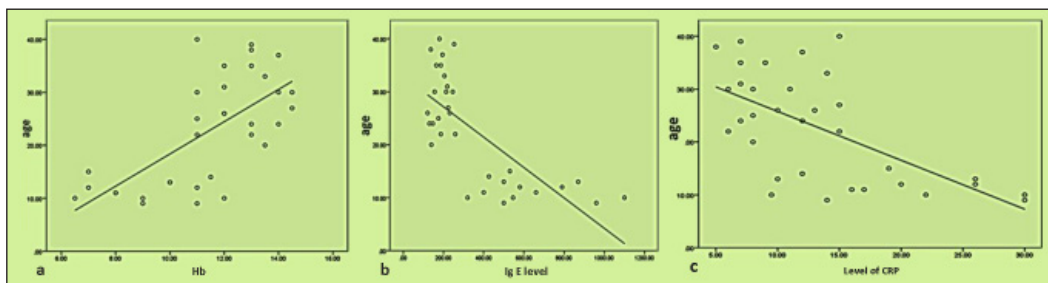


Figure 2. Correlation between age profile and biomarkers was performed by Spearman correlation coefficient. Open symbols and lines represent the total schistosomiasis infected population. Age versus haemoglobin (Hb) (a), IgE level, (b), and C-reactive protein (CRP) level (c).

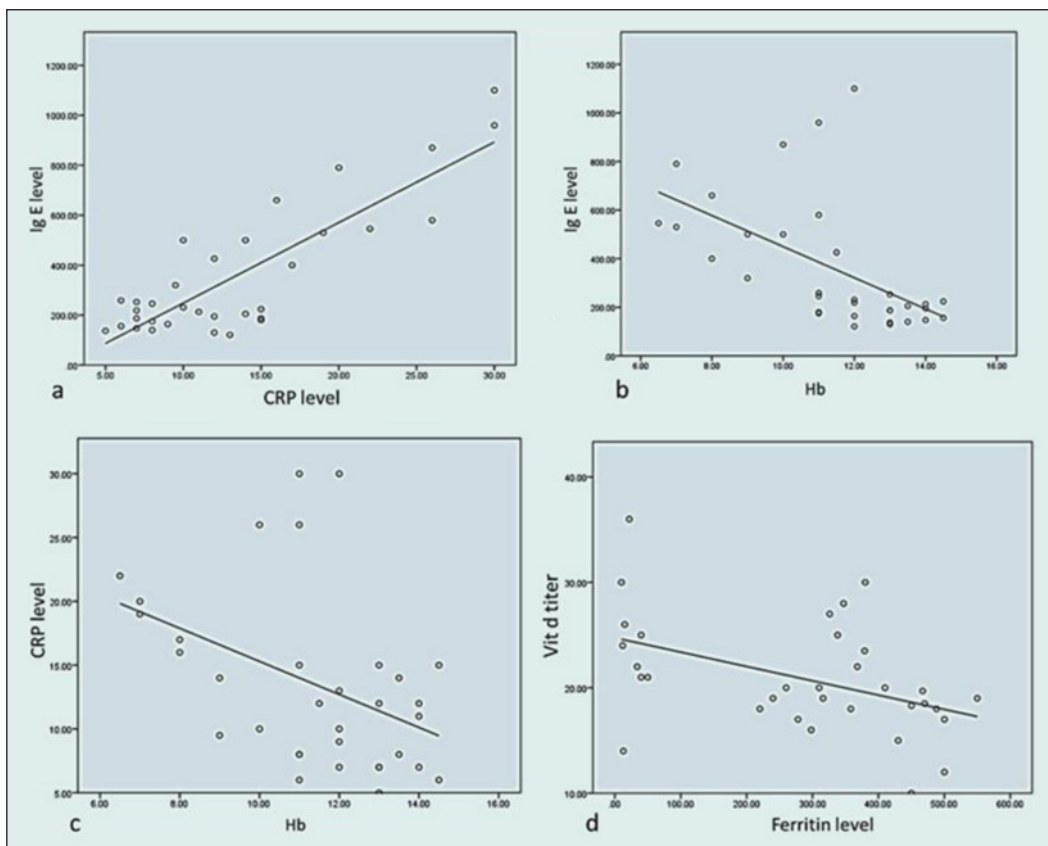


Figure 3. Interaction patterns of various biomarkers were statistically studied using Spearman correlation coefficient. Open symbols and lines represent the whole schistosomiasis infected population in the study. (a) IgE versus C-reactive protein (CRP) levels, (b) IgE versus hemoglobin (Hb), (c) CRP levels versus Hb, and (d) vitamin D levels versus ferritin levels (iron stores).

in inverse relationships with hemoglobin (* $P < 0.005$) as shown in Figure 3-b and 3-c respectively. Further relationships concerning biomarkers measured in this study with each other for example, vitamin D level with ferritin in Figure 3-d, were statistically significant (* $P > 0.005$).

DISCUSSION

Schistosomiasis is a significant parasitic infection creating disease burden throughout many of the world's developing nations (Youssef and Uga, 2014). In this study group, active *S. mansoni* and *S. haematobium* infections constituted 1.75% and 1.5% consecutively and chronic schistosomiasis was detected in 5% that simulate percentiles published previously in Egyptian studies (Barakat, 2013 and Sharazly *et al.*, 2016). These results might be attributed to schistosomiasis control projects that started in Egypt since 1953 in the form of Praziquantel mass treatment and snail control measurements. Interestingly, construction of Aswan High Dam that was accomplished in 1967 did not affect prevalence of schistosomiasis as it was expected (Barakat, 2013).

High worm burden was observed in 14.29% and 33.33% of the active *S. mansoni* and *S. haematobium* infections successively. Infection intensity relies greatly on the age of study population being peak at early childhood to decline later in adulthood (Reilly *et al.*, 2012). According to The World Health Organization (WHO) in 2002 this evidence is attributed to the early contact of the child with the infested water.

Human C-reactive protein (CRP) showed high levels during active schistosomiasis of this study population to act as a link between innate and adaptive immune systems (Osei-Bimpong *et al.*, 2007 and Feldman *et al.*, 2013). CRP is a serum-soluble pattern recognition receptor that uptake schistosomes' released antigens (Golam *et al.*, 2014) and interacts with Fc gamma receptors on leucocytes and activates complement pathway (Pascual *et al.*, 2005

and Tron *et al.*, 2008). Moreover, CRP polarizes T cells towards Th2 and inhibits Th1 differentiation (Zhang *et al.*, 2015 and Golam *et al.*, 2014) simulating the action exerted by the secreted schistosome soluble egg antigens (SEA) on T-cells (Tron *et al.*, 2008 and El-Aal *et al.*, 2015).

Chronic schistosomiasis in older patients had low CRP levels. Despite the described pivotal role of CRP in active *Schistosoma* infection, later on it exerts down regulatory effect on inflammation (Reeves, 2007 and Feldman *et al.*, 2013). This action runs in one track with other multiple immune modulatory mechanisms exerted by adult and egg stages of the schistosomes (Negrão-Corrêa *et al.*, 2014). Feldman *et al.* (2013) considered low CRP serum levels to be a sign of resolution of inflammation.

IgE is a key antibody in active *Schistosoma* infection in young age groups that acts to defeat the parasite (de Jesus *et al.*, 2004). Concerning our results active *S. haematobium* infection induced higher levels of IgE if compared with active *Schistosomiasis mansoni*. This may explain why the light worm burden (Pinot de Moira *et al.*, 2013) in *S. haematobium* was 66.67% while in active *S. mansoni* light worm burden constituted 57.14%.

In chronic schistosomiasis IgE serum levels declined profoundly which mimic results of a previous study reported by Stavitsky, (2004). McMahon and Kolstrup, (1979) and Herwaldt *et al.* (1995) related decline of immune responses to the elimination of adult worms and *Schistosoma* soluble egg antigens especially under influence of Praziquantel therapy. However, Negrão-Corrêa *et al.* (2014) reported low IgE serum levels in early infection that upgrades during chronicity.

Anemia in the form of low hemoglobin values were recorded in active *S. haematobium* and *S. mansoni* infections in contrary to chronic schistosomiasis in older age. Such sequel might be attributed to the extracorporeal blood loss, autoimmune hemolysis and elevated hepcidin serum levels (Friis *et al.*, 2003 and Coutinho *et al.*, 2007). In

addition, schistosomes had appeared to ingest red blood cells including reticulocytes (Zussman *et al.*, 1970 and Jones *et al.*, 2007).

Ferritin in the present study had increased unabatedly in some cases of active *S. haematobium* and *S. mansoni* infections. Immune contexts including tumor necrosis factor (TNF) and interleukin-1 (IL-1) upgrade in response to stress of disease activity and thus induce incorporation of iron (Fe) into ferritin (Tsuji *et al.*, 1991). Ruddell *et al.* (2009) recommended increasing the cut off points for ferritin in such populations.

Yet, low ferritin levels were also observed in considerable percentiles of patients with active schistosomiasis. This might be evidenced to the acute phase protein, hepcidin, that inhibits iron transportation by binding to the iron export channel ferroportin (Nemeth *et al.*, 2004) on the plasma membrane of macrophages of the reticulo-endothelial system and the basolateral surface of the intestinal enterocytes (Papanikolaou *et al.*, 2005, Rossi, 2005 and El Saftawy *et al.*, 2019). And thus iron becomes sequestered intracellular without passing into portal circulation and thereby, dietary iron absorption becomes reduced (Drake *et al.*, 2007).

In chronic schistosomiasis ferritin levels were shown to be within normal values. Nevertheless previous studies deduced anemia of chronic disease in schistosomiasis relying on the effect of pro-inflammatory cytokines especially IL-6 to upgrade hepcidin and thus induce anemia (Dienz and Rincon, 2008 and de Mast *et al.*, 2009). Drake *et al.*, (2007) suggested ferritin to accelerate fibrosis and high intracellular iron to expose hepatocytes to oxidative stress and hence collagen deposition occurs in a vicious circle. Therefore, it had been suggested by some authors that utilization of dietary iron supplementation to resolve anemia associated with chronic schistosomiasis may unexpectedly promotes fibrosis and worsen the long term disease outcomes (McDonald *et al.*, 2010).

Regarding data collected hypovitaminosis-D was demonstrated in both active and chronic stages. Vitamin D deficiency deprives patients of the anti-

inflammatory effects of the vitamin biomolecule required during the active stage (Liefwaard *et al.*, 2015). Shi *et al.* (2017) highlighted the afflicting role of hypovitaminosis D to induce fibrosis in chronic disease. This was approved by a study on animal models conducted by Bonventre, (2013) who concluded the anti-fibrotic properties of vitamin D. On the other hand previous studies described vitamin D as an immunomodulatory biomarker that induces cytokines polarization towards transforming growth factor- β (TGF- β) (Cantorna and Mahon, 2004; Reilly *et al.*, 2012 and Lee *et al.*, 2014) that in turn induces collagen deposition and fibroblast activation (Griffin *et al.*, 2003 and Beilfuss *et al.*, 2015).

Platelets range within normal in active and chronic schistosomiasis. Similarly, Da'dara and Skelly, (2014) observed that adult *Schistosoma* in spite of being large intravascular parasites, stressful on the vascular endothelium and can disturb blood flow platelets activation and thrombus formation don't appear to occur. Moreover, Fuentes and Palomo, (2013) described several mechanisms including degradation of Adenosine diphosphate (ADP) by enzyme-like tegumental molecules that hinders platelet-activation, production of prostaglandins that inhibit platelet aggregation in addition to induction of fibrinolysis by several tegumental proteins.

Statistics revealed that CRP and IgE were in a significant direct relationship as their bioactivity occurs under the same Th2 immunological mode (Feldman *et al.*, 2013 and de Jesus *et al.*, 2004). By contrast, vitamin D was in an inverse relationship with both CRP and IgE. Harrison, (2015) and Kruija and Zanenb, (2016) explained in their studies the deep influence of Th1 cytokines on the bioconversion of 25 (OH) vitamin D₃ to its active metabolite 1, 25 (OH) vitamin D₃ inside the macrophage while depressing the inflammatory biomarkers, CRP and IgE.

CONCLUSION

The study focused on the potential effect of a group of biomarkers on the morbidity caused

by *Schistosoma* infection. Higher levels CRP and IgE in active *Schistosoma* infection were associated with high risk of developing anemia in spite of their role in defeating infection. Investigating ferritin levels may provide deep insight for the iron status in both active and chronic infections to handle properly the further appropriate therapeutic measures. Hypovitaminosis-D is a hidden long run health problem throughout the course of the disease. However, its exact effect on liver fibrosis is still a matter of debate. Schistosomiasis doesn't appear to affect platelet counts.

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