Blood culture and Serum Cytokine profile associated with Hepatic Fibrosis in *Schistosoma mansoni* Infected Individuals

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Abstract: In the current study, we tried to evaluate the levels the T-helper-1 (TH-1) cytokine IFN-gamma (IFN- γ) and the TH2 cytokines (IL-4, IL-10 and IL-13) in both peripheral blood mononuclear cell culture (PBMC) following in vitro stimulation with Schistosoma soluble egg antigen (SEA) and serum of patients infected with S. mansoni and their relation to hepatic fibrosis induced by S. mansoni. Thirty-one S. mansoni infected individuals with and without liver fibrosis were analyzed for cytokine profiles. According to ultrasound evaluation of liver fibrosis, our study subjects were classified into Group 0, Group I, Group II, and Group III. Cytokines were measured in S. mansoni Soluble egg antigens (SEAs) stimulated whole blood culture supernatants and serum using commercial kits. Subjects in the hepatic fibrosis groups showed significant increase in IFN- γ, IL-10, and IL-13 ($p<0.05$) in comparison to non-fibrotic group. On the other hand, IL-4 was non-significantly elevated in the fibrosis group in comparison to the nonfibrotic group ($p >0.05$). Our results suggest that high serum and blood culture level of IL-13 recorded in fibrotic patients is associated with development of Schistosoma mansoni induced liver fibrosis.
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1. Introduction:

Schistosomiasis is the second most important parasitic disease in terms of public health impact (**Tucker et al., 2013**). It is estimated that at least 20 million out of 120 million symptomatic cases (**Chitsulo et al., 2004**) are having severe morbidity (**Crompton, 1999**). In Africa only including Egypt, almost 200 million individuals are infected with schistosomiasis (**Steinmann et al., 2006**).

The clinical picture and complications caused by Schistosoma species are variable according to the species-specific tropism of the egg laying adults**.** Schistosoma haematobium inhabits the vessels of the bladder causing urinary schistosomiasis, while both Schistosoma mansoni (S. mansoni) and Schistosoma japonicum (S. japonicum) reside in mesenteric veins of intestinal tract resulting in hepatosplenic schistosomiasis that may develop later to liver fibrosis and portal hypertension, if untreated (**Anthony et al., 2010**).

In endemic areas, most clinical cases of S. mansoni are often unrecognized. However, in a subset of infected individuals, severe hepatic fibrosis occurs due to excessive deposition of extracellular matrix in portal spaces in response to trapped Schistosoma eggs leading to presinusoidal fibrosis and portal hypertension. In such individuals, complications like splenomegaly, ascites, and esophageal varices may occur leading to life threatening hemorrhage (**Richter, et al 2015**).

Th-1 cells secrete IFN-γ and IL-2 that promote cell-mediated immunity, while the cytokine secretion of Th-2 cells (IL-4, -5, and -10) provide B cell help for antibody production (**Mosmann et al., 1986**).Both responses cross-regulate each other, as IFN- γ down-regulates Th2 cell development, while IL-4 and -10 antagonize Th1 cell differentiation (**Mosmann and Sad, 1996**) .

Schistosomiasis morbidity is induced by the eggs not directly by the adult (**Burke et al 2009**). After infection, not all produced eggs are excreted by the host and some become lodged in the intestines, liver or in the urogenital system. These eggs release highly immunogenic and cytolytic secretions (**Fairfax et al 2012**) and induce host granulomatous and fibrotic reaction that is mainly characterized by predominance T helper-2 cytokines (IL-4, IL-5, and IL-13), eosinophils and activated macrophage (**Booth et al 2004**).

Schistosomiasis induced pathology is characterized by a granulomatous reaction with subsequent fibrosis (**Pearce and MacDonald, 2002**), which is dependent mainly on Th2 mediated cytokines including IL- 4, IL-5, and IL-13

(**Schwartz, et al 2014**). This pathology induced by S. mansoni is attributed to unregulated CD4+T cell immune responses directed against the trapped ova in the hepatic sinusoids **(Wynn et al 2004).**

IL-13 is a pro-fibrotic cytokine essential for the development of Schistosoma induced hepatic fibrosis, which represent the main cause of morbidity and mortality in chronic schistosomiasis **(Fallon et al 2000) and (De Jesus et al., 2004)**.

IFN- γ has been identified as antifibrogenic cytokine (**Duncan and Berman, 1985**). It has been shown to confer protection against fibrosis in S. mansoni infected individuals (**Henri et al 2002**). IFN-γ inhibits the synthesis of extracellular molecules by the hepatic stellate cells (**Mallat et al 1995)** and enhances the expression of matrix metalloprotease (MMP) gene (**Tamai, et al 1995**). Low levels of IFN-γ were associated with severe periportal fibrosis in S. mansoni infected people (**Booth et al 2004**).

IL-10 has been assumed to play an important role in the regulation of CD4+ Tcell responses induced by S. mansoni. and failure to down-modulate these responses has been suggested as a mechanism for the development of hepatosplenic disease (**Malaquias et al 1997) and (Araujo et al 1996**).

Interleukin-4 is a central effector cytokine of Th2 cells, and key differentiation factor, which promotes the development of this subset of CD4+ lymphocytes. IL-4 induces B-cell class switching to IgE production, and up-regulates MHC class II. Th2 lymphocytes which produce IL-4 and IL-5 could mediate the formation of granuloma lesion and contributes to pathogenesis of hepatic fibrosis **(Sher *et al.,* 1992). Henderson et al., (1992)** reported that IL-4 and IL-5 are important cytokines for generation, expansion and maintenance of Schistosoma egg granuloma and it was suggested that a possible anti pathology vaccine could be produced based on reducing the presence of IL-4.

A peripheral blood mononuclear cell (PBMC) is any blood cell having a round nucleus such as lymphocyte, monocyte or macrophage**.** These blood cells are essential components in the immune system to attack infection. The lymphocyte population consists of T cells, B cells and NK cells **(Janeway *et al.,* 2001).** These cells are extracted from whole blood using ficoll, a hydrophilic polysaccharide which separates blood into layers, with monocytes and lymphocytes forming a buffy coat that contains the PBMCs under a layer of plasma.

The aim of the present study is to evaluate the levels of IFN-γ, IL-4, IL-10, and IL-13 cytokines in both serum and an in-vitro peripheral blood mononuclear cell (PBMC) culture stimulated with soluble egg antigen (SEA) of patients infected with Schistosoma mansoni. Also, we aim to relate their levels to the degree of hepatic fibrosis.

2. Subjects & Methods

Study subjects:

This study was conducted from October 2017 to October 2018 on patients attending the outpatient clinic of Tropical Medicine Department, Zagazig University Hospitals, Egypt. **Thirty-one patients** proven to be infected with S. mansoni were included in our study with age range from 18 to 63 years old (**23** males and **8** females).

Informed consent was obtained from all participants. The inclusion criteria for subjects were: positive Kato-Katz or rectal snip parasitological examinations for Schistosoma egg negative patients after repeated stool examination, negative history of therapy for schistosomiasis in the last 1 year. While The exclusion criteria for subjects were: age <4 years or >65 years, positive serology for human immunodeficiency virus, human T-cell leukemia virus type 1, or hepatitis virus types B and C, and combined infection with urinary schistosomiasis.

Parasitological detection of S. mansoni infection:

Stool samples from subjects were examined for S. mansoni infection by the Kato- Katz method **(Martin and Beaver, 1968).** Rectal snip (**Shoeb *et al.,* 1966)** was used to detect Schistosoma egg for repeated stool negative patients. Control negative subjects were selected by negative results for Schistosoma eggs, living in nonendemic areas for schistosomiasis and no history of infection.

Ultrasound evaluation for S. mansoni liver fibrosis:

S. mansoni induced liver pathology was detected using a conventional portable ultrasound instrument (ultrasonic diagnostic instrument model EUB-200; Hitachi, Tokyo, Japan, with portable Aloka SSD 500 Echo camera and 3.5-MHz convex probe).

Ultrasonographic examination was achieved using World Health Organization criteria created in 1993 (**WHO,1993)** for classification of hepatic fibrosis as follows: Grad**e 0 (G0):** Normal liver with no thickening of the wall of peripheral portal vein branches (PPB). PPB diameter is 2–3 mm. **Grade I (GI):** Small stretches of fibrosis around secondary portal branches. This patchy fibrosis usually yields a "fishes in the pond" appearance. PPB diameter is 4 mm. **Grade II (GII):** Continuous and patchy thickening of PPB. Most second order branches appear as long segment of fibrosis; PPB diameter is 5– 6mm. Gallbladder wall thickness may be >4 mm.

Grade III (GIII): Wall thickening of nearly all PPB and fibrosis reaches the surface of the liver. Gallbladder wall thickness is usually above normal (2–4mm).

According to the result of parasitological detection of Schistosoma *mansoni* egg and the presence of periportal fibrosis determined by ultrasonographic examination, the individuals included in our work were classified into:

1- *Schistosoma mansoni* negative individuals: Control healthy group.

2-*Schistosoma mansoni* positive patients: they were classified into 4 groups according to the levels of hepatic fibrosis diagnosed into *G0 **(n: 16)**, **control diseased:** no signs of periportal fibrosis, *GI **(n: 5):** mild periportal fibrosis, *GII **(n: 6):** moderate periportal fibrosis and *GIII **(n: 4):** severe periportal fibrosis. The groups included in our statistics were (G0, GI. GII, GIII respectively).

From all the studied individuals, 15 ml of blood was obtained separated into two parts.10 ml was assigned for blood culture and the other 5 ml were allotted for serum separation and both were yielded for cytokine evaluation.

Purification and culture of PBMC from fresh blood samples according to **Gazzinelli et al., (1983):** Was performed in the Medical Microbiology department, Zagazig faculty of Medicine as follow**:**10 ml of fresh heparinized blood was diluted with 10 ml sterile phosphate buffered saline **(PBS)** in 50 ml falcon tube. Each 7 ml of diluted blood were slowly layered on top of three 15ml falcon tubes with histopaque layer**.** Carefully, the tubes are centrifuged at 400 x g for exactly 30 minutes at room temperature (25°C) with switched off brake to avoid turbulence and loss of layers. The PBMC containing interphase (opaque) was identified above the barrier between Ficoll (colorless) and plasma (yellow). Carefully, the interface of (PBMCs) from all 3 sample tubes was pooled into one 15 ml conical tube for washing using PBS (the tube is centrifuged for exactly 10 minutes, until the visible pellet of PBMC is formed in the bottom of each tube then the PBMC pellet is pipetted and re-suspended in 15 ml PBS (the washing cycle is repeated until the supernatant become clear)**.** About2 ml Roswell Park Memorial Institute medium, commonly referred to as (**RPMI**) culture media is added to PBMCs pellet in preparation for counting and assessing its viability using Trypan blue dye using haemocytometer. Viable PBMCs will appear clear; while non-viable PBMCs will be blue (40% loss of cells, the sample will be ineffective). PBMC were cultured at 1×10^6 cells (the optimum concentration of cells/ ml culture media tested and found to induce cytokine response in patients not controls) in a total volume of 1 ml of complete RPMI 1640 (90% RPMI + 10% FBS + Gent/Pencilin stock 200µl) in the presence of SEA **(Theodor Bilharz Institute, Giza, Egypt)** at a concentration of 25 µg/ml (the optimum concentration of SEA that found to induce cell proliferation from Schistosomiasis patients but not from the control). After 48h incubation (time point previously been shown to be optimal for the measurement of cytokines. Except for IL-4 only which have been harvested after 24 hours) at 37°C in 5% CO_2, the supernatants were collected and immediately frozen at −80°C for the later measurement of the cytokine levels produced by the stimulated PBMC and cytokines were titrated by ELISA.

PBMC count was adjusted to 1×10^6 cells in a total volume of 1 ml of RPMI. 1640 according to the formula: Concentration X Volume = Concentration X Volume.

Cytokine analysis was performed in serum and whole blood culture supernatants using enzyme-linked immunosorbent assay-based prediction kits for detection **IFN-γ** (The Biosource IFN-γ EASIA (Biosourse Europe S. A. Zoning industriel, Rue de I" Industrie 8-B1400. NIVELLES, (Belgium) and for **IL-4, IL-10 and IL-13** (Orgenium Laboratories' ELISA; Ani Biotechoy, Orgenium laboratories Business unit, Finland). The cytokines were measured using the mentioned kits according to instructions.

Statistical Analysis:

IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. was used for statistical analysis. The Mann–Whitney test was used to determine differences in mean cytokine levels between the non-fibrotic and fibrotic groups. One-way ANOVA test was used for comparison between means of different fibrosis grades. **P- values**<0.05 were considered significant.

3. Results:

Characteristics of the study participants.

As shown in **Table 1**, the mean age of G0 (32.4±7.09), GI (43.00±3.16), GII (53.50±9.04) and GIII (60.50±2.38). The mean age difference between the four groups varied significantly (p **< 0.05**).

As regard to sex of our study participants, although there were more male (**n= 23**) than female participants (**n =8**), the difference in sex between the groups was not significant (p> **0.05**).

Cytokine levels in different grades of hepatic fibrosis:

Cytokine levels in serum **(Table 2)** and blood culture supernatants **(Table 3)** of 31 participants were determined in relation to fibrosis. Mean level of

(IFN-γ, IL-10, and IL13) in serum and blood culture supernatants of our study groups were significantly elevated in participants with fibrosis than those without fibrosis (**p<0.05**). While no significant elevation was found in IL-4 serum and blood culture level in fibrosis than non-fibrotic groups (**p>0.05**).

Table (1): Age and sex of study participants in different grades of hepatic fibrosis.

	G0 (18-45) n=16	GI (40-48) n=5	GII (40-63) n=6	GIII (58-63) n=4	P- value
Mean age±SD	32.4 ±7.09	43.00 ±3.16	53.50 ±9.04	60.50 ±2.38	0.000 (< 0.05)
SEX -Male -Female	 11(68.75%) 5 (31.25%)	 4 (80%) 1(20%)	 5 (83.33%) 1 (16.66%)	 3(75%) 1(25%)	0.911 (> 0.05)

*** The mean difference is significant at the 0.05 level**

Table (2): Mean serum cytokine levels (pg./ml) in the (G0) and fibrosis groups (GI, GII and GIII).

Serum cytokine	Non-fibrosis G0 N=16	Fibrosis (GI, GII, GIII) N=15	P value
IFN- γ	11.13	21.20	0.001(<0.05%)
IL-4	15.75	16.27	0.89 (>0.05%)
IL-10	11.38	20.93	0.003 (<0.05%)
IL- 13	8.50	24.00	0.00 (<0.05%)

Table (3): Mean peripheral blood mononuclear cells (PBMC) cytokine levels (pg./ml) in (G0) and fibrosis groups (GI, GII and GIII).

Blood culture cytokine	Non-fibrosis G0 N=16	Fibrosis (GI, GII, GIII) N=15	P value
IFN- γ	11.19	21.13	0.00 (<0.05%)
IL-4	15.78	16.23	0.89 (>0.05%)
IL-10	11.38	20.93	0.00 (<0.05%)
IL- 13	8.50	24.00	0.00 (<0.05%)

NB: The Mann–Whitney test was used to compare differences in mean cytokine levels in subjects without fibrosis and those with fibrosis **(table 2 and table 3**).

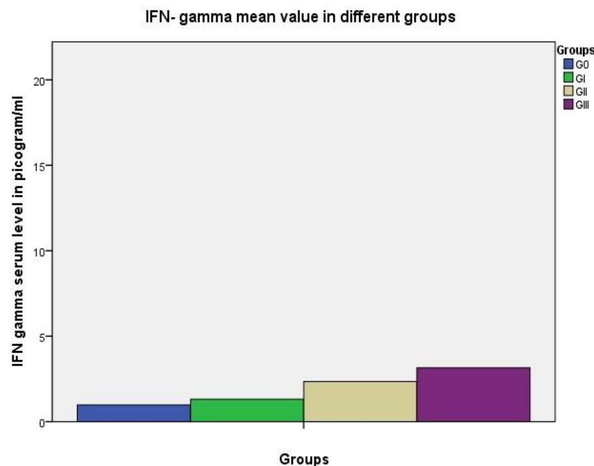

Fig 1a. Serum **IFN -γ** mean value in non-fibrotic group (G0), mild fibrosis (GI), moderate fibrosis group (GII) and severe fibrosis group, (GIII). **A highly significant** P- value **(<0.001%)**, the mean difference is significant at the 0.05 level.

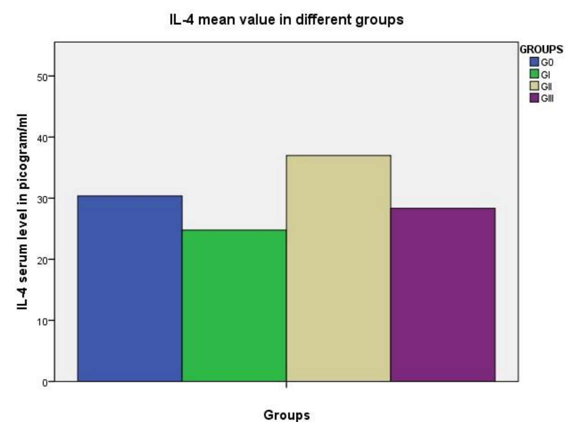

Fig 1b. Serum IL-4 mean value in non-fibrotic group (G0), mild fibrosis (GI), moderate fibrosis group (GII) and severe fibrosis group (GIII). Non-significant P value (**>0.05%**).

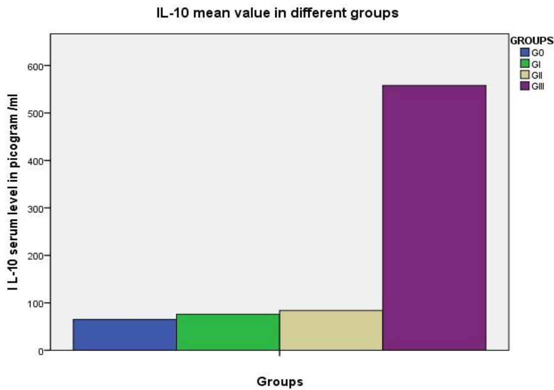

Fig 1c. Serum IL-10 mean value in non-fibrotic group G0, mild fibrosis GI, moderate fibrosis group GII and severe fibrosis (GIII). **A highly significant P- value (<0.001%)**.

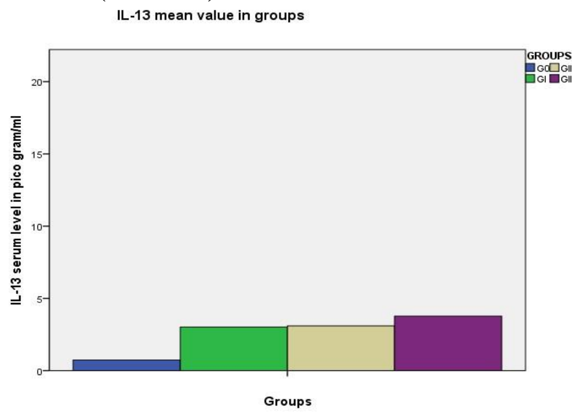

Fig 1d: Serum IL-13 mean value in non-fibrotic group G0, mild fibrosis GI, moderate fibrosis group GII and severe fibrosis (GIII). A highly significant **P- value (<0.001%)**.

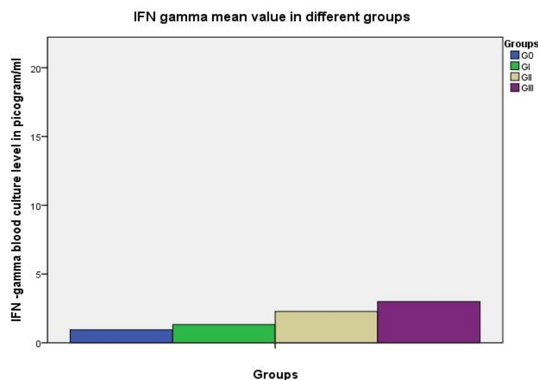

Fig. 2a: IFN -γ mean value in PBMC in non-fibrotic group (G0), mild fibrosis (G1), moderate fibrosis group (GII) and severe fibrosis (GIII). A **highly significant** P -value **(<0.001%)**.

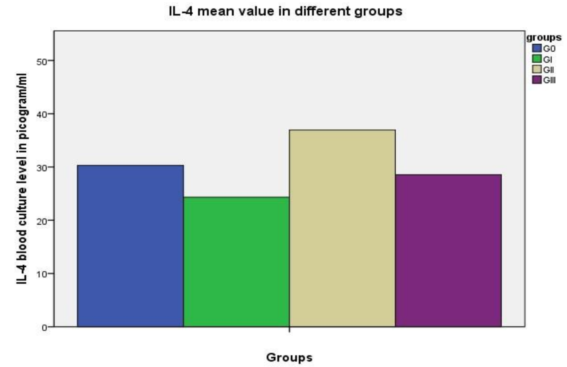

Fig. 2b: IL-4 mean value in PBMC in non-fibrotic group G0, mild fibrosis G1, moderate fibrosis group GII and severe fibrosis GIII. Non-significant P value **(>0.05%)**.

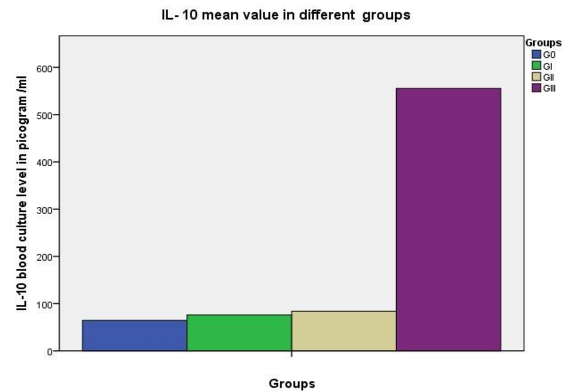

Fig. 2c: IL-10 mean value in PBMC in non-fibrotic group (G0), mild fibrosis (GI), moderate fibrosis group (GII) and severe fibrosis (GIII). A **highly significant P** -value **(<0.001%)**.

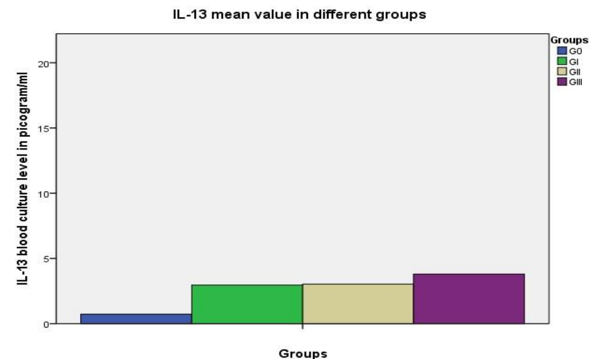

Fig. 2d: IL-13 mean value in PBMC in non-fibrotic group (G0), mild fibrosis (GI), moderate fibrosis group (GII) and severe fibrosis group (GIII). **A highly significant P-** value **(<0.001%)**.

4. Discussion:

Hepatic fibrosis is a pathology caused by numerous etiologies including infections like schistosomiasis (**Poelstra 2016**) duo to accumulation of Schistosoma eggs in the liver (**Zoni et al.,2016**). This process is associated with the production of profibrotic cytokines that modulates its development (**Leask, Abraham, 2004).** Cytokine response is an important factor inclined in developing the Schistosoma induced pathology in humans **(King *et al.,* 2001).**

Because of its documented role in main hepatic pathology induced by *S. mansoni* infection, soluble egg antigens (SEAs) of S. *mansoni* egg is widely used for stimulation of in vitro blood culture. **El Zayadi (2004)** concluded that granuloma formation is a cell-mediated immune response dependent on $CD4^+$ T cells sensitized to Schistosoma egg antigens. Thus, the outcome of host responses to *Schistosoma* eggs in the liver is advanced portal fibrosis with dense collagen deposition in the expanded portal tracts**.**

In the present work, we investigated expression of the Th-1 cytokine (IFN- γ) and Th-2 cytokines (IL4, IL -10 and IL-13) in positive cases of human hepatic fibrosis induced by Schistosoma mansoni infection**.** The cytokine levels in serum and blood culture supernatants of 31 participants were evaluated in relation to fibrosis.

In our study the mean age difference across the four groups varied significantly ($p < 0.05$). (**23**) males and (**8**) females were included in the study. The difference in sex between the groups was not significant ($p > 0.05$).

IFN- γ inhibits the activity of hepatic stellate cells (HSC) and so affecting negatively the proliferation and synthesis of collagen and extracellular matrices important for fibrosis to occur (**Duncan and Berman 1985), (Mallat et al 1995), (Rockey and Chung. 1994**).

The mean IFN-γ level in serum and blood culture supernatants were significantly elevated in fibrosis groups (GI, GII and GIII) than non-fibrotic one (G0) (**$p<0.05$**). Similar finding was reported by **Talaat *et al.,* (2007)** they found a general increase in IFN-γ production in response to SEA stimulation compared to normal controls in *S. mansoni* infected patients. **Morais *et al.,* (2008)** also found high level of IFN-γ in (GIII) hepatic fibrosis and suggested that IFN-γ level increase in to control the progress of hepatic fibrosis in chronic patients.

On the other hand, **Mutengo et al., (2018)** found that individuals with severe schistosomal liver fibrosis produced less of IFN-γ than the non-fibrosis group but was not statistically significant. Moreover, **Booth et al (2004**) and **Henri et al** (**2002)** reported higher levels of INF- γ were associated with reduced risk of fibrosis in human schistosomiasis. In the same context, lower levels of INF- γ were linked to a higher risk of periportal fibrosis schistosomiasis patients (**Arnaud et al 2008**). Molecular studies revealed that a polymorphism in the INF- γ receptor 1 (IFNGR1) gene that result in reduced functionality of the receptor, may lead to increased susceptibility to severe fibrosis (**Dessein et al 2004**).

Our results revealed non-significant increase in IL-4 mean level in serum and blood culture of fibrosis groups than non-fibrosis group ($p>0.05$). Similar results were obtained by **Talaat *et al.,* (2007)** who recorded poor response of *S. mansoni* infected patients to produce IL-4 after *Invitro* stimulation of PBMCS of *S. mansoni* infected patients with SEA antigen. **Martins-leite *et al.,* (2008)** also reported that no association was found between **IL**-4 and *S. mansoni* periportal fibrosis. Similar results were obtained by **Mutengo et al., (2018)** who found non statistical significance in IL-4 median level between non-fibrotic and liver fibrosis groups of S. mansoni infected patients.

In our study, the mean level of IL- 10 in serum and blood culture supernatants were significantly elevated in subjects with fibrosis than those without fibrosis (**$p<0.05$**). Similar finding was obtained by **De jesus *et al.,*** (**2004)** who found high levels of IL-10 in subjects with GIII compared to GI and GII hepatic fibrosis. In contrast with our results, **Henri *et al.,* (2002)** recorded low IL-10 level in cases of advanced fibrosis. Moreover, **Alves-*oliveira et al.,* (2006)** reported no association between *Schistosoma mansoni* periportal fibrosis and IL-10 level in PBMC cultures supernatants. Also, several studies found that increased risk of severe fibrosis was observed when IL-10 production by blood mononuclear cells from schistosomiasis patients was low (**Booth et al., 2004, Arnaud et al., 2008 and Mutengo et al., 2018**) suggesting its protective role against hepatic fibrosis. The anti-inflammatory and immunosuppressive actions of IL-10 which regulate the inflammation process could explain these findings (**Bataller et al 2003**).

Concerning IL -13 our results revealed high significant increase in its mean level in serum and blood culture in fibrosis groups than non-fibrosis group ($p<0.05$). This comes in agreement with findings of **Alves-Oliveira et al (2006)** where a significant association between high IL-13 levels and severe hepatic fibrosis in S. mansoni infected individuals was detected. On the contrary, no associations were found between this cytokine and the different stages of fibrosis in hepatosplenic

patients with S. mansoni infection in study by **Brandt et al., (2010**).

From the results obtained in our work compared to the others work, it is obvious that the literature still lack works such ours which elucidate the precise role of host cytokine profile implicated in development of liver fibrosis caused by S. mansoni infection. This response determines the development of hepatic fibrosis during schistosomiasis as the increased expression or lack of expression of certain cytokines regulate the hepatic fibrosis process.

Conclusion:

In the present study, our results have shown that high levels of IFN- γ, IL-10, and IL-13 were associated with development of Schistosoma mansoni induced liver fibrosis. Our findings of significant higher IL-13 in the fibrotic groups than the nonfibrotic one confirmed its role in fibrosis process enforcing its central involvement in schistosomal liver fibrosis with previous studies that also concluded that result.

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3/5/2019