Schistosomiasis mansoni: ultrasound-evaluated hepatic fibrosis and serum concentrations of hyaluronic acid

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Schistosomiasis mansoni is a fibrogenic liver disease that constitutes a major health problem in north-eastern Brazil. Although one common manifestation of the disease, periportal fibrosis (PPF), can be assessed by ultrasonography by well-trained physicians, the necessary equipment and personnel are not always readily available. Serum markers, including hyaluronic acid (HA), have been used as alternative means of measuring fibrosis. Recently serum concentrations of HA have been evaluated in 77 Brazilians (61 cases of schistosomiasis mansoni and 16 healthy controls) and compared against the ultrasound-evaluated PPF in the same subjects. The HA was measured using a non-competitive fluorescence-based assay, while the PPF was explored using a portable ultrasound scanner (SSD-500; Aloka, Tokyo) and graded, as patterns A–F, according to the World Health Organization's 'Niamey protocol'. In general, the serum concentrations of HA were found to be positively correlated with the severity of the PPF. The mean concentration of HA in the sera of the 16 controls was significantly lower than that recorded in the schistosomiasis cases who showed PPF of patterns D or E ($P < 0.001$ for each). The cases who showed pattern-C PPF also had significantly less HA in their sera than the cases with PPF of patterns D or E ($P < 0.001$ for each), and the cases with pattern-D fibrosis had significantly lower HA concentrations in their sera than the cases with PPF of pattern E ($P < 0.001$).

In an analysis based on a receiver-operating-characteristic (ROC) curve, an HA concentration of 20.2 µg/litre of serum was identified as a threshold that could be used to distinguish moderate cases of PPF (i.e. patterns C or D) from the more advanced cases (i.e. patterns E or F), with a sensitivity of 60% and specificity of 65%.

In conclusion, it appears that serum concentrations of hyaluronic acid could be used as markers for periportal fibrosis in patients with schistosomiasis mansoni.

Although the human morbidity and mortality caused by schistosomiasis mansoni have been reduced over the last 25 years by chemotherapy, the disease still represents a significant health hazard, particularly in

north-eastern Brazil (Katz and Peixoto, 2000; Araújo *et al.*, 2007).

It is believed that between 2% and 7% of humans infected with *Schistosoma mansoni* go on to develop hepato-splenic disease, which is associated with portal hypertension and digestive bleeding brought on by the rupture of oesophageal varices or hypertensive

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gastropathy (Richter *et al.*, 1992; Chitsulo *et al.*, 2000; Ferraz *et al.*, 2003).

Periportal fibrosis (PPF) or Symmers' fibrosis is a pathognomonic hepato-splenic lesion characterised by broad fibrous bands that reach into the branches of the portal vein, spreading from the vein's entry into the liver through to the divisions of the fourth-order branches (Andrade, 2008). The eggs of *S. mansoni* are deposited in the mesenteric veins and may subsequently become impacted in the portal venules, where they trigger a granulomatous inflammation that evolves into PPF (Andrade *et al.*, 1997).

In the pathophysiology of portal hypertension in schistosomiasis mansoni, the primary outcomes are PPF-attributable resistance to blood flow in the pre-sinusoidal circulation of the branches of the portal vein and splenic-vein hyperflow resulting from an enlarged spleen (Raia *et al.*, 1991; Vezozzo *et al.*, 2006; Maia *et al.*, 2007). As the pressure levels in the portal vein are correlated with severity of PPF, the assessment of such fibrosis — using liver biopsy, ultrasonography and/or serum markers (Santos *et al.*, 2005; Parise *et al.*, 2006; Rossi *et al.*, 2007) — is of particular interest in clinical practice (Richter *et al.*, 1992). In schistosomiasis mansoni, a liver wedge biopsy is the most accurate method of assessing PPF but requires major surgery. Unfortunately, a fine-needle biopsy does not always present a true histopathological picture because PPF, although diffuse, varies in intensity throughout the hepatic parenchyma (Dimmette, 1955). Ultrasonography is an important tool for assessing schistosomiasis mansoni because it allows enlargement of the left hepatic lobe, reduction of the right lobe, periportal thickening and splenomegaly to be detected (Domingues *et al.*, 1993; Richter *et al.*, 2001; Vezozzo *et al.*, 2006; Maia *et al.*, 2007). In 1996, a World Health Organization convention in the Nigerien capital city of Niamey proposed that the PPF seen in schistosomiasis mansoni be classified into six patterns (known as patterns A–F), with patterns A

and F representing the least and most severe forms of the fibrosis, respectively (WHO, 1996). Although this 'Niamey protocol' is now employed in general practice, it requires ultrasound equipment and qualified examiners that are not readily available in all areas where schistosomiasis mansoni is endemic. The search for simpler but accurate methods for assessing PPF continues.

Hepatic stellate cells are the principal mediators of fibrosis and, when activated by tumour necrosis factor, platelet-derived growth factor and fibrogenic cytokines (tumour growth factor- β , angiotensin II and leptin), are transformed into the proliferating contractile cells that constitute the essence of the fibrotic response to hepatic aggression (Friedman, 2004; Bataller and Brenner, 2005; Morais *et al.*, 2006). As well as the proteins responsible for fibrogenesis, other molecules originating from the degradation of the extracellular matrix [e.g. hyaluronic acid (HA), type-III pro-collagen, type-IV collagen and laminin] have been used to assess liver fibrosis (Santos *et al.*, 2005; Rossi *et al.*, 2007; Marinho *et al.*, 2010).

Since, during fibrogenesis, HA synthesis in the stellate cells is increased and there is a reduced clearance of HA from the circulation, serum concentrations of HA are usually elevated in fibrotic liver diseases (Friedman, 2004; Bataller and Brenner, 2005), although there are few relevant data on HA concentrations in schistosomiasis mansoni. The main aim of the present study was to evaluate HA concentrations in the sera of Brazilians with schistosomiasis mansoni and see if they were good markers of the severity of PPF, which was assessed in the same subjects using ultrasonography and the 'Niamey protocol'.

PATIENTS AND METHODS

The 61 cases of schistosomiasis mansoni included in the study had all attended the Gastro-enterology Clinic at the *Hospital das*

Clínicas of the *Universidade Federal de Pernambuco* (UFPE), in Recife, in north-eastern Brazil. When first seen at the clinic, all 61 had a history of water contact in an endemic area for schistosomiasis mansoni, had ultrasonographic pictures compatible with PPF, and were found stool-positive for *S. mansoni* eggs. All had been treated with praziquantel at least 6 months before the blood samples investigated in the present study were collected. Subjects were excluded from the study if they presented markers for hepatitis B or C (see below), had a body mass index (BMI) of $>30 \text{ kg/m}^2$, consumed $>210 \text{ ml}$ ethanol/week, and/or suffered from diabetes, cirrhosis and/or a neoplastic disease.

As controls, blood samples were also collected from 16, apparently healthy employees of the *Centro de Pesquisas Aggeu Magalhães* (also in Recife). Shortly before blood samples were collected from them, all 16 had been found stool-negative for *S. mansoni* eggs and seronegative for the markers of hepatitis B and C (see below). All had BMI of $<30 \text{ kg/m}^2$ and were similar to the cases in terms of their socio-economic status.

Ethical Approval

The study protocol was approved by the Ethics Committee of the UFPE's Health Sciences Centre, and all the cases and controls investigated gave their written informed consent.

Laboratory Tests

A blood sample of about 10 ml was collected, from a peripheral vein of each subject, into a Vacutainer® tube (BD Diagnostics, Franklin Lakes, NJ) and sent to the Central Laboratory at the UFPE's *Hospital das Clínicas*. The sera were separated from the blood samples by centrifugation before a subsample of each serum was checked, for antibodies to the core antigen of the hepatitis B virus and antibodies to the hepatitis C virus, in commercial microparticle enzyme immuno-assays (Abbott

Laboratories, Abbott Park, IL). A further, 1-ml subsample of each serum was frozen at -20°C and sent to the Department of Biochemistry (Discipline of Molecular Biology) of the *Universidade Federal de São Paulo*, in São Paulo, where each subsample was tested in a non-competitive fluorescence-based assay that can detect HA concentrations ranging from 0.2–500 $\mu\text{g/litre}$ (Köpke-Aguiar *et al.*, 2002; Martins *et al.*, 2003). The assay used employed HA binding protein (isolated from bovine cartilage) immobilized in microwell ELISA plates (Martins *et al.*, 2003).

Ultrasonography

Each case and control was given an abdominal scan, by the same examiner (A.L.D.), with an SSD-500 ultrasound scanner (Aloka, Tokyo) fitted with a 3.5-MHz convex transducer. During each scan, the characteristics of the liver parenchyma and the surfaces of the liver and spleen were explored and any PPF detected was classified according to the 'Niaméy protocol' (WHO, 1996).

Statistical Analysis

Odds ratios (with their 95% confidence intervals), χ^2 tests and logistic regression were used, as appropriate, to explore and compare the qualitative variables, while analysis of variance was used to investigate the mean values of the quantitative variables. The Levene test was used to assess the homogeneity of the variances, Tukey's test being used subsequently when homogeneity was verified and the Tamhane test being used when the variances were not found to be homogeneous.

A receiver-operating-characteristic (ROC) curve was used to establish the cut-off point for the serum HA concentration that best differentiated cases with moderate PPF from those with severe fibrosis.

The Excel 2000 software package (Microsoft) and version 8.0 of the SPSS package (SPSS Inc, Chicago, IL) were used for the data storage and analysis. A *P*-value of

≤ 0.05 was considered indicative of a statistically significant difference or association.

RESULTS

The 16 controls (10 women and six men) had a mean (S.D.) age of 34.3 (9.5) years (range=21–57 years). The 61 cases of schistosomiasis mansoni (36 women and 25 men) were, in general, slightly older, with a mean (S.D.) age of 46.7 (12.3) years (range=23–65 years). Forty-two (69%) of the cases had experienced previous episodes of upper gastro-intestinal bleeding, and 27 (44%) had each had a splenectomy.

The serum concentrations of HA found in the 16 controls and 61 cases are summarized in Table 1 and the Figure. The mean concentration of HA in the sera of the controls was significantly lower than that recorded in the schistosomiasis cases who showed PPF of patterns D or E ($P < 0.001$ for each) (but similar to that seen in the cases with pattern-C or pattern-F PPF). The cases who showed pattern-C PPF also had significantly less HA in their sera than the cases with PPF of patterns D or E ($P < 0.001$ for each), and the cases with pattern-D fibrosis had significantly lower HA concentrations in their sera than the cases with PPF of pattern E ($P < 0.001$). No examples of pattern-A or pattern-B fibrosis were observed.

When the subjects were considered as three groups — healthy controls, cases with

moderate fibrosis (patterns C and D) and cases with severe fibrosis (patterns E and F) — it became clear that mean serum HA concentrations increased significantly ($P < 0.001$) as the PPF became more severe (Table 1). Exploration of an ROC curve indicated that the use of a threshold of 20.2 μg HA/litre of serum gave the best differentiation between the cases with moderate PPF and those with more severe PPF (Table 2), achieving a sensitivity of 60% [with a 95% confidence interval (CI) of 43.8%–76.2%], a specificity of 65% (CI=47.1%–83.7%), a positive predictive value of 70% (CI=53.6%–86.4%), and a negative predictive value of 55% (CI=37.3%–72.4%).

DISCUSSION

In many areas where schistosomiasis mansoni is endemic, liver biopsies carry too much risk and the equipment and personnel required for accurate ultrasonography are not readily available. The main aim of the present study, in which ultrasonography was used as the 'gold standard' for classifying PPF, was to explore the value of serum HA as a marker for PPF.

Ricard-Blum *et al.* (1999) found that serum concentrations of HA (but not those of type-I collagen or type-III pro-collagen) were markedly higher in (ultrasonography-evaluated) cases of PPF than in healthy controls, and suggested that serum concentrations of

TABLE 1. Serum concentrations of hyaluronic acid in the 77 adult Brazilians investigated in the present study

Pattern of periportal fibrosis (WHO, 1996)	No. of subjects	Hyaluronic acid ($\mu\text{g}/\text{litre}$)	
		Mean and (S.D.)	Range
None (healthy controls)	16	6.2 (2.8)	2.2–12.8
C (peripheral fibrosis)	4	4.8 (1.5)	3.0–6.0
D (central fibrosis)	22	17.0 (9.9)	2.0–34.0
E (advanced fibrosis)	27	37.3 (31.0)	6.0–129.0
F (very advanced fibrosis)	8	36.4 (23.6)	15.0–82.0
C or D	26	15.1 (10.1)	2.0–34.0
E or F	35	37.1 (29.2)	6.0–129.0

FIG. A 'box-and-whisker' plot of the concentrations of hyaluronic acid detected in the sera of 16 healthy controls and 61 cases of schistosomiasis mansoni with periportal fibrosis of patterns C, D, E or F.

HA could be used to assess morbidity in schistosomiasis mansoni. More recently, Marinho *et al.* (2010) reported how serum concentrations of HA (but not those of type-IV collagen) could be used to separate patients with light PPF from those with intense fibrosis. They also reported a positive correlation between serum HA concentrations and the

prevalence of portal hypertension (as indicated by relatively large portal- and splenic-vein diameters) in patients with PPF caused by schistosomiasis mansoni. Although these results contrast with those of Burchard *et al.* (1998), who found no correlation between serum concentrations of HA (or type-III procollagen or laminin) in cases of schistosomiasis mansoni and the results of ultrasound scans, most of the cases investigated in this earlier study only had mild fibrosis.

In other studies of Brazilian cases of schistosomiasis mansoni, the severity of PPF (as assessed with ultrasound) has been found to correlate with 'aspartate aminotransferase to platelet ratio indexes' (APRI; Lambertucci *et al.*, 2007) and with serum concentrations of immunoglobulin G (Correia *et al.*, 2009).

In the present study, a serum HA concentration of about 20.2 µg/litre was found to be the best HA threshold for

TABLE 2. The patterns of periportal fibrosis (WHO, 1996) seen in the cases of schistosomiasis mansoni found to have >20.2 or ≤ 20.2 µg hyaluronic acid/litre of serum

Hyaluronic acid (µg/litre)	No. of subjects showing pattern:		Odds ratio and (95% confidence interval)	P
	E or F	C or D		
>20.2	21	9	2.8 (1.0–8.1)	0.053
≤ 20.2	14	17	1	

distinguishing patients with moderate PPF from those with severe fibrosis. In their earlier study, Köpke-Aguiar *et al.* (2002) found that a similar HA threshold (20 µg/litre of serum) could be used to separate schistosomiasis patients with and without portal hypertension, while a much higher threshold (80 µg/litre) could be used to differentiate patients with cirrhosis from those with portal hypertension caused by schistosomiasis mansoni. Among Sudanese cases of schistosomiasis mansoni, Pascal *et al.* (2000) found that serum concentrations of HA increased as the severity of PPF increased, and Eboumbou *et al.* (2005) found that cases had about 22.4 µg HA/litre of serum if they did not have PPF and up to 91.2 µg HA/litre if they had severe PPF. Curiously, the HA concentrations detected by Pascal *et al.* (2000) were generally higher than the corresponding values reported by Eboumbou *et al.* (2005) or observed in the present study. It seems possible that the cases of schistosomiasis mansoni investigated in the present study had even higher serum concentrations of HA before they were treated (the blood samples investigated in the present study were collected at least 6 months after the cases had been given praziquantel). The concentrations of HA in the sera of patients with schistosomiasis mansoni tend to fall a few weeks after treatment with praziquantel (Hassanein *et al.*, 1997; Ricard-Blum *et al.*, 1999). Many of the cases investigated in the present study had also had their spleens removed and it is possible that the splenectomies led to reductions in serum HA. In a study of patients with schistosomiasis mansoni in a tertiary-care hospital in Brazil, Wyszomirska *et al.* (2005) observed post-splenectomy reductions in the serum concentrations of type-IV collagen.

The measurement of PPF via serum markers, such as HA, would seem to have a wide application, especially in endemic areas where ultrasonography is difficult or impossible. In schistosomiasis mansoni, the early detection of hepatic involvement is crucial,

not only to impede the development of more severe morbidity but also to guide the epidemiological control of *S. mansoni*. The measurement of serum HA concentrations may also be useful during post-treatment follow-up, to monitor the regression of any PPF.

Further studies involving many more patients and other areas of endemicity will be necessary in order to confirm the present results and see if they are applicable world-wide.

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