Targeting molecular signaling pathways of Schistosoma haematobium infection in bladder cancer

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Since 1911 epidemiological evidence indicates that S. haematobium is associated to squamous cell carcinoma of the bladder. However, the mechanisms of this interaction are not clearly defined. Using normal epithelial cells, S. haematobium parasite extracts were able to induce cancer-like phenotypes such as proliferation, apoptosis, migration, invasion and tumorigenesis. The parasite extracts on normal urothelium also presented carcinogenic and mutagenic ability. To further elucidate the biological effects of this parasite, new estrogenic molecules were identified in its extracts. These estrogens are also present in the sera of Schistosoma-infected patients, and they have the ability to repress ER transcriptional activity both in estrogen responsive MCF7 cells and normal urothelial HCV29 cells. This review will present some of the recent studies of mass spectrometry of S. haematobium extracts and sequence analysis of bladder tissue treated with the same extracts. Finally the molecular and cellular events that might be responsible for schistosomiasis-related bladder cancer will be discussed.

Schistosomiasis: General Aspects

Anthropophilic Schistosoma species currently infect more than 200 million people in 76 countries worldwide, according to the World Health Organization, in the endemic areas of Africa, the Caribbean, Central America, South America, East Asia and the Middle East. Prevalence is thought to be rising mainly due to increasing travel by people from industrialized countries to these endemic regions for business or leisure.¹

Most broad generalizations about schistosomiasis state first that it is a rural disease and second that it affects the poor populations. The changing global demography requires that the first assumption be reconsidered. Urban schistosomiasis is a reality rather than a potential threat. Urban schistosomiasis is the cumulative outcome of local transmission and migration.² Movements of displaced persons due to conflict, drought and famine are currently playing an important role in the changing epidemiology of schistosomiasis in these countries: (1) Laos/Cambodia/Thailand; (2) Ethiopia/Somalia/Sudan; (3) Mozambique/Zambia/Malawi; Angola/Zaire.³

Schistosomiasis Life Cycle and Infection

The life cycle of human schistosomes begins once cercariae have entered the body. They are called schistosomula, which migrate through the tissues to invade blood vessels. The organisms are transported to the lungs and then to the liver, where they mature into an adult worm within six weeks before descending to their final positions in the venous circulation. Adults of S. haematobium are mostly found in the venous plexi of the bladder, prostate and uterus, while S. intercalatum, S. japonicum, S. mansoni and S. mekongi are observed in the portal, inferior and superior mesenteric veins. Maturity of female worms depends on the presence of a mature male because they form pairs, with the female lying enclosed within a groove formed by the male.⁴⁻⁸

Adult worms have a mean life span of 5–10 years, with females releasing 300–3,000 eggs per day. Mature parasites can live in human tissues for more than 30 years.¹ Eggs are deposited in the terminal venules of the bladder (S. haematobium), intestine and rectum (S. intercalatum, S. japonicum, S. mansoni, S. mekongi), where they mature to contain a miracidium over the next 10 days. The schistosomulum releases proteolytic enzymes, which facilitate larval movement through the tissues into either the genitourinary tract or the gastrointestinal tract. Eggs are passed in human urine and excrement and motile miracidia are released upon contact with fresh water. Miracidia then actively seek out snail hosts in which they develop first into mother and daughter sporocysts by asexual division and then into cercariae over four to six weeks. Cercariae then leave the snail and penetrate the human skin on contact with the assistance of their glandular secretions.⁶⁻⁷

Mortality in human schistosomiasis is thought to be around 500,000 every year and is related to complications of

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fibro-occlusive disease secondary to the immune stimulus of Schistosoma ova and end organ damage. There is a great lack of information in the real numbers, mainly because the countries where prevalence is higher have insufficient or inexistente registries.

Clinical features of human schistosomiasis depend on the species, developmental stage and site of infection in the body. This can be summarized into three major syndromes: (1) cercarial dermatitis, (2) acute schistosomiasis or Katayama fever and (3) chronic fibro-obstructive disease.

**Schistosomiasis: Aspects of Epidemiology**

Schistosomiasis is an ancient scourge of mankind, depicted graphically in papyri from Pharaonic Egypt and known from human remains over 2000 years old from China. Human schistosomiasis is caused by one of six species: *Schistosoma haematobium*, *Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma intercalatum*, *Schistosoma malayensis* and *Schistosoma mekongi*. Schistosomiasis was originally called bilharzia in homage to Theodor Bilharz, a young German pathologist who first described the disease and its association with Schistosoma, working at Cairo in Egypt. Blood-dwelling Trematoda (phylum Platyhelminthes) of the genus Schistosoma cause this chronic and debilitating disease. In a different manner of other flatworms, *Schistosoma* are confined to residing in blood vessels of the genitourinary or gastrointestinal tracts. They present a heteroxenic life cycle, requiring an invertebrate as secondary host, water-dwelling snails of genus Bulinus, Oncomelania or Biomphalaria. Geographic distribution and maintenance of human infection by Schistosoma is dependent on and limited by the presence of a suitable snail host.

Schistosoma adult worms are sexually distinct, a characteristic that separates them from other flukes that are hermaphrodites. However, males present a ventral infolding from the ventral sucker to the posterior end forming the gynecophoric canal, where females are kept during copulation (copulatory groove). Schistosoma life cycle differs from that of other flatworms also by the infecting process of the definitive host: the penetration of cercariae through the skin (per cutem) rather than through oral ingestion (per os). Human Schistosoma can infect other vertebrates and provide an animal reservoir of infection, although it is of epidemiologic significance only for *S. japonicum* and possibly *S. mekongi*. Snails of the genera Biomphalaria, Bulinus, Neotricula and Oncomelania are the principle intermediate hosts for *S. mansoni*, *S. haematobium*, *S. mekongi* and *S. japonicum*, respectively.

Human schistosomiasis infects people in the endemic areas of Africa, the Caribbean, Central America, South America, East Asia and the Middle East. Prevalence is thought to be increasing. The most severely affected countries in Africa are Angola, Chad, Congo, Egypt, Ghana, Kenya, Madagascar, Malawi, Mali, Mozambique, Nigeria, Senegal, Sudan, Tanzania, Uganda, Zambia and Zimbabwe. Yemen has the most infected people in the Middle East. Brazil is the most affected country in the Americas, with 25 million people living in endemic areas and an estimated 3 million infected.

*S. haematobium* is endemic in 53 countries in the Middle East and most of the African continent, including the islands of Madagascar and Mauritius. The infection is unlikely to be of public health significance in Lebanon, Oman, Syria, Tunisia and Turkey because transmission is low or nonexistent. A disputed and ill-defined focus exists in India and requires further confirmation.

*S. mansoni* is found in 54 countries, including the Arabian Peninsula, Egypt, Libya, Mauritania, Somalia, Sudan, sub-Saharan Africa, Brazil, the Caribbean (except Antigua, Guadeloupe, Martinique, Montserrat and St. Lucia), Suriname and Venezuela.

*S. japonicum* is endemic in China, the Sulawesi province of Indonesia, and the Philippines. China is the most affected country, with an estimated 900,000 people infected. The parasite has been eradicated from Japan since 1982.

*S. intercalatum* has been reported from 10 countries in central and western Africa. *S. mekongi* is confined to Cambodia and Laos where the borders run along the Mekong River. Neotricula snails have been reported in Southern China, but no reports of *S. mekongi* exist from these areas. *S. malayensis* and its intermediate host Robertsiella snails were found in a small jungle focus in Malaysia infecting aboriginal people (WHO).

According to Wright, schistosomiasis was recognized to be endemic in 71 countries, although neither India nor Mauritius was included among these. Before that time transmission of schistosomiasis had ceased in Cyprus and Portugal. Cyprus and Portugal were known, during the first half of the last century, as the only autochthonic endemic focus of schistosomiasis in Europe. Since that time no new or old infections were declared in St. Martin and Israel and are no longer included. Since 1972, additional countries reporting schistosomiasis are Equatorial Guinea, India, Jordan, Malaysia, Mauritius, Montserrat, Oman and São Tomé e Príncipe. As of 1989 schistosomiasis is endemic in 76 countries.

Before 1972 transmission of schistosomiasis had ceased in Portugal. Nowadays, schistosomiasis is not endemic in Portugal. However, between 1961 and 1974, the country was involved in African colonial wars, which led to the dispatching of Portuguese military personnel to Angola, Mozambique and Guinea-Bissau, all with endemic *S. mansoni* and *S. haematobium*. Upon the return of military personnel to Portugal, medical services were provided. In the only report in 1971, stools and urine from 3206 of the returnees were examined microscopically, and 3.4% were found to be positive for *S. mansoni* and 2.8% to be positive for *S. haematobium*. This was evidence that Portuguese repatriates had been exposed to parasites, and more would probably have been found to be infected had they been examined by trained personnel.

**Schistosomiasis haematobia**

More people are infected with *S. haematobium* than with the other schistosomes. Of ~112 million cases of *S. haematobium* infection in sub-Saharan Africa, 70 million are associated with hematuria, 18 million with major bladder wall pathology, and 10 million with hydronephrosis leading to severe kidney
damage. In many patients, the deposition of the *S. haematobium* parasite ova in the bladder leads to squamous cell carcinoma of the bladder. *S. haematobium* is classified as a Group 1 carcinogen by the World Health Organization’s International Agency for Research on Cancer although the cellular and/or molecular mechanisms linking *S. haematobium* infection with cancer formation have yet to be defined (see below). Moreover, as many as 75% of women infected with *S. haematobium* also suffer from female genital schistosomiasis (FGS) of the lower genital tract. FGS results from deposition of the schistosome eggs in the uterus, cervix, vagina and/or vulva, with ensuing host inflammatory responses comprised of granulomas, fibrosis and pathological localized blood vessel formation. FGS increases susceptibility to HIV/AIDS. A recent study of Zimbabwean women aged 20 to 49 years with FGS revealed a 3-fold risk of HIV infection relative those without FGS.

**Schistosoma haematobium and its Association with Squamous Cell Carcinoma of the Bladder**

Not all schistosoma eggs are excreted from the body, and up to 50% can embryolise to other body areas, leading to a host immune reaction and granuloma formation. Granulomas begin to form with maturation of the miracidium at six days and are focal within two weeks. The most common sites are the liver for *S. intercalatum*, *S. japonicum* and *S. mansoni* and the bladder for *S. haematobium*. Other areas less commonly affected include the lungs, central nervous system and kidneys. During infection by *S. haematobium*, eggs are deposited in the mucosa and submucosa of the bladder and lower ureters. Granulomas are very cellular and form intraluminal polypliod lesions that can lead to hydro-nephrosis. Lesions tend to necrotize, ulcerate and bleed. With age, the lesions become acellular, fibrotic and calcify and are termed sandy patches. Calcification may lead to bladder deformation, ureteric obstruction, secondary infections, hydropnephrosis, chronic pyelonephritis and renal failure. Carcinoma of the bladder is a long-term sequela of chronic infection.

Bladder cancer is one of the most severe complications of chronic schistosomiasis. Case report studies indicate that individuals with schistosomiasis may develop bladder cancer earlier than uninfected people. The severity and frequency of the sequela or urinary schistosomiasis and of its complications (urothelial cancers) depend on the intensity of infection, worm burden and tissue egg burden, and the duration of infection.

Squamous cell carcinoma of the urinary bladder has been associated with *S. haematobium* infection in many parts of Africa. The epidemiologic association is based both on case-control studies and on the close correlation of bladder cancer incidence with prevalence of *S. haematobium* infection within different geographic areas. It has been estimated a Schistosoma-associated bladder cancer incidence of 3–4 cases per 100,000. A parasite-tumor linkage is further suggested by the predominance of squamous cell (as opposed to transitional cell) morphology of bladder carcinomas seen in *S. haematobium*-endemic areas, and by the frequent association of tumors with parasite ova and egg-induced granulomatous pathology in involved bladder tissues.

Another factor that may play a major role in bladder carcinogenesis in schistosomiasis patients is the presence of continuous physical irritation and inflammation produced by Schistosoma eggs in the bladder mucosa. The adult *S. haematobium* worms inhabit the veins of the perivesical plexus, where the female lays eggs. Some eggs pass through the bladder mucosa and are excreted in the urine. Other eggs are trapped in the tissue. A chronic inflammatory reaction is initiated, with the invasion of histiocytes and other inflammatory cells into the bladder, the formation of granulomas and eventually fibrosis. In addition, the eruption of the eggs through the mucosa stimulates reparative urothelial hyperplasia and cell turnover. Moreover, in association with schistosomiasis, cancer may originate throughout the bladder but rarely in the trigone, a frequent site of tumor origin in non-Schistosoma cases. The tumors are often first seen in an advantage stage, arising from the posterior bladder wall and vault. The trigone is only affected in 8.5% of the cases. The reason accounts for the striking deformities in the ureters, including medial deviation, a straight lumbar course, and in the pelvic segment a bowed appearance with medial and cranial displacement at the vesico-ureteric junction. Stasis and dilatation of the upper urinary tract are present in the absence of mechanical obstruction and these changes in the upper urinary tract are due to functional derangement of the ureters.

Human bladder cancer is the fifth to the seventh most common cancer in western countries. In many tropical and subtropical areas, however, it is the first among all types of cancer, mainly due to the endemic parasitism. Felix et al. has shown that the occurrence of transitional cell carcinoma (TCC) of the bladder has supplanted the squamous cell carcinoma (SCC) in Egypt following a major decline in the prevalence of urinary schistosomiasis. Such decline in the pattern of this infection suggests the importance of Schistosoma-associated bladder cancer that, elsewhere, may be more widespread than is presently thought.

The mechanisms underlying the association between *S. haematobium* and SCC are largely unknown. Firm understanding of the pathobiologic features of this disease is necessary. We will next describe the latest studies involving these pathobiologic features with the use of in vitro and in vivo essays and their future role in the direction of treatment.

**The effects of Schistosoma haematobium total antigen in normal mammalian cells and its involvement in tumorigenesis**. Hepatic lesions induced by *S. haematobium* immature male worms were observed in the golden hamster. These lesions and the nature of the local immune response were characterized by examining the hepatic inflammatory infiltrate. The induction of hepatic fibrosis and hepatitis, characteristic of egg granulomas, induced by male adult worms of *S. haematobium* alone is now demonstrated. Granulomas of schistosome-infected hamsters are composed of numerous macrophages, eosinophilic granulocytes, lymphocytes and fibroblasts. Similar dominant cell types were characterizing the granulomatous-like reaction around the worms, thus closely resembling the hepatic granulomas induced by schistosome eggs although without the concentric layers. Macrophages, fibroblasts and lymphocytes, and eosinophils were found to infiltrate along the outer layer of the hepatic induced
granuloma-like. These histopathology appearances represent an early advanced feature of granuloma formation.\textsuperscript{41}

There is no report in the literature that associates *S. haematobium* immature worms to hepatic fibrosis. But in *Schistosomiasis mansoni*, Baki et al. reported hepatic fibrosis and histopathological lesions in mice experimentally infected with male *Schistosoma mansoni*. These authors revealed that, from the 25th week post-infection, a diffuse fibrosis affected the main branches of the portal vascular system following the host inflammatory reaction, associated with the proliferation of myofibroblasts in situ.\textsuperscript{32}

An increase of fibrotic deposit occurred during chronic unisexual infection suggesting that antigenic substances secreted by adult schistosomes, in the absence of any eggs, might initiate periportal and perisinusoidal fibrous reaction. This study provided further experimental evidence for the role that schistosome worms, and their derived antigens, may play in the pathology of the infection of schistosomiasis and prompted to study the effects of *S. haematobium* total antigen in normal cells.

Cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis.\textsuperscript{43} To begin investigating the effect of *Schistosoma haematobium* total antigen (Sh) on cell viability and proliferation, CHO cells were seeded on 96-well plates, starved overnight, treated with increasing concentrations of Sh for 24 h, cultivated for 24, 48 and 72 h and then analyzed by MTS assay. The growth curve shows that treated cells proliferated significantly faster and more than control cells.\textsuperscript{44} Increased cell proliferation is the result of cell cycle progression driven by Sh, and therefore analyzed cell cycle progression with flow cytometry. This method revealed that the cell growth induction by Sh was a result of an increase of G\textsubscript{1}/M- and S-phase and a decrease of G\textsubscript{0}/G\textsubscript{1}-phase, as compared to control.\textsuperscript{44} Whether Sh affect the expression of cell cycle-related proteins was further examined. Expression of p27, a cyclin dependent kinase inhibitor (CDKI) was analyzed in CHO cells after Sh treatment. Immunoblotting for p27 was performed in CHO cells. p27 protein was down-regulated in Sh-treated CHO cells.\textsuperscript{44} In accordance, the results of Thuwajit et al. reported that excretion/secretion (ES) products of *Clonorchis sinensis* egg, another liver fluke also associated with cholangiocarcinoma, induced cellular proliferation in mouse fibroblasts NIH-3T3 cells. Kim et al.\textsuperscript{46} used the human epithelial cell line HEK293 to examine the in vitro effect of ES products of *Clonorchis sinensis*, another liver fluke also associated with cholangiocarcinoma, on the growth of bile duct epithelial cells. The cells proliferated in the presence of ES products in a dose-dependent manner.\textsuperscript{46}

To analyze apoptosis, CHO cells were seeded on 96-well plates, starved overnight, treated with increasing concentrations of Sh for 24 h, cultivated for 24, 48 and 72 h and then analyzed by TUNEL assay. CHO cells showed an increasing number of apoptotic cells per field in the control compared to cells treated with 50 μg/ml of Sh.\textsuperscript{44} In this study cell counts show that treated cells markedly decreased apoptosis in comparison to control cells.

Since the Bcl-2 family of proteins is regarded as key regulator of apoptosis, the influence of Sh on Bcl-2 expression levels in CHO cells was subsequently investigated. Bcl-2 is perceived as anti-apoptotic.\textsuperscript{45} In this study Sh exposure led to significant increase in Bcl-2 expression levels when compared to controls. The percentage of cells Bcl-2 positive in the control group was 50% whereas in the Sh-treated group was 53%.\textsuperscript{44} In agreement, apoptosis is rarely observed in either *O. viverrini*-infected hamster liver or in biliary cells that are co-cultured with flukes in vitro. Biliary cell damage by *O. viverrini* likely stems from the actions of oxygen radicals such as nitric oxide (NO). NO not only induces DNA damage but has been reported to mediate DNA repair inhibition. Moreover, NO has also been demonstrated to inhibit apoptosis. All of these manifestations facilitate carcinogenesis. Anti-apoptosis has been described in infection-associated cancers such as those caused by *Helicobacter pylori*. In the case of *O. viverrini* infection, DNA damage is caused in biliary epithelial cells while apoptotic mechanisms are disregulated, resulting in genetic alterations which may become fixed, leading to malignant transformation.\textsuperscript{48}

Cell motility and tissue invasion was suggested to be one of the essential alterations in cell physiology that collectively dictate malignant growth.\textsuperscript{43} To test whether Sh induces invasion in vitro, invasion was measured using Boyden chambers. Membranes in the chambers are coated with Matrigel to mimic basement membrane. Movement of cells through the membrane requires not only migration but also degradation of matrix, a critical step in the invasion process. These results show that treatment of cells with Sh stimulated invasion compared to controls.\textsuperscript{44} Understanding the mechanisms that lead to the invasion of host tissues by a growing population of neoplastic cells is a major task of cancer research.\textsuperscript{49} In the present work we have used in vitro assays to evaluate migration and invasion of Sh-treated cells and found them increased in comparison to controls. As Oliveira and collaborators\textsuperscript{50} have stated, the Matrigel invasion assay used in this study is by no means the equivalent of invasion in the in vivo situation. These investigators have repeatedly emphasized that all elements of the ecosystem need to be taken into account when analyzing the phenotype of cells. Nevertheless, the Matrigel assay we used contains some of the elements of the in vitro ecosystem of invasion, such as collagen type IV, laminin and entactin. Furthermore, this assay also covers some of the cellular activities implicated in invasion, like three-dimensional migration through matrices and proteolysis.\textsuperscript{50} It is now demonstrated that Sh induce invasion of CHO cells through the use of Matrigel invasion assay. This could lead to extracellular matrix degradation and subsequent invasion of cancer cells, suggesting a role for Sh in later stages of bladder carcinogenesis.

Taken together, the effects observed in Sh-treated cells seem to be interconnected. Through its effects in cell biology, *S. haematobium* total antigen is likely to participate in a number of carcinogenesis mediated processes, such as increased cell proliferation and loss of p27, decreased apoptosis and increased expression of Bcl-2 and increased migration and invasion, all of which are processes needed for cancer cell survival. The effects of Sh on the cell cycle may contribute to the high proliferation rate and accumulation of genetic changes during schistosomiasis associated tumorigenesis. Loss of p27 may be the reason for the uncontrolled proliferation seen in Sh-treated cells and could be involved in bladder cancer-associated schistosomiasis.\textsuperscript{44}
The involvement of Sh in tumorigenesis was also investigated. A xenograft model was employed to show that Sh has the potential to induce tumor development. Mice injected with Sh-treated CHO cells developed a solid, large multilobulated mass in the interscapular region. These tumors grew very fast and ulcerated overlying the skin. All tumors corresponded to sarcomas. There were no tumor metastases. The morphology of CHO cells is both epithelial-like and fibroblast-like. These results suggest that Sh may induce the transformation of the fibroblast-like component of CHO cells into sarcomas. This result is not surprising since it has been shown that egg granulomas isolated from livers of mice infected with *Schistosoma mansoni* or *S. japonicum* and cultured in vitro released a fibroblast-stimulating substance which stimulated proliferation in resting dermal fibroblasts. This liver pathology is the terminal event of a complex pathophysiologic cascade involving interactions between fibroblasts and both host and parasite products and is associated with prominent accumulations of fibroblasts, reinforcing the tumors observed.

Alterations and subsequent mutations in urothelium of CD-1 mice induced by *Schistosoma haematobium* total antigen. CD-1 mice were used to show that Sh has a carcinogenic ability. Therefore, the effects of Sh in CD-1 mice normal bladder were investigated after intravesical instillation of the parasite antigens. The bladders were analyzed histopathologically 20 and 40 weeks after treatment. Intravesical instillation of *S. haematobium* total antigen induces the development of urothelial dysplasia and inflammation.

Bladder histopathology of animals instilled with Sh was characterized by extensive diffuse or nodular infiltrate composed predominantly of lymphocytes admixed with variable number of eosinophils and by absence of umbrella cells, nodular hyperplasia and dysplasia. The nodular hyperplasia exhibiting budlike proliferated urothelial cells extending into the lamina propria. After 20 weeks of treatment 30% of these animals displayed urothelial dysplasia a LGIUN (Low Grade Intra Urothelial Neoplasia), whereas after 40 weeks of treatment we found 70% of the bladders with this LGIUN, a non-invasive malignant flat lesion in the urothelium of these animals. No bladder histopathological changes were observed in animals from control groups. No microscopic changes were seen in the spleen, lung, kidney and liver in samples obtained from all animals. These results suggest that Sh induced the malignization of the urothelium. Although helminths have been implicated in the etiology of human cancer, the role of the mechanisms by which parasites induce malignant transformation of the host cells is unclear. *Schistosoma haematobium* is considered to be carcinogenic to humans, with sufficient evidence for its role in causing carcinoma of the bladder. According to Herrera and Ostrosky-Wegman, parasites might initiate carcinogenesis by direct action of genotoxic factors, either secreted by them or produced during the inflammatory response. Activation of inflammatory cells may cooperate to induce cancer in many cases of infection- and inflammation-associated cancers.

To further study specifically the carcinogenic alterations caused by Sh in the bladders of the previous CD-1 mice, gene mutations were investigated in these bladders. Carcinoma of the bladder frequently harbors gene mutations that constitutively activate the receptor tyrosine kinase-Ras pathway and therefore activating mutations in *KRAS* gene were studied. Twenty percent of the dysplastic bladders presented a mutation in codon 12 of *Kras* gene. Two (out of 10) of the bladders with dysplasia presented a *KRAS* mutation in codon 12 of exon 2. The parasite extract of *S. haematobium* has carcinogenic ability possibly through oncogenic mutation of *KRAS* gene.

One cannot state at this point that *kras* mutation detected in dysplastic bladders induced by Sh treatment is an initiating event. The presence of this mutation could have one of two explanations: either the parasite total antigen has carcinogenic molecule(s) sufficiently genotoxic and, consistent with the mutation observed, caused direct attack on the ras gene leading to uncontrolled proliferation of the cells and causing dysplasia in the bladders treated with the parasite antigen; or the parasite antigens are only mitogenic for the urothelial cells causing increased proliferation that resulted in the *kras* activation via non-carcinogen-mediated pathways. Few studies were carried out to investigate the role and frequency of activated ras in schistosomiasis-associated bladder cancer. The mutations were not detected in two previous studies. These findings indicate that the advanced stage of schistosomiasis-associated bladder cancer may not involve a high frequency of detectable mutations within the ras genes. It is known that more than 90% of the SCC in the schistosome-infected bladder was advanced (stages T3 and T4) at the time of diagnosis. However, this does not exclude the possibility that activation in ras could be an important factor in the initiation as well as promotion and progression of schistosomiasis-associated bladder cancer.

**S. haematobium Infection and the Host Endocrine System**

Hormones regulate a variety of cellular and physiological functions of organisms, such as growth, reproduction and differentiation. The mechanisms by which host hormones act on parasites have recently been investigated, and some parasite molecules that are involved in transregulation have been identified and characterized.

In the case of schistosomes, hormonal signals from the host seem to have a major influence on larval homing, survival, growth and sexual maturation. Moreover, this is a two-way dialog and helminth parasites have a particularly marked effect on the fecundity of the host.

**Nuclear hormone receptors comprise a large superfamily of transcription factors whose activity is under hormonal control.** These receptors are characterized by a central DNA-binding domain, which interacts with specific hormone response elements located near the target gene promoter and by two distinct activation function (AF) domains that contribute to the transcriptional activity of these receptors.

The estrogen receptor (ER), a member of the steroid receptor family, mediates the stimulatory effects of estrogens and the inhibitory effects of anti-estrogens in estrogen target cells. ER mediates the biological effects of estrogens in a variety of target
tissues and ER-regulated genes are involved in many biological processes, including cell growth and differentiation, morphogenesis and programmed cell death. The ligand binding to estrogen receptor stimulates gene transcription via interaction with the estrogen response element (ERE).

Estrogens receptors are known to mediate important physiological functions, including reproduction, metabolism, maintenance of bone density and growth of estrogen-responsive tumors, including breast and endometrial cancers. There are two nuclear receptors, ERα and ERβ, that mediate the physiologic response to estrogen. They share several functionally conserved structural domains such as the regions responsible for DNA binding, dimerization, ligand binding and ligand-dependent transactivation of gene expression. ERα and ERβ have different transcriptional activities in certain ligand, cell type and promoter contexts and also exhibit distinct tissue- and cell type-specific expression patterns. ERα is found predominantly in the normal pituitary, breast, uterus, vagina, testis, liver and kidney, whereas ERβ is mainly expressed in thyroid, ovary, prostate, skin, bladder, lungs, gastrointestinal tract, bone and cartilage. ER was found to localize to the urethral epithelium but has been reported to be absent from transitional epithelium.

The knowledge that schistosomes have estradiol receptors as long been attained. This molecule able to bind estradiol has been previously described in schistosomes. It could be used by the parasite as a likely mechanism for the protective effect of this hormone in infected mice and hamsters.

Recent experimental evidence suggests that schistosomes can not only evade immune responses actively, but also exploit the hormonal microenvironment within the host to favor their establishment, growth and reproduction. It is generally believed that infection by schistosomes leads to modifications in the hormonal status of their hosts and that this is the result of a secondary reaction of the host to infection, one that is not directly mediated by parasite-derived factors.

The role of estrogens produced by schistosomes. After identifying the expression of an E2-related molecule by Sh, the ability of this estrogenic molecule produced by S. haematobium to interact in vitro with ER in the estrogen responsive MCF7 cell line was tested. Expression of ERα and ERβ were analyzed in MCF-7 cells after estradiol, ICI and Sh treatment. Real-time PCR analysis for ERα, ERβ and endogenous control gene GADPH were carried out by using RNA derived from either vehicle-treated (control), estradiol-treated (E2), ICI-treated (ICI) or Sh-treated in MCF-7 cell cultures. Incubation with estrogen clearly increased both ERα and ERβ expression. Furthermore, both ER isoforms were downregulated by Sh treatment in this cell culture, either alone or in the presence of ICI in comparison to control.

Nuclear hormone receptors regulate gene expression by interacting directly with DNA response elements. To confirm Sh-mediated downregulation of ER transfection of CHO cells with a reporter gene construct containing an estrogen response element (ERE) was carried out. Sh effectively reduced the transcriptional activity of the ER. When cells are treated simultaneously with Sh and ICI there is a synergistic effect in the reduction of luciferase activity. Sh was also able to significantly reduce ER transcriptional activity in the presence of E2 as revealed by comparing Sh+E2 with E2 results.

Liquid chromatography coupled to electrospray ionization mass spectrometry (LC-ESI-MS) was used to identify new estrogenic molecules produced by Schistosoma haematobium. Figure 1 depicts chromatograms obtained for biological samples by LC-ESI-MS. As seen, both the Schistosoma extract (Fig. 1A) and the plasma from a schistosome-infected individual (Fig. 1B) present four main peaks with retention times of approximately 20, 22, 24 and 26 min. Interestingly, these four peaks were not observed for the plasma from a healthy donor (Fig. 1C).

The structural assignment for the four components is depicted in Scheme 1. The species detected after MS2 fragmentation of
Figure 1. LC-ESI-MS analysis of (A) Schistosoma extract, (B) serum from a Schistosoma-infected individual and (C) serum from a healthy donor. (Figure extracted from Botelho et al.24)
Sh modulates the concentration of ER transcripts in the estrogen-responsive MCF-7 cells, by promoting its downregulation in a similar way as the estrogen inhibitor ICI. Sh clearly downregulates ERα and ERβ in MCF7 cells. The estrogen receptor (ER) exists in two isoforms ERα and ERβ with a different distribution in the body and different functions, which are not clearly identified yet. Physiological effects of estrogen are mediated through both ERα and β. Importantly, ERα- and ERβ-dependent pathways regulate distinct and largely non-overlapping sets of genes. Whereas ERα is essential for most of the estrogen-mediated

Scheme 1. Components 1, 2, 3 and 4 detected by LC-ESI-MS (scheme extracted from Botelho et al.24).
increase in gene expression, ERβ mediates the large majority (nearly 90%) of estrogen-mediated decreases in gene expression.80 Additionally, it is possible that Sh-induced ERα and β downregulation serves to control physiological responses in estrogen target tissues, which ultimately serves to limit the expression of estrogen responsive genes by the parasite.

These results are consistent with the existence of an estrogenic molecule that antagonizes the activity of estradiol. This molecule was identified and characterized by Mass Spectrometry. New estrogenic molecules previously unknown are present in schistosome worm extracts and sera of schistosome-infected individuals. The detection method developed by LC-MS analysis specific for estrogenic molecules, revealed that these new estrogens are present in schistosome worm extracts. At the light of these findings, we believe that the species detected on the LC-MS analysis of both the Schistosoma extract and the plasma of a Schistosoma-infected human were formed by reactions of estrogen-quinones with DNA. An interpretation of these findings was built at the light of well-established metabolic pathways for estrogens. It is known from the literature that estradiol is mainly metabolised by (1) oxidation of the hydroxyl function at the C17 position (via steroid dehydrogenase) to yield estrone and (2) hydroxylation by (1) oxidation of the hydroxyl function at the C17 position (via cytochrome P450 enzymes, occurring preferentially on C2, C4 and C16.9,82 When hydroxylation takes place on the steroid aromatic ring A, catechol-estrogens are produced.83 Furthermore, the proposed molecular structure of these new estrogenic molecules explains the cellular effects of Sh demonstrated previously in reference 26. These molecules show a putative binding affinity with ER and they probably are able to antagonize estradiol activity and downregulate ER.

To our knowledge there are no studies in the literature reporting the presence of genes involved in estrogen pathways in S. haematobium. As the genome sequences of the closely related African schistosome S. mansoni84 and the more distantly related Asian species S. japonicum85 are now available we can expect that schistosomes will express all the genes necessary to synthesize estrogenic molecules. In this regard, it is noteworthy that the genomic information suggests the presence of an integral hypothalamic-pituitary-thyroid in S. japonicum. It was also confirmed that S. japonicum has receptors for steroid hormones such as progesterin, progesterone and estrogen. In addition it possesses intricate pathways for processing steroid hormones to form other sex hormones. For example, there are putative enzymes present that could convert the female hormones progesterone and pregnenolone to estriol, oestrone, androsterone and testosterone. Hence, schistosomes might use these pathways during their parasitic existence.85 Finally, the absence of information on these issues in specific relation to S. haematobium supports the recent call for urgent progress towards sequencing the genome, transcriptome and proteome of S. haematobium.86

Since the repression of ER in MCF7 cells by Sh was observed, the hypothesis that Sh would also have the same effect in urothelial cells was tested. Therefore HCV29 cells, a cell line of normal urothelial cells, and CD-1 mice were used to evaluate the expression of ER in the bladders of Sh-instilled animals.87 Expression of ERα and ERβ were analyzed in HCV29 cells after estradiol, ICI and Sh treatment. Real-time PCR analysis for ERα, ERβ and endogenous control gene GADPH were carried out by using RNA derived from either vehicle-treated (control), estradiol-treated (E2), ICI-treated (ICI) or Sh-treated in HCV29 cell cultures. Incubation with estrogen clearly increased both ERα and ERβ expression (Fig. 2). Furthermore, both ER isoforms were downregulated by Sh treatment in this cell culture, either alone or in the presence of ICI in comparison to control. Interestingly, Sh incubation extensively reduced ERα and ERβ expression, even in the presence of estrogen (Fig. 2).

A total of 30 bladders, 10 controls and 20 treated animals, were analyzed for the ER expression. The urothelium was always negative for ER protein expression in all animals of the four groups.87 The controls of Group 2, sacrificed after 20 weeks, showed positive staining in the urethral epithelium and in the smooth muscle cells (SMC) in 80% (4 out of 5) of the bladders (Table 1 and Fig. 3A–D). The treated bladders were negative for ER expression in the urethral epithelium (Table 1 and Fig. 3E) and SMC (Table 1 and Fig. 3F) in 60% (6 out of 10) of the bladders of mice belonging to Group 1, which were sacrificed after 20 weeks. The bladders of the animals in this group that were considered positive for ER expression (40%) exhibited a weak immunostaining. The treated animals of Group 3, sacrificed after 40 weeks, were negative for urethral epithelium and SMC in 50% (5/10) of the bladders. The other half showed a pattern of staining similar to the positive bladders of Group 1 (Table 1). Group 4, controls sacrificed after 40 weeks, showed positive ER expression in the urethral epithelium and in the smooth muscle cells (SMC) in 60% (3 out of 5) of the bladders.87

This work addressed the regulation of expression of S. haematobium antiestrogenic activity as an important mechanism used by the parasite to influence ER transcription. The benefit that this ER repression may provide to the parasite could be explained by the following hypothesis: estradiol treatment as been shown to increase the smooth muscle density of the bladder in female rats.88 In our study we observed downregulation of expression of ER in the smooth muscle of Sh-treated bladders.88 During
Figure 3. Immunohistochemistry staining for ER in the bladders of mice. (A and B) urothelium of a control animal showing negative staining for ER; (C) urethral epithelium of a control animal showing stained nuclei (400x); (D) SMC of a control animal also with positive staining (400x); (E) urethral epithelium of Sh-treated mouse with negative expression (400x); (F) SMC lining the urethral epithelium of Sh-treated mouse also negative for ER expression (400x). (Figure extracted from Botelho et al.87)
infection with *S. haematobium* the parasite eggs must cross the bladder mucosa to reach the lumen of the bladder to be excreted, in order to survive and continue its life cycle. A thinner smooth muscle layer in the bladder mucosa of infected individuals could possibly increase the number of eggs released.

On another level, the repression of ER during *S. haematobium* infection could be a collateral damage of the infection. Nevertheless ER repression could have implications in the long term sequela of bladder cancer associated with this infection. As we described above, four estrogenic molecules have been identified in the parasite and in the sera from infected patients that are capable, according to their molecular structure, to bind ER and exert the repression effect observed. May the prolonged exposure to estrogens correlate with an increased risk for bladder cancer? At the light of these findings, we propose that the exposure to estrogens correlate with an increased risk for bladder cancer associated with this infection. As we described above regarding proliferation, apoptosis, tumorigenesis and the pathway of the estrogenic molecules present in the *S. haematobium* extracts could explain the link between this parasite and the carcinogenic effect of this estrogen adduct-mediated pathway of the estrogenic molecules present in *S. haematobium* extracts could explain the link between this parasite and squamous cell carcinoma of the bladder, as well as the findings described above regarding proliferation, apoptosis, tumorigenesis, dysplasia and gene mutations caused by extracts of *S. haematobium* in normal cells and normal urothelium.

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**Table 1.** Expression of ER in Sh-instilled bladders and controls

| Group  | Evaluated structures | Number of samples showing the stated estrogen-receptor immunoreactivity (%) |
|--------|----------------------|--------------------------------------------------------------------------|
|        |                      | Negative | Weak (+) | Moderate (+++) | Strong (+++) |
| 1 (n = 10) | Urothelium          | 10 (100) | -        | -               | -            |
|         | Urethra              | 6 (60)   | 4 (40)   | -               | -            |
|         | Smooth muscle        | 6 (60)   | 4 (40)   | -               | -            |
| 2 (n = 5) | Urothelium          | 5 (100)  | -        | -               | -            |
|         | Urethra              | 1 (20)   | -        | 3 (60)          | 1 (20)       |
|         | Smooth muscle        | 1 (20)   | -        | 3 (60)          | 1 (20)       |
| 3 (n = 10) | Urothelium        | 10 (100) | -        | -               | -            |
|         | Urethra              | 5 (50)   | 5 (50)   | -               | -            |
|         | Smooth muscle        | 5 (50)   | 5 (50)   | -               | -            |
| 4 (n = 5) | Urethra              | 2 (40)   | -        | 2 (40)          | 1 (20)       |
|         | Smooth muscle        | 2 (40)   | -        | 2 (40)          | 1 (20)       |

Table extracted from Botelho et al.97 *p* = 0.01 urethra Group 1 vs. Group 2; *p* = 0.01 smooth muscle Group 1 vs. Group 2; *p* = 0.03 urethra Group 3 vs. Group 4; *p* = 0.03 smooth muscle Group 3 vs. Group 4.

*Correlation between expression of estrogen-receptor immunoreactivity (ER) and smooth muscle 1 (20) 3 (60) 1 (20) 4 (50) 2 (40) 1 (20) 2 (40) 1 (20) 2 (40) 1 (20) 2 (40) 1 (20) 2 (40) 1 (20) 2 (40) 1 (20) 2 (40) 1 (20)
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