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M

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Angiogenesis in Schistosomiasis: Contribution to cancer and disease

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Dissertação submetida à Escola Superior de Saúde para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica em Saúde – Ramo em Biotecnologia, realizada sob a orientação do Professor Doutor Rúben Fernandes, Professor Adjunto com Agregação da Escola Superior de Saúde (ESS), Instituto Politécnico do Porto (P.PORTO) e Doutora Mónica C. Botelho, do Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA), Departamento de Promoção da Saúde e Doenças Crónicas, Porto, Portugal.

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Abstract

Background: Schistosomiasis is associated immunologic reactions to Schistosoma eggs trapped in tissues. Antigens released from the egg stimulate a granulomatous reaction involving lymphocytes, macrophages and eosinophils that results in clinical disease. Schistosomose is considered the second most devastating parasitic disease after malaria in the world. Its etiological agents are related to the carcinogenic process in the bladder and fibrosis in the liver. Recent advances in the fields of molecular biology and epidemiology have led to significant revelations to clarify the relationship between infectious agents and cancer and have given valuable insights into the molecular basis of carcinogenesis.

Aims: Furthermore, to better understand these mechanisms, we evaluated the role of inflammation (IL-6), angiogenesis (CD31), lymphangiogenesis (LYVE-1) and carbohydrate metabolism in an animal model infected with *S. mansoni* eggs through histochemical, histoenzymological, and immunohistochemical approaches.

Results: The results obtained in this study indicate that schistosomiasis influences the release of proinflammatory factors like IL-6 in significant amounts in the liver, which may be related to the amount of granulomas obtained in tissue histological analysis. Angiogenesis was increased in infected mice. Microvessel Density (MVD) is also increased. The expression of LYVE-1 in liver and spleen indicated an increase in lymphangiogenesis when compared to controls. *Schistosoma* eggs presented large amounts of fibrotic tissue and promoted a significant decrease of glycogen in the infected tissue.

Conclusions: Infection caused by eggs of *S. mansoni* increases inflammation, lymph/angiogenesis and influences cellular metabolic processes for the maintenance and / or development of the parasite, and for a cellular repair response, where neovascularization promotes these pathways.

Keywords: *Schistosoma* eggs, Angiogenesis, Lymphangiogenesis, Inflammation, Schistosomiasis

Resumo

Introdução: A schistosomose é uma infeção associada a ovos de *Schistosoma* em tecidos. Os antigénios libertados pelo ovo estimulam uma reação granulomatosa envolvendo linfócitos, macrófagos e eosinófilos que resultam em doença clínica. Schistosomose é considerada a segunda doença parasitária, a seguir à malária no mundo. Os seus agentes etiológicos estão relacionados ao processo carcinogénico na bexiga e à fibrose no fígado. Avanços recentes nos campos da biologia molecular e epidemiologia levaram a demonstrações significativas para esclarecer a relação entre agentes infeciosos e cancro e forneceram informações valiosas sobre a base molecular da carcinogénese.

Objetivos: Neste sentido, para melhor compreender estes mecanismos, avaliamos o papel da inflamação (IL-6), angiogénese (CD31), linfangiogénese (LYVE-1) e metabolismo (glicogénio) num modelo animal infetado com *S. mansoni* mediante ensaios histoquímicos, histoenzimáticos e imunohistoquímicos.

Resultados: Os resultados obtidos neste estudo indicam que a schistosomose influencia a libertação de fatores pró-inflamatórios como IL-6 em quantidades significativas para o fígado, o que pode estar relacionado à quantidade de granulomas obtidos na análise histológica do tecido. A expressão de CD31 demonstrou uma quantidade significativa para MVD que está diretamente relacionada com angiogénese. A expressão de LYVE-1 no fígado e baço, quando comparados aos controles, indica um aumento da linfangiogénese. Os granulomas apresentaram grandes quantidades de matriz fibrótica e promoveram uma diminuição significativa de glicogénio no tecido infetado.

Conclusão: A infeção causada por *S. mansoni* aumenta a inflamação, linfa/angiogenese e influencia os processos metabólicos celulares para a manutenção e / ou desenvolvimento do parasita e para uma resposta tecidular, onde a neovascularização detém um papel central.

Palavras-chave: Ovos de schistosoma, Angiogénese, Linfangiogénese, Inflamação, Schistosomose

Publication Under the Scope of This Thesis

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Abbreviations

3Rs	Reduce, Reuse, Recycle
AIDS	Acquired Immune Deficiency Syndrome
ANG	Angiogenin
Bcl-2	B-cell lymphoma 2
bFGF	basic Fibroblast Growth Factor
CD4	Cluster of Differentiation 4
CD31	Cluster of Differentiation 31
CD34	Cluster of Differentiation 34
CHO	Chinese Hamster Ovary Cells
DAB	3'3-Diaminobenzidine
DALYs	Disability-Adjusted Life Years
DNA	Deoxyribonucleic Acid
EC	Endothelial Cells
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Directive
GTP	Glucose Transporter Proteins
FELASA	Federation of European Laboratory Animal Science Association
HC	Histochemistry
HCC	Hepatocellular Carcinomas
HCV	Hepatitis C Virus
HGF	Hepatocyte Growth Factor
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HUVEC	Human Umbilical Vein Endothelial Cell
IARC	International Agency for Research on Cancer
IgG-B	Immunoglobulin G-B
IHC	Immunohistochemistry
IL1-α	Interleukin 1 Alpha
IL1-β	Interleukin 1 Beta
IL4	Interleukin 4

IL5	Interleukin 5
IL6	Interleukin 6
IL12	Interleukin 12
IL13	Interleukin 13
IPSE	Inducing Principle of Schistosoma Eggs
LYVE-1	Lymphatic Vessel Endothelial Hyaluronan Receptor 1
M2	Macrophages 2
Mm2	Square Millimetre
MVD	Microvascular Density
NO	Nitrogen Oxide
P27	Cyclin-dependent kinase
PAS	Periodic acid-Schiff
PECAM-1	Platelet Endothelial Cell Adhesion Molecule 1
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RNOS	Reactive Nitrogen Oxide Species
ROS	Reactive Oxygen Species
SCC	Squamous Cell Carcinoma
SEA	Soluble Egg Antigen
SEM	Standard Error of the Mean
SPSS	Statistics Offer the Self-Service
TCC	Transitional Cell Carcinoma
TH1	T helper cell 1
TH2	T helper cell 2
TNF-α	Tumor Necrosis Factor Alpha
UB	Urinary Bladder
UGS	Urogenital Schistosomiasis
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand Factor
WHO	World Health Organization

Structure of the document

- CHAPTER 1 - In this chapter, we encompass the disease caused by *Schistosoma* ssp. with the purpose of demonstrating the damages caused by the parasite and the causes of an infection with its worst prognosis, furthermore, in this chapter, we address the objectives of our study and the means that were developed to study schistosomiasis in mice.

- CHAPTER 2 - In this study, we enlighten the relationship between angiogenesis in cancer caused by schistosomiasis. For this purpose, we sought to understand the vascularization process in *S. haematobium* infection in the development of bladder cancer in people infected by the parasite through a literature review.

- CHAPTER 3 - In this chapter, we propose to study the role of angiogenesis in inflammation caused by schistosome infection. We have study the infection caused by *S. mansoni* eggs in the process of fibrosis, inflammation and vascularization in the livers and spleens of mice infected by the parasite in comparison to controls by means of morphological analysis through both histochemical and immunohistochemical procedures, such as H&E, Trichrome Masson's, and IL-6, CD31, LYVE-1 expression,

- CHAPTER 4 – In this chapter, we evaluated the role of schistosomiasis in central host metabolism by assessing glycogen storage in the liver of *S. mansoni*-infected mice using the PAS technique.

- CHAPTER 5 - In this chapter we present the discussion of the results obtained in the carried out experiments.

- CHAPTER 6 – In this chapter, we seek to promote a basis for future studies based on the research conducted for the development of this dissertation

Chapter I

General Introduction

1. The-state of the art

Schistosomiasis is a chronic and debilitating parasitic disease is caused by trematode parasites of the genus *Schistosoma* includes some 20 species, of which 7 have been associated with human infection. *Schistosoma japonicum, S. haematobium* and *S. mansoni* account for most of the social and economic burden. In terms of morbidity and mortality, schistosomiasis has been ranked second only to malaria among parasitic diseases. Disease burden assessments for schistosomiasis, based on the extent of end organ damage and the associated morbidities related to malnutrition and chronic inflammation, indicate that the annual number of disability-adjusted life years (DALYs) lost is around 70 million. This figure is comparable with the DALYs lost through HIV/AIDS and exceeds that of malaria or tuberculosis. Despite this, schistosomiasis remains underdiagnosed and undertreated, leading to its inclusion within the World Health Organisation's (WHO) Neglected Tropical Diseases list. Three major species - *Schistosoma mansoni, S. japonicum,* and *S. haematobium* - cause severe disease in humans. *S. mansoni* and *S. japonicum* are responsible for intestinal schistosomiasis, while *S. haematobium* causes urinary schistosomiasis. *S. japonicum* is distributed in the People's Republic of China, Indonesia, and the Philippines, whereas *S. mansoni* has a wider spread involving Africa, the Middle East, South America, and the West Indies. *S. haematobium* has a distribution similar to that of *S. mansoni* but does not occur in South America or in the West Indies. In addition, *S. mekongi* and *S. intercalatum* are two species with local importance, causing intestinal schistosomiasis in the Mekong River basin of Southeast Asia and in Middle and West Africa, respectively (1, 2 ,3).

Global statistics for mid-2003 suggest that almost 800 million individuals were at risk of schistosomiasis, 207 million were infected, 120 million suffered from clinical disease and 20 million exhibited severe morbidity. It's believed that are currently over 200 million people throughout the world infected with at least one species it Schistosoma helminth. There are 76 endemic countries, but the greatest burden (≥ 1 million infections) occurs in 30 African countries and Brazil. Approximately 20 to 50 million people experience substantive disability due to their infections. The map

bellow, published in Acta Tropica, depicts this global distribution including those areas where schistosomiasis has been eliminated (4).

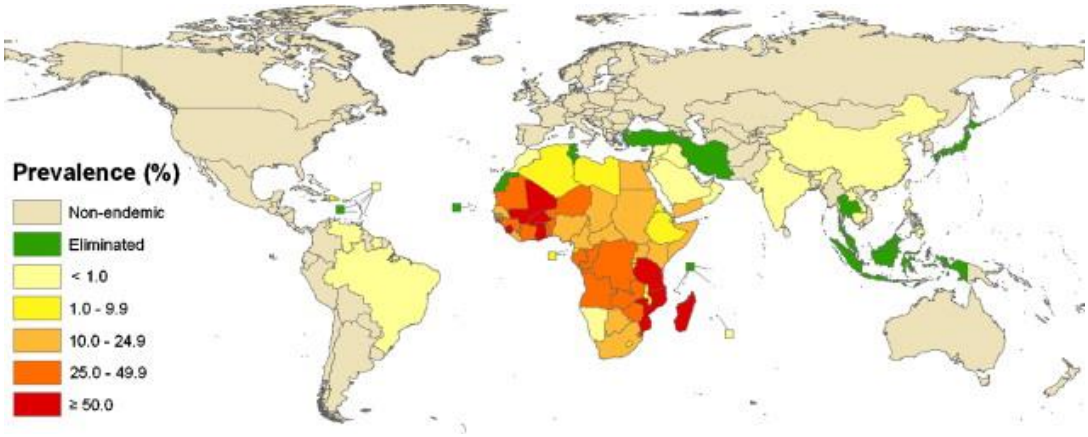

Figure 1. Current global distribution of schistosomiasis, stratified according to country-specific prevalence estimates

The life cycle of the schistosoma undergoes through two hosts: one intermediate and the other definitive. Initially eggs contained in the feces or urine of a human host and may be thrown to aquatic environments. Then eggs developed into a ciliated aquatic larva called miracidia. Miracidia temporarily establishes himself into a specific type of planorbide snail and then changing into cercaria. Cercariae penetrate actively through the epidermis, when people contact with contaminated water. After penetration, larvae reach the bloodstream, where they are transported to the intestine and subsequently displaced to the liver or bladder, settling and reproducing sexually (5).

Recent advances in the fields of molecular biology, epidemiology and infectious diseases have led to significant revelations to clarify the relationship between cancer and infective agents. *Schistosoma haematobium* and *S. japonicum* infections have been found to be strongly associated with bladder cancer and hepatocellular carcinomas (HCC), respectively. The international agency for research on cancer (IARC) considers *S. haematobium* infection a definitive cause of urinary bladder cancer with an associated 5-fold risk. (6).

Hepatocellular carcinoma (HCC) is one of the most common cancers world-wide; it accounts for more than 90.0% of all primary hepatic tumors. Clinical signs of schistosomiasis are dependent on the maturation stage of parasites and their eggs. In humans, acute infection is characterized by a debilitating febrile illness (Katayama fever) that usually occurs before the appearance of eggs in the stool, having a peak 6–8weeks after infection. In chronic disease, eggs trapped in various tissues evoke the formation of granulomatous inflammation composed of macrophages, epithelioid cells, giant cells and surrounded by T lymphocytes, which along with the ensuing fibrosis cause the majority of pathological conditions (6, 7).

In chronic infection with *Schistosoma*, the severe pathology, including liver fibrosis and splenomegaly, is caused by the immune response to the parasite eggs rather than the parasite itself. Parasite eggs induce a Th2 response characterized by the production of IL-4, IL-5 and IL-13, the alternative activation of macrophages and the recruitment of eosinophils (8). Schistosome-derived lipids and the schistosomal cytokine IL-4-inducing principle of *S. mansoni* eggs (IPSE) may be responsible for altering the host immune response from a Th1- to a Th2-type (9).

During the acute stage (5 to 7 weeks) of infection, immune responses are largely of the CD4+ Th1 type, associated with increased numbers of classically activated macrophages producing interleukin-12 (IL-12), IL-6, tumor necrosis factor alpha (TNF-α), and nitric oxide (NO). The early Th1 phase is followed by a short period of mixed Th1 and Th2 responses, which shifts to a dominant Th2 response by 9 to 12 weeks post-infection coincident with a shift in macrophage phenotype to M2, which are tissue-resident macrophages. M2 macrophages play a direct and critical role in fibrosis, maintenance of granulomas, tissue repair, and host survival (10, 11).

Angiogenesis, the formation of new endothelial vessels from pre-existing post-capillary venule, is a characteristic feature of inflammatory diseases, wound repair and cancer. Vascular endothelial growth factor (VEGF)-A is a representative proangiogenic factor. Traditionally, VEGF-A is largely associated with angiogenesis in cancer. The angiogenic activity depends on the balance or imbalance between angiogenic and angiostatic mediators. A large number of pro-angiogenic factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor

(bFGF), hepatocyte growth factor (HGF) and angiogenin (ANG) are often over expressed in tumors. Remodeling and degradation of the surrounding stroma is essential to start an angiogenic phenotype. These stromal changes facilitate recruitment and activation of leucocytes, fibroblast and endothelial cells. While granulomas are traditionally considered to be avascular structures, schistosome granulomas should be seen as an inflammatory condition that initiates a variable degree of wound healing response in which angiogenesis and fibrosis are highly involved (12, 13, 14).

Schistosomes, long-lived adult worms, continually bathed in blood, absorb nutrients directly on the surface of the body and also by ingesting blood in the intestine. Recent proteomic analyzes of the body surface revealed the presence of hydrolytic enzymes, solute and ion transporters, emphasizing their metabolic importance. The definition of molecular mechanisms for the uptake of selected metabolites (glucose, certain amino acids and water) establishes it as a vital nutrient acquisition site (15).

It is recognized that glucose is the common currency of cellular metabolism and all cells import glucose across their hydrophobic surface membranes using glucose transporter proteins (GTPs). Four glucose transporters (SGTP1, 2, 3, and 4) have been identified in *S. mansoni*, of which SGTP1 and SGTP4 display glucose transport activity. SGTP1 is present in a number of life stages (eggs, cercariae, schistosomula and adult female and male worms) while SGTP4 is detected only in the intra-mammalian forms, where it appears after the transformation of the cercariae into schistosomula and the appearance of the double membrane of the adult worm tegument (16).

In Schistosomiasis, an important consequence of liver injury is stimulated glycolysis, which is manifested by a reduction in the levels of plasma glucose, liver glucose, and glycogen (17). Glycogen degradation and replenishment occur through the body of the parasite, confirming inhibition of aerobic respiration and stimulation of anaerobic glycolysis through hexokinase, a rate-limiting enzyme of glycolysis (17). This metabolism of glucose through glycogen degradation should help in maintaining a low internal free glucose concentration and thus promote sufficient glucose diffusion to deeper tissues.

2. Aims and Goals

Schistosomiasis is a parasitic disease that is considered a major source of mortality in the developing countries. *Schistosoma mansoni* infection leads to liver fibrosis, which is represented by small focal areas of chronic inflammation. This fibrosis is found in the periovular granulomas (18).

The main goal was to evaluate the morphological variables of angiogenesis in the context of *S. mansoni* infection. Thus, studying inflammation and neovascularization of liver and spleen blood and lymphatic vessels in mice infected with *S. mansoni* eggs is of paramount importance.

In order to establish an association between inflammatory factors and microvasculature associated with this pathology, an animal model was used to determine several parameters associated with schistosomiasis infection. To accomplish this goal, we proposed to:

a) To evaluate and quantify the collagen fibers formed around the eggs through the technique of staining of Masson's trichrome in liver and spleen tissues;

b) To evaluate the inflammation status associated with infection of *S. mansoni* eggs by measuring levels of IL-6 (pro-inflammatory marker) also in liver and spleen;

c) To evaluate the alterations that promotes vascularization of both liver and spleen tissues by measurement of cluster of differentiation (CD) 31 levels (angiogenic marker) and Lymphatic Vessel Endothelial Hyaluronan Receptor 1 (LYVE-1) levels (lymphangiogenic marker).

Furthermore, the molecular mechanisms underlining Schistosomes metabolism and subsequent metabolism necessary to nourish growth and fertility may provide new avenues for the development of new interventions for the control of schistosomiasis. To establish this association, it was conducted by PAS (Periodic acid-

Schiff) the evaluation of central carbohydrate metabolism. To achieve this goal, we intended to:

d) Evaluate alterations in the pool of carbohydrates in liver tissues
e) Evaluate the glycogen liver depletion

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Chapter II

Angiogenesis in *Schistosoma haematobium*-associated bladder cancer

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Abstract

Schistosoma haematobium, a parasitic flatworm that infects more than 100 million people, mostly in the developing world, is the causative agent of urogenital schistosomiasis, and is associated with a high incidence of squamous cell carcinoma (SCC) of the bladder. During infection eggs are deposited in the bladder causing an intense inflammatory reaction. Angiogenesis is defined as the formation of new blood vessels from pre-existing ones and is recognized as a key event in cell proliferation and carcinogenesis and spread of malignant lesions. A growing amount of evidence points to angiogenesis playing a key role in schistosomiasis associated bladder cancer. Thus, identifying biomarkers of this process plays an important role in the study of cancer. Here, we review recent findings on the role of angiogenesis in bladder cancer and the growth factors that induce and assist in their development, particularly SCC of the bladder associated to urogenital schistosomiasis.

Keywords: Schistosomiasis, Urothelial Carcinoma, Blood Vessels, Urogenital Schistosomiasis, Angiogenic Markers

1. Schistosomiasis

Schistosomiasis is a neglected tropical disease transmitted to humans from freshwater snails. It is caused by a blood fluke of the genus Schistosoma. Schistosomiasis is considered the most important of the helminthiases and the second most important parasitosis, after malaria, causing high rates of morbidity and mortality. As of 1989 schistosomiasis was endemic in 76 countries (1). Recent WHO report declared schistosomiasis endemic in 78 countries (2). *S. haematobium* is endemic in 53 countries in the Middle East and most of the African continent, including the islands of Madagascar and Mauritius. Due to successful eradication programs the infection is no more of significant public health significance in Egypt, Lebanon, Oman, Syria, Tunisia and Turkey because transmission is low or nonexistent (3). A disputed and ill-defined focus exists in India and requires further confirmation (4). After more than 50 years in which no more autochthonous cases of schistosomiasis were recorded in Europe *S. haematobium* infection has recently emerged in Corsica (5). This disease affects 200 million people worldwide. From these, 20 million have severe disease and 120 million are considered symptomatic. Risk of infection affects 600 million others including travellers from developed countries (6). This review focuses on the morphological variables and prognosis in carcinoma of the urinary bladder associated with schistosomiasis, and the fact that the appearance and formation of angiogenesis alters the course of cancer development, in the context of *S. haematobium* infection. The present work attempts to integrate a variety of studies and experimental approaches with *S. haematobium* models, while giving particular emphasis to the in vitro studies that have contributed to expanding our understanding of the mechanisms of action of growth factors and formation of new vessels in urinary bladder cancer. In particular, we suggest that the presence of eggs of *S. haematobium* plays a key role in angiogenesis and contribute to the development of urinary bladder cancer (Figure 2).

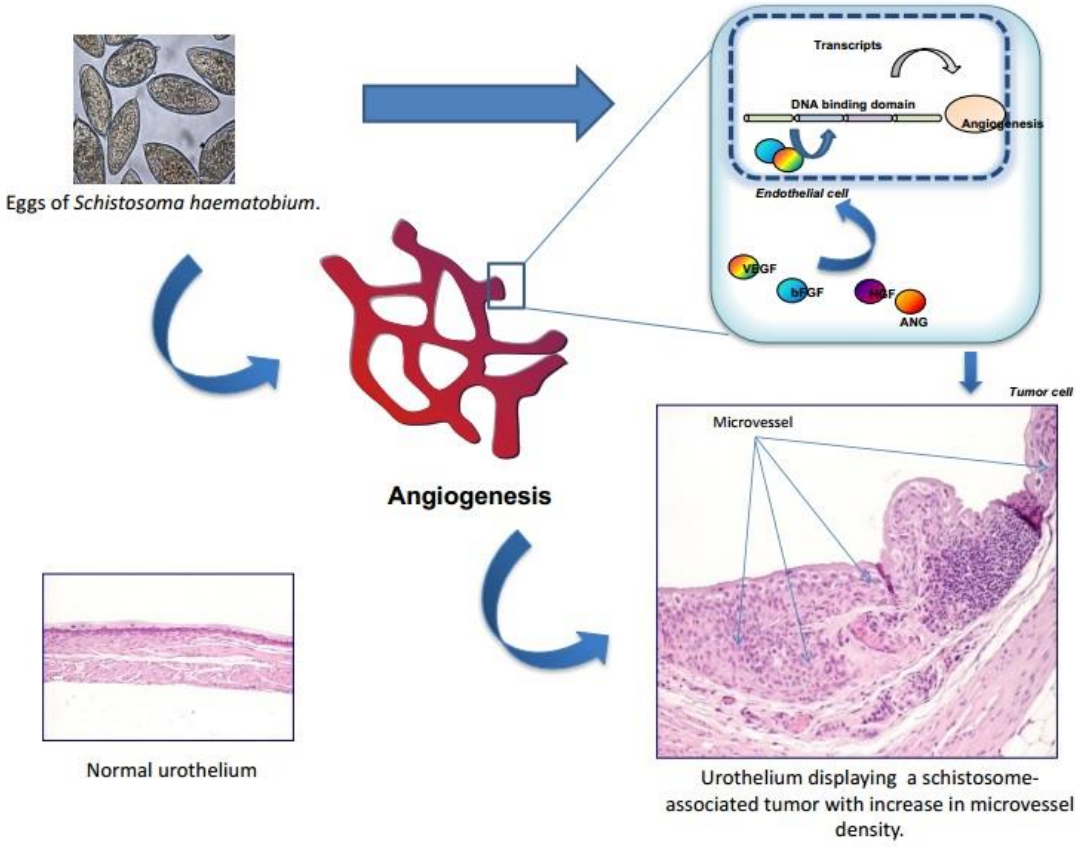

Figure 2. Schematic representation of angiogenesis associated with urogenital schistosomiasis. 448 Microphotographs from urinary bladder sections stained with Hematoxylin and Eosin (400X).

1.2. Urogenital Schistosomiasis

Three major species of schistosomes are the agents of human schistosomiasis – *Schistosoma japonicum* and *Schistosoma mansoni* cause intestinal schistosomiasis in East Asia, Africa, South America and the Caribbean, while *S. haematobium*, occurring widely throughout Africa and the Middle East, causes urogenital schistosomiasis. Recent recalibration of health burdens revealed that in the range of 4.5–70 million disability adjusted life years (DALYs) are lost to schistosomiasis. More people are infected with *S. haematobium* than with the other schistosomes combined. Of 112 million cases of *S. haematobium* infection in sub-Saharan Africa, 70 million are associated with hematuria, 18 million with major urinary bladder wall pathology, and 10 million with hydronephrosis leading to kidney damage (7 ,8, 9). In many patients, deposition of *S. haematobium* parasite ova eventually leads to squamous cell carcinoma (SCC) of the urinary bladder (10, 11). Accordingly, *S. haematobium* has been

classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC) (12, 13).

1.3. *S. haematobium*-associated urinary bladder cancer

SCC is a malignant, poorly differentiated neoplasm. SCC is the common form of urinary bladder cancer in rural Africa where *S. haematobium* is prevalent (14, 15). By contrast, the majority of urinary bladder cancer in developing countries and regions not endemic for urogenital schistosomiasis is transitional cell carcinoma (TCC), which arises from the transitional epithelium lining of the urinary bladder. The parasite eggs trapped in the urinary bladder wall release antigens and other metabolites (presumably evolved to expedite egress to the urine, and hence to the external environment). The phenomenon leads to haematuria and to chronic inflammation, in turn increasing the risk of SCC of the urinary bladder. The epidemiological association between SCC of the urinary bladder with schistosomiasis haematobia is based both on case control studies and on the correlation of urinary bladder cancer incidence with prevalence of *S. haematobium* infection within diverse geographic locations. The incidence of urogenital schistosomiasis-associated SCC is estimated in 3–4 cases per 100 000 (16). *Schistosomiasis haematobia* is a chronic infection. The adult, egg-producing schistosomes live for many years, re-infections frequently occur, and schistosomiasis associated urinary bladder SCC appears relatively early, often by the mid-decades of life (TCC usually presents in the later decades of life). In its most recent monograph, IARC confirmed that chronic infection with *S. haematobium* causes cancer of the urinary bladder (13). The cellular and molecular mechanism linking infection with *S. haematobium* and cancer is usually related to adult parasite invasion in the venous plexus around the urinary bladder, the eggs released by worms, cause chronic granulomatous inflammation of the mucosa and submucosa of the urinary bladder. Chronic granulomatous inflammation and irritation subsequently leads to the development of squamous metaplasia of the transitional epithelium. Chronic granulomatous inflammation also leads to fibrosis in the urinary bladder that causes urinary stasis and super-infection by bacteria. The bacteria convert the nitrates and nitrites in dietary nitrosamines, which are then excreted in urine. These nitrosamines are carcinogenic and acting on metaplastic epithelium, promote

subsequent progression of squamous cell carcinoma. The infection can spread and involve the ureters and kidneys, causing chronic obstructive disorders and kidney failure (17). Several models have been proposed to explain the genesis of urinary bladder cancer induced by Urogenital Schistosomiasis (UGS). Some attribute initiation of carcinogenesis to low doses of nitrosamines and / or other environmental carcinogens associated with the infection. In other models, it is suggested that UGS-induced carcinogenesis is due to exposure to tobacco smoke, industrial and agricultural dyes and vitamin A deficiency (18). However, the mechanism by which infection contributes to carcinogenesis is still unresolved. Recent contributions suggest a crucial role of *S. haematobium*: Chinese Hamster Ovary cells (CHO) cells experimentally treated with parasite antigens show increased proliferation, cell migration and invasion, decreased apoptosis, increased Bcl-2 expression and reduced p27 expression. Altogether, these biological processes are characteristic of tumorigenesis and tumor cell survival (19). Further, intravesical administration in a murine model of *S. haematobium* extract induce urothelial dysplasia (20), implying that infection by *S. haematobium* induce malignant transformation of the urothelium, even in the absence of nitrosamines. Previous reports of our group revealed that schistosomes produce estrogen metabolites called catechols and that these molecules can be used as biomarkers for the detection of schistosomiasis-associated urinary bladder cancer (21, 22, 23). Based on this scientific evidence, and the discovery of parasitic origin of estrogen metabolites, becomes fundamental to understanding the role of this parasite as initiator of carcinogenesis (24).

1.4. Angiogenesis and lymphangiogenesis in urinary bladder cancer

Angiogenesis, or the formation of new endothelial sprouts from preexisting postcapillary venules, is a well-known characteristic of inflammatory diseases, wound repair and cancer (25, 26). Accordingly, angiogenesis is a process in which endothelial cells migrate and divide to form new capillaries, providing support for tumor progression. As such, much attention has been focused on pathological significance and detailed mechanism of the vascular system and angiogenesis in

cancer. Moreover, the spread of tumor cells through the bloodstream and lymphatic system plays an important role in metastases development (26). A large number of pro-angiogenic factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF) and angiogenin (ANG) are often over expressed in tumors (27). Several studies have indicated that angiogenic activators play an important part in the growth and spread of tumors. On immunohistochemical examination, the VEGF family and their receptors were found to be expressed in about half of the human cancers investigated (28). These factors are known to affect the prognosis of adenocarcinomas that have developed in the uterine cervix, (29), endometrium, (30), ovary (31), and stomach (32). In addition, a significant correlation between the expression of VEGF and prognosis has been described in colorectal cancer (33), breast cancer (34), lung cancer (35), head and neck squamous cell carcinoma (36), Kaposi sarcoma (37), and malignant mesothelioma (38). These studies also indicated that the levels of angiogenic factors in tissue reflect the aggressiveness with which tumor cells spread, and thus have predictive value in the identification of the high-risk patients with poor prognosis. Metastatic spread to regional lymph nodes is an early step in systemic dissemination of tumors, being usually associated with poor survival (39). Moreover, exacerbated angiogenesis together with presence of lymph node metastasis are poor prognostic factors for transitional cell carcinoma and urinary bladder carcinoma (40). While both the blood and lymphatic vascular systems have been implicated, preclinical experimental systems supported by clinical evidence suggest the most common pathway of initial metastasis is through the lymphatic system (41). In recent years, several works discuss the importance of pathological lymphangiogenesis in urinary bladder cancer and in its importance in the invasiveness towards adjacent muscle tissue (42, 43). Similarly, recent reports evidence the activation of VEGF signaling that controls and promotes lymphangiogenesis by several parasites such as filariasis and leishmaniasis agents (44, 45). Although lymphangiogenesis is shown to be increased in both urinary bladder cancer and in infection caused by parasites, it is a question needed to be answer weather lymphangiogensis would be involved in urinary bladder cancer associated with schistosomiasis.

1.5. Schistosomiasis and angiogenesis

Angiogenesis plays a complex and extraordinary role in schistosomiasis. This statement may seem a paradox, since schistosomes are intravascular parasites that cause damage by destroying the blood vessels (46). Angiogenesis plays an important role during the formation of periovular granulomas as well as in the genesis of schistosomiasis fibrosis. From the point of view of general pathology, schistosomal periovular granulomas are dynamically similar to the healing of wounds, with the production of granulation tissue, which becomes increasingly less vascularized with time (47). It has been demonstrated that intact live eggs, excretory/secretory products of eggs and the extracts of homogenized eggs stimulate the proliferation and migration of endothelial cells. Formation of endothelial capillary-like outgrowths, was stimulated by egg extracts (48). The effects mediated by eggs of schistosomes revealed that the soluble egg antigen induces endothelial cell proliferation and upregulates vascular endothelial growth factor (VEGF) (46). Loeffler and collaborators (49) investigated the effects of *Schistosoma mansoni* soluble egg antigen (SEA) on angiogenic processes: proliferation, tube formation, and apoptosis of human umbilical vein endothelial cells (HUVECs). In this study SEA increased HUVEC tube formation and decreased HUVEC apoptosis after serum and growth factor deprivation. These authors showed that messenger RNA for vascular endothelial growth factor (VEGF) increased 2-fold in SEA-treated HUVECs. Their findings suggest that products secreted by schistosome eggs may promote angiogenesis by up-regulating endothelial cell VEGF (49). Other authors analyzed VEGF levels in sera from people diagnosed with schistosomiasis. These patients had significantly high VEGF levels compared with healthy people (50, 51). Therefore, this angiogenic capacity has been suggested as an early marker of preneoplastic and neoplastic lesions in schistosomiasis associated SCC (52). Several growth factors and other molecules produced by the schistosome itself have been reported to be associated with tumor growth, progression and survival of urinary bladder cancer. Moreover, tumor microvessel density (MVD) is thought to be the most useful prognostic marker for cancer development, the relapse-free survival and overall survival (53). El-Sobky and collaborators (2002) found a significant relationship between angiogenesis and

tumor grade. These findings suggest that assessing angiogenesis using the MVD provides an independent predictor of survival in patients with schistosome-associated carcinoma of the urinary bladder (54). Studies to quantify the concentration of angiogenic factors in cases of SCC of the urinary bladder associated to schistosomiasis may be of great clinical importance for urinary bladder cancer detection, assessing their stage and level of development. The methodologies that can be performed to evaluate angiogenesis associated with *Shistosoma haematobium* infection are by means of microscopy / immunochemical, ELISA, PCR based techniques and other molecular biology techniques that can be used to evaluate vascularization markers in both samples of infected patients and in animal models. Such markers may include CD31, CD34, vonWillebrand factor (vWF) and VEGFRs. Angiogenic markers showed significant association with clinical stage (27). It was reported that basic fibroblast growth factor (bFGF) increased significantly in urinary bladder squamous cell carcinoma cases. These authors found that bFGF and hepatocyte growth factor (HGF) significantly correlated with tumor grade (27). Understanding the growth factors that influence the progression of angiogenesis and lymphangiogenesis during infection by schistosomes becomes feasible from the point of view of a possible intervention in the spread of tumor cells. Moreover, cancers are genetically diversified using different exposures, DNA repair effects and cellular origin, which may suggest that a particular exposure (parasite antigen) can lead to a cascade of events that promote cancer in susceptible hosts, and that angiogenic factors, as reported in *S. haematobium*, could be used as diagnostic and prognostic markers of urinary bladder cancer in UGS. An in the case of Symmers' fibrosis associated to schistosomiasis, angiogenesis inhibitors 238 are indicated as an effective tool for the treatment of this liver fibrosis (54).

2. Concluding remarks and future perspectives

In the last years' bladder cancer associated schistosomiasis has been a field under extensive investigation. Increasing knowledge in the field has opened up important new perspectives with respect to how this type of cancer is perceived. Among them there are crucial findings on the hallmarks of cancer and the contribution of these to the carcinogenic process, specially angiogenesis and lymphangiogenesis. On the basis of these results a new mechanistic approach to the development of schistosomiasis associated bladder cancer arose, enabling us to further comprehend their underlying molecular mechanisms and contribution to the development of this type of cancer. Studies are needed to identify and characterize angiogenic and lymphangiogenic markers in carcinoma of the urinary bladder associated to UGS. Tumor induction, proliferation, invasion and metastasis represent a complex and incompletely understood series of events (55). Thus, the development of additional biological markers of prognosis for tumor angiogenesis and lymphangiogenesis, may add information to the initial risk assessment. It is to be hoped that the rapidly increasing volume of information in these field can be used in the future to develop specific and more effective anti-cancer therapies, not only towards schistosomiasis associated bladder cancer but also other types of cancer.

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Chapter III

Schistosomiasis mansoni increases murine hepatic and splenic fibrosis, lymph/angiogenesis, and IL-6 production

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Abstract

Experimental studies have demonstrated the occurrence of angiogenesis, blood vessels formation from pre-existing vessels, in the initial phase of granuloma formation and during fibrosis progression in chronic hepatic schistosomiasis.

The present work aims to demonstrate the relationship of *S. mansoni* eggs infection in the liver and spleen of mice, with the increase of vascularization and inflammation in these tissues, being the analysis made by tissue-specific biomarkers of inflammation (Il-6) and vascularization (CD31 and LYVE-1), as well as evaluate and quantify periovular fibrosis presented in liver tissues by technique Masson's trichrome.

In the present study, infection caused by schistosome eggs involves several processes mediated by the release of cytokines produced by eggs, thus we demonstrated the infection enhances the fibrosis and inflammation in hepatic-splenic axis in mice. Furthermore, we found that infected animals are more prone to develop increased neovascularization not only in blood vessels but also in lymphatic vessels. Accordingly, the present study brings new insights for the comprehension of the mechanisms underlining tissue regeneration but also the route of infection associated to schistosomes.

1. Introduction

Schistosomiasis is one of the most widespread parasitic infectious diseases that affect human. Globally an estimated 200 million people are currently infected and an estimated 800 million people are at risk in 74 countries with an annual death of about 20,000 patients. The disease is widely distributed in Africa, Western Asia, South America, and South West Asia affecting a high proportion of populations, especially children in developing countries causing major socioeconomic and public health consequences (1, 2).

Most human infections are linked *to S. haematobium*, strongly associated with bladder cancer and *S. mansoni*, which has been linked to many case reports of liver cancer, colorectal cancer, prostate cancer, and giant follicular lymphomas (3).

Schistosoma mansoni infection leads to liver fibrosis, which is represented by small focal areas of enduring inflammation. These fibrosis immoderations of the extra-cellular matrix are placed in the periovulares granulomas (4).

Experimental studies have demonstrated the occurrence of angiogenesis, blood vessels formation from pre-existing vessels, in the initial phase of granuloma formation and during fibrosis progression in chronic hepatic schistosomiasis. Paradoxically, a recent work demonstrated an occurrence of angiogenesis during fibrosis regression months after curative treatment (5). Angiogenesis involves primarily endothelial cells in the capillaries, but a concomitant stimulation of smooth muscle cells and pericytes leads to the formation of a more mature, vascular network. Hence the *S. mansoni* entity, with the ability to stimulate both endothelial and smooth muscle cells, is an even more effective vascular growth factor (6). The proliferative changes in the liver blood vessels, fibroblasts and hepatocytes are thought to be due to a paracrine secretion of endogenous host growth factors or to a direct effect of egg-derived factors. Granuloma-derived mitogens were found to stimulate endothelial and smooth muscle cells, as well as fibroblasts. The notion of a direct effect is supported by the observation that an egg-secreted factor stimulates endotelial cell proliferation (6).

Inflammation-induced lymphangiogenesis is a hardwired program designed to address drastically altered tissue needs during pathogen invasion, tissue expansion and injury, or other disturbances of homeostasis as a result of molecular imbalances and physical causes. The lymphatic system is primarily responsible for balancing high levels of interstitial fluid and removing massive amounts of immune cells mobilized to inflamed tissues in response to the aforementioned effects (7).

The present work aims to evaluate and quantify periovular fibrosis present in the liver in association with *S. mansoni* infection. Also to understand the relationship of *S. mansoni* infection in the liver and spleen of mice, with inflammation and blood/lymph vessels in these tissues. To this purpose we evaluated expression of IL-6, CD31 and LYVE-1 in livers and spleens of infected mice.

To our knowledge this is the first report addressing IL-6 expression related to *S. mansoni* infection.

2. Materials and Methods

2.1. Animal Experiments

This experiment followed the guidelines and recommendations of FELASA (Federation of European Laboratory Animal Science Associations) and the European Directive 2010/63/EU related to animal protection in scientific studies were followed, especially the reduction principle of the 3Rs for animal experiments.

Six-week-old CD-1 mice were provided by Charles River (Barcelona, Spain). Animals spent 1 week being acclimated under routine laboratory conditions before starting the experiments. They did not receive any treatment prior to the study.

Animals were fed standard balanced food and water *ad libitum*. All the animals were maintained at the National Institute of Health (Porto, Portugal) in rooms with controlled temperature (22% ± 2°C) and humidity (55% ± 10°C) and continuous air renovation. Animals were housed in a 12 h light/12 h dark cycle (8 am–8 pm). All animal experiments were performed in accordance with the National (DL 129/92; DL 197/96; P 1131/97) and European Convention for the Protection of Animals used for Experimental and Other Scientific Purposes and related European Legislation (OJ L 222, 24.8.1999).

2.2. Experimental infections

Twelve 12 CD-1 mice from which 8 were experimentally infected with 50 cercariae *S. mansoni*. Mice were infected by member's extremities and tail immersion, respectively. The control animals consisted of 10 littermates. The cercariae were obtained by shedding of snails infected with miracidia.

2.2. Histochemistry

2.2.1. Hematoxylin and Eosin Stain (H&E)

Deparaffinization in section of xylene 10 minutes, hydrated in absolute alcohol, 90% and 70% at 3 minutes each, stain in hematoxylin solution for 20 seconds and apply eosin for 2 min, then wash briefly in running water. Dehydrated through 70% alcohol, 90% and 100% clean in xylene 10 minutes and mounted with *entellan*.

2.2.2. Masson's trichrome Stain

Sections from liver were stained by Masson's trichrome Stain, used for the detection of collagen fibers in tissues. For Formalin fixed tissue, for 1 hour at 56 C to improve staining quality, stained in Biebrich scarlet-acid fuchsin solution for 10-15 minutes, differentiated in phosphomolybdic-phosphotungstic acid solution for 10-15 minutes or until collagen is not red, transferred sections directly (without rinse) to aniline blue solution and stain for 5-10 minutes and differentiated in 1% acetic acid solution for 2-5 minutes. Expected results: In this technique, it is expected that collagen fiber stained blue, muscle, cytoplasm and keratin are stained in red, and nuclei are contrasted in black.

2.3. Immunohistochemistry

2.3.1. Inflammation markers

Sections of liver and spleen were stained for the pro-inflammatory IL-6. Tissue sections were incubated overnight, at 4ºC in a humidified chamber, with a primary rabbit polyclonal antibody anti-human IL-6 (ab6672, Abcam plc, UK; 1:600 dilution)

followed by the biotinylated goat anti-rabbit antibody IgG-B (sc-2040, Santa Cruz Biotechnology, Inc., USA; 1:200 dilution) (Table 1).

2.3.2. Blood and lymphatic vessels

Sections from liver and spleen were stained for CD31, a specific endothelial vessel marker. Tissue section were incubated overnight, in a humidified chamber, at 4ºC, with the polyclonal rabbit antibody anti-mouse CD31 (ab28364, Abcam plc, UK; 1:200 dilution) followed by the biotinylated antibody goat anti-rabbit IgG-B (sc-2040, Santa Cruz Biotechnology, Inc., USA; 1:200 dilution).

Lymphatic vessel evaluation was performed in sections of liver and spleen, which were stained for the specific lymphatic vessel marker LYVE-1. Tissue sections were incubated, thirty minutes at room temperature, with the rat anti-LYVE-1 monoclonal antibody (sc-80170, Santa Cruz Biotechnology, Inc., USA; 1:200 dilution) followed by the biotinylated rabbit anti-rat antibody IgG (DK-E0468, Dako, Denmark; 1:400 dilution). For all the parameters evaluated, the color development procedure after secondary antibody incubation was done for approximately three minutes with 3,3'-Diaminobenzidine (ab-94665, Abcam plc, UK) chromogen and further counterstaining with hematoxylin (Table 1).

Lymph/Angiogenesis evaluation

For CD31 IHC sections, the stained area and the number of vessels present in the analyzed fields allowed to calculate the microvascular density (MVD), represented as number of vessels per mm^2.

For LYVE-1 IHC technique, it was analyzed the number of vessels stained by this antibody the results represent a comparison of their presence in the liver infected and control.

2.4. Image Analysis

Histological sections were analyzed altogether 17 liver tissue and 15 spleen tissue with optical microscope Nikon Eclipse 50i (Nikon Instruments Inc.) and images captured with attached Nikon DS-Fi1 digital microscope camera (Nikon Instruments, Inc.), being this camera connected to a computer. Eighty-five pictures of the liver and spleen for the each IHC and HC technique from the mice studied were taken, at 400X optical amplification in Nikon Nis Elements Viewer 3.22.15 (Nikon Instruments Europe B.V.) software.

For further bioinformatics analysis, five pictures were obtained for each tissue of most stained areas. Those captured images were analyzed with Image J (1.49u, Wayne Rasband, National Institute of Health, (USA) software. This software allowed to calculate the stained areas, obtained by the revelation process, of each field. To do this evaluation it was used the color deconvolution plugin for image separation by its different colors visualized in the IHC and HC technique. For CD31 IHC it was also counted the number of vessels present on those fields. For the LYVE-1 IHC technique it was analyzed all the liver tissue present in the histological slide to count the vessels that were marked within this IHC technique and the vessels that were possible to count without staining.

2.5. Statistical Analysis

For statistical analysis, the normality test for Shapiro-Wilk was applied. With all the collected data, and for each IHC technique and HC, it was calculated the mean ± standard error of the mean (SEM). For non-parametric distributions such as Masson's trichrome stain and immunochemical expression of LYVE-1 and CD31, Mann-Whitney test was applied. For parametric distributions was applied T-test as the case of IL-6.

The statistical analysis was performed with IBM SPSS (Statistic Package for the Social Sciences, version 24) and results were considered statistically significant when *p* values were <0.05.

Table 1. List of antibodies used in the immunohistochemistry experiments and their respective commercial references.

	Evaluation	Antibody	Dilution	Commercial Reference
Primary Antibody	Inflammation	Rabbit polyclonal antibody anti-human IL-6	1:600	ab6672, Abcam plc, UK
	Vascularization	Rabbit polyclonal antibody anti-mouse CD31	1:200	ab28364, Abcam plc, UK
		Rat monoclonal anti-LYVE-1 antibody	1:200	sc-80170, Santa Cruz Biotechnology, Inc., USA
Secondary Antibody		Biotinylated goat anti-rabbit antibody IgG-B	1:200*	sc-2040, Santa Cruz Biotechnology, Inc., USA
		Biotinylated Rabbit anti-Rat antibody IgG	1:400	DK-E0468, Dako, Denmark

* The dilution of the secondary biotinylated antibody was the same for the several immunohistochemistry techniques.

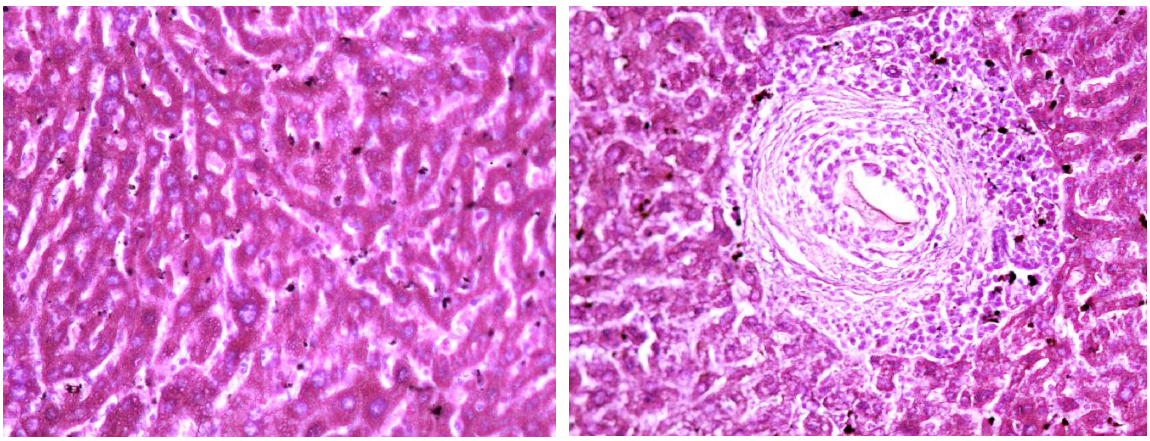
3. Results

As stated previously, the present study aims to evaluate formation of fibrosis in periovular granulomas, the glycogen levels in the tissues, inflammation, blood and lymphatic microvasculature, of liver and spleen in mice infected with *S. mansoni* eggs. It was performed histochemistry assays of Masson's trichrome, and for performed immunohistochemistry assays, was applied IL-6, CD31 and LYVE-1, respectively.

3.1. Microscopic Morphology

Liver and spleen stained by H&E allowed to observe the presence granulomas in liver. In the liver, we observed the hepatocytes and their stained nuclei arranged in sinusoidal laminas between the tissue (Figure 3 A), observed the granulomas formed by *S. mansoni* eggs, with an average of 42 granulomas per liver tissue (Figure 3 B), in the spleen, observed a dense connective tissue covered by a large concentration of lymphocytes, which no granulomas are present (Figure 3 C and D).

A **B**



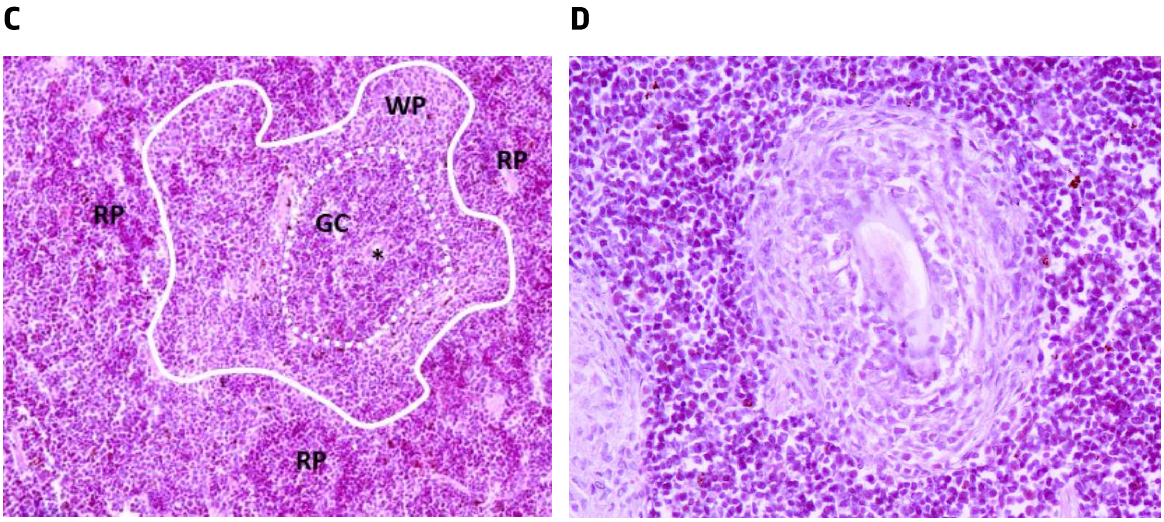


Figure 3. Liver (A and B) and Spleen (C and D); section taken at 200X amplification. In the fig. (3 A) it can be observed the Kupffer cells (stained in black) that are positioned between sinusoids and the space of Disse. In the fig. (3 C) it can also be observed spleen red pulp (RP) and white pulp (WP) surrounding the germinative center (GC) and central arteriole (*) in section 100X In the image (3B and D) it is observed a Hepatic and a Spleen periovular granuloma respectively (amplification of 200x). Stain: Hematoxylin-Eosin.

3.2. Fibrosis

The evaluation of the parasite infection-related periovular fibrosis in liver was assessed by Masson's Trichrome stain. Microscopic observation of the experiments revealed the presence of granulomatous periovular areas of liver tissue.

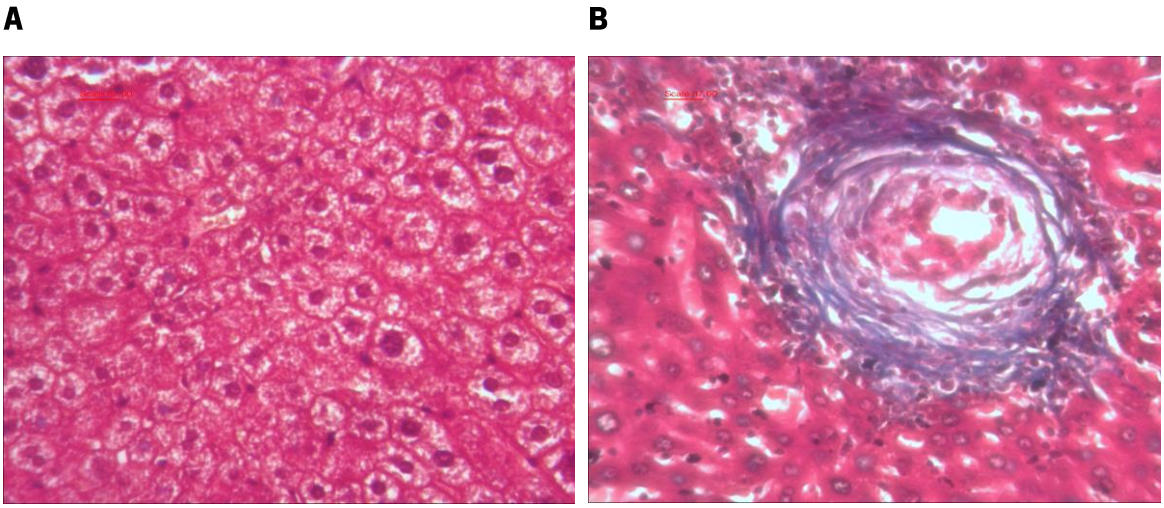


Figure 4. Liver of control (A) and infect (B) mice; Microphotographs taken at 400X amplification. Stain: Masson's Trichrome.

After image analysis of Masson's Trichrome, it was observed higher stained areas in periovular zone (134683 ± 4637 µm²) of infected livers when compared with the control tissue (1160 ± 24 µm²), with a statistically significant result ($p < 0.05$) for formation fibrosis in the liver.

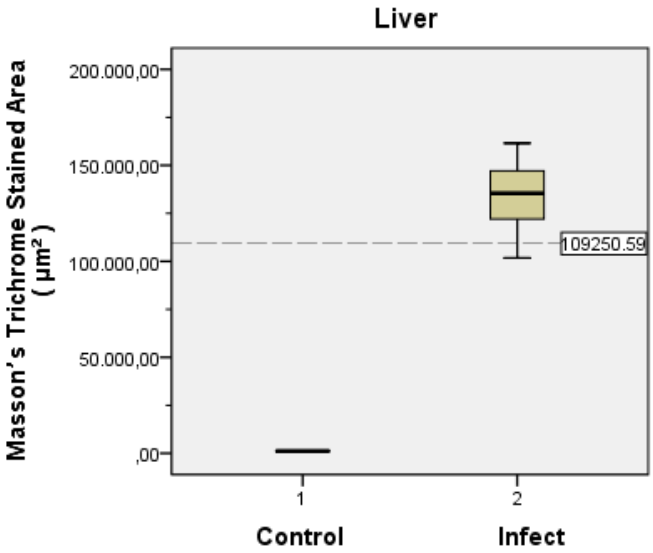


Figure 5. Masson's Trichrome quantitative histochemistry for liver fibrosis. Significant difference ($p < 0.05$). Data obtained by ImageJ bioinformatics (1.49u) and analyzed by SPSS (IBM v. 24) statistic package.

3.4. Inflammation

Evaluation of the inflammation grade in liver and spleen in the infection environment around parasite eggs was assessed by IL-6 IHC.

Microscopic observation revealed the high levels of IL-6 expression in the periovular areas of liver and dispersed on tissue (Figure 6 A and B). Contrariwise, the expression of IL-6 in the spleen presented no differences and the expression of this interleukin its distribution over the tissue was dispersed (Figure 6 C and D).

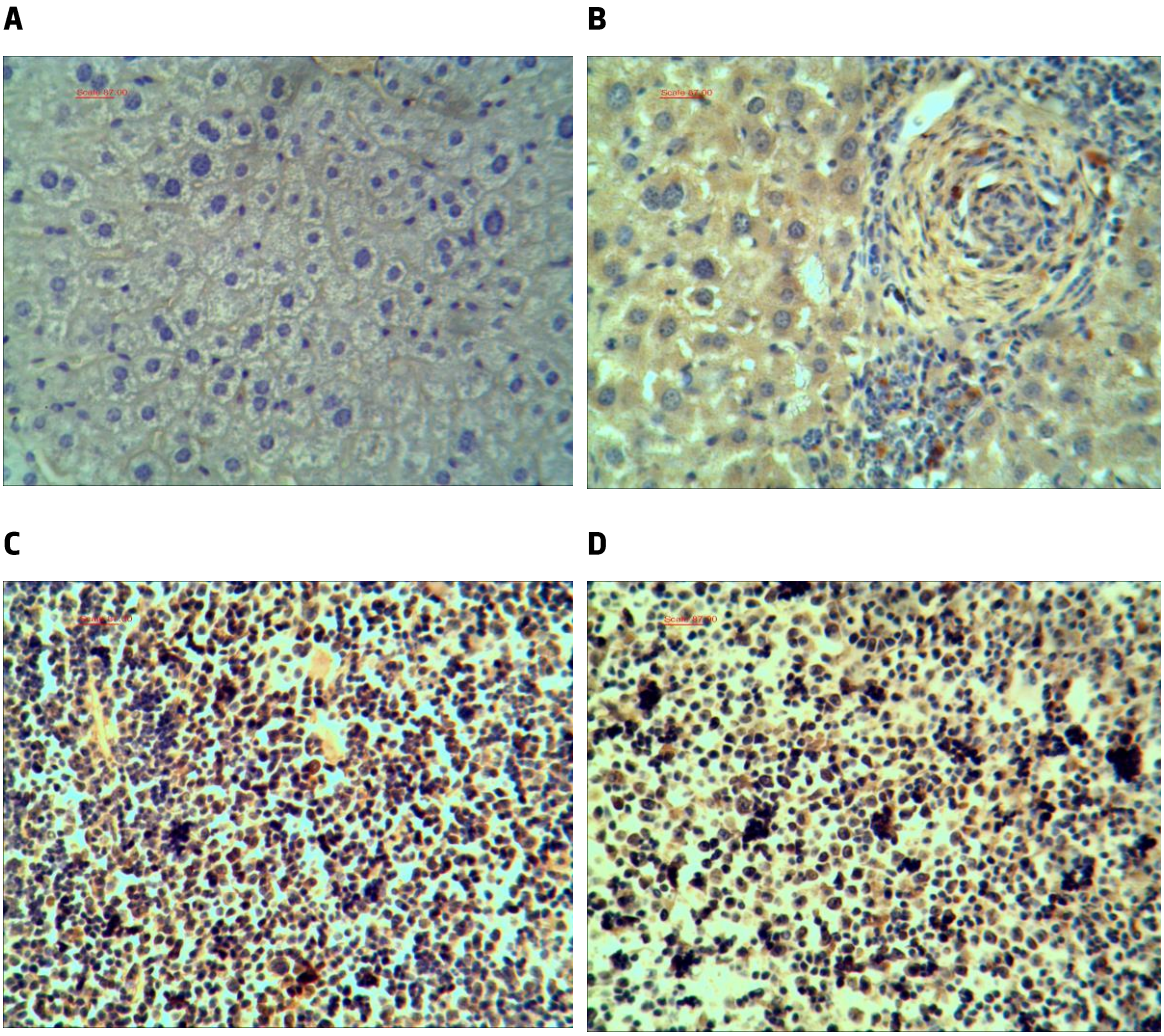


Figure 6. Representative IL-6 expression by immunohistochemistry for liver (A and B) and spleen (C and D). A and C are control tissues. B and D are infected tissues. Amplification 400X. Brownish stain denote IL-6 expression.

After image analysis of IL-6 staining, it was observed that liver presented higher stained IL-6 areas for infected tissue (121352 ± 9163 μm^2), when compared with control (65274 ± 6973 μm^2) with a statistically significant ($p < 0.05$).

Inversely the expression levels of IL-6 in the spleen of infected mice when compared with controls (59076 ± 6934 μm^2 and 65274 ± 6973 μm^2 respectively) presented non-significant differences ($p = 0{,}175$).

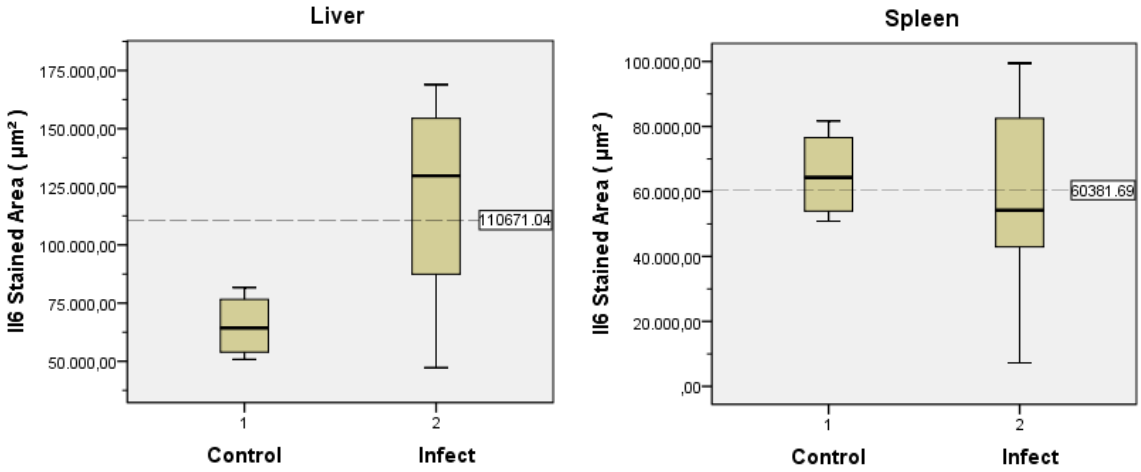


Figure 7. IL-6 quantitative immunohistochemistry for liver and spleen. Significant difference ($p < 0.05$) in liver. Data obtained by ImageJ bioinformatics (1.49u) and analyzed by SPSS (IBM v. 24) statistic package.

3.5. Vascular Biology

The shistosome eggs antigens (SEA) may induce the formation of new vessels. Hereby we present the results of SEA-induced neovascularization, not only in blood vessels but also in lymphatic vessels.

3.5.1. Blood vessels

Blood vessel evaluation in the liver (Figure 8 A and B) and spleen (Figure 8 C and D) was accomplished by CD31 IHC assay. CD31 stain allows the identification of blood vessels endothelial cells and is, for that reason and histological biomarker of blood vessels.

3.5.1.1. CD31 expression in liver and spleen

IHC allowed the image bioinformatics quantification of the CD31 expression in blood vessels both in liver (Figure 8 A and B) and spleen (Figure 8 C and D). It was

observed the stained endothelial cells in both tissues but must of the identified vessels were present in the hepatic tissue accordingly with the expected.

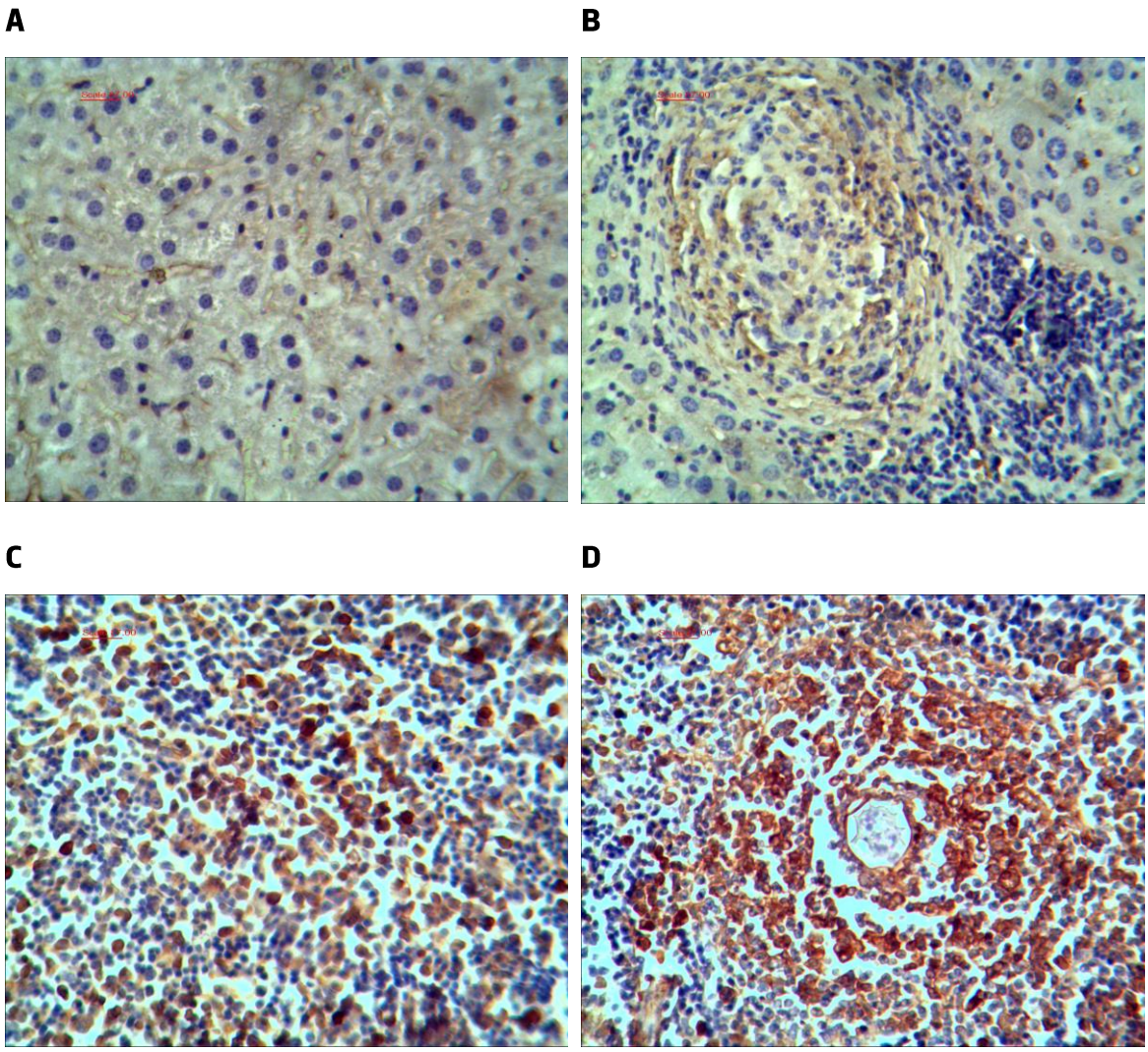


Figure 8. Representative CD31 expression by immunohistochemistry for liver (A and B) and spleen (C and D). A and C are control tissues. B and D are infected tissues. Amplification 400X. Brownish stain denote CD31 expression.

Regarding CD31 expression (Figure 9) in the analyzed tissues it was observed non-significant difference between the infected liver when compared to controls (71246 ± 3780 μm^2 and 68328 ± 3813 μm^2 respectively).

Concerning the spleen, our results demonstrate an increased CD31 expression for the infected tissue when compared to the control (86600 ± 3986 µm2 and 68328 ± 3813 µm2).

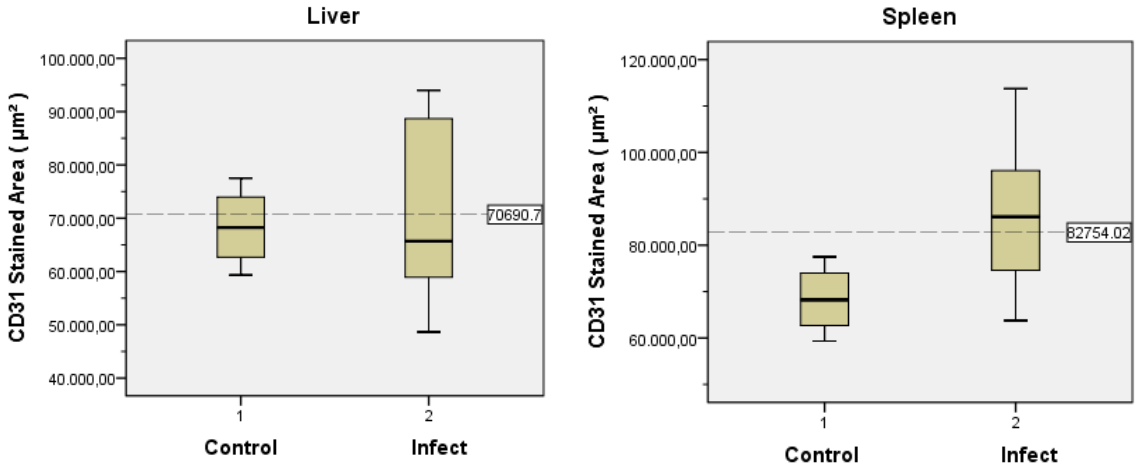


Figure 9. CD31 quantitative immunohistochemistry for liver and spleen. Significant difference ($p < 0.05$) in spleen. Data obtained by ImageJ bioinformatics (1.49u) and analyzed by SPSS (IBM v. 24) statistic package.

3.5.1.2. Microvascular density (MVD)

The number of vessels stained by CD31 IHC allowed to calculate the microvascular density (MVD) in liver in mice. For MVD in infected liver, it was presented a mean value of 333 ± 12 vessels/mm^2. These values when compared to the control, with a mean value 77 ± 19 vessels / mm^2. The MVD was significantly higher ($p < 0,05$) when compared in infected animals when compared to control group. The spleen infected it was presented a mean value of 217 ± 20 vessels/mm2 when compared with control 77 ± 18 vessels / mm2. MVD was significant for $p < 0,05$ but smaller than the MVD of the liver.

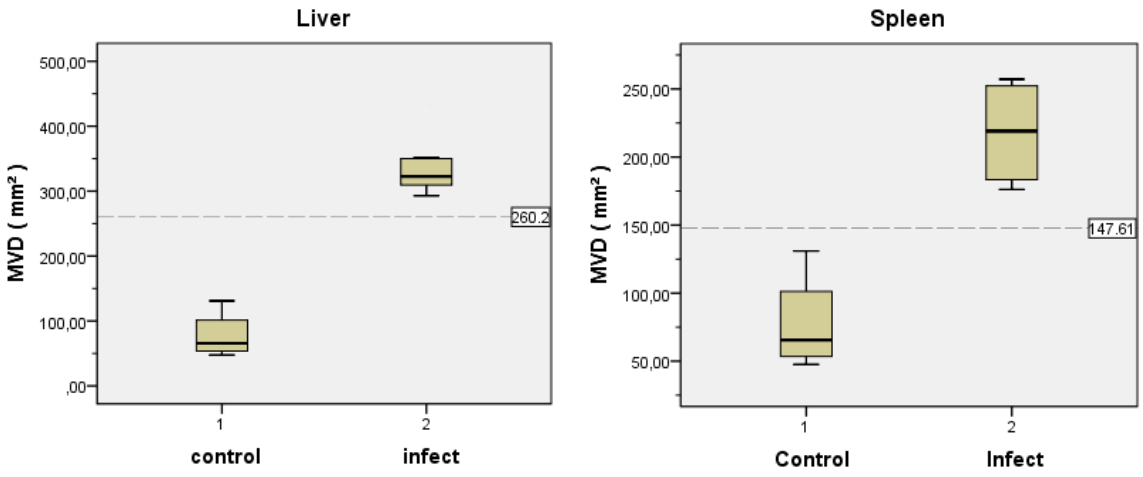


Figure 10. Microvessel density (MVD) for liver and spleen. Significant difference in both tissue ($p < 0.05$) . Data obtained by ImageJ bioinformatics (1.49u) and analyzed by SPSS (IBM v. 24) statistic package.

3.5.2. Lymphatics vessels

Lymphatic vessel assessment was performed by LYVE-1 IHC. Lymphatic endothelial cells hyaluronan receptor 1 (LYVE-1) is considered a lymphatic vessel biomarker since it is expressed in al lymphatic vessel endothelial cells. It is as glycoprotein and it acts as a receptor which binds to both soluble and immobilized hyaluronan. This glycoprotein may function in lymphatic hyaluronan transport. LYVE-1 IHC was performed in both liver and spleen.

In what concerns to the liver, this organ presented a lesser number of lymphatic vessels with a diffuse pattern of distribution in the liver except. However, our results demonstrate that its distribution is more concentrated around the granulomatous areas (Figure 11 B). Regarding to spleen the pattern of distribution demonstrates that lymphatic vessels are disperse between leukocytes (Figure 11 D).

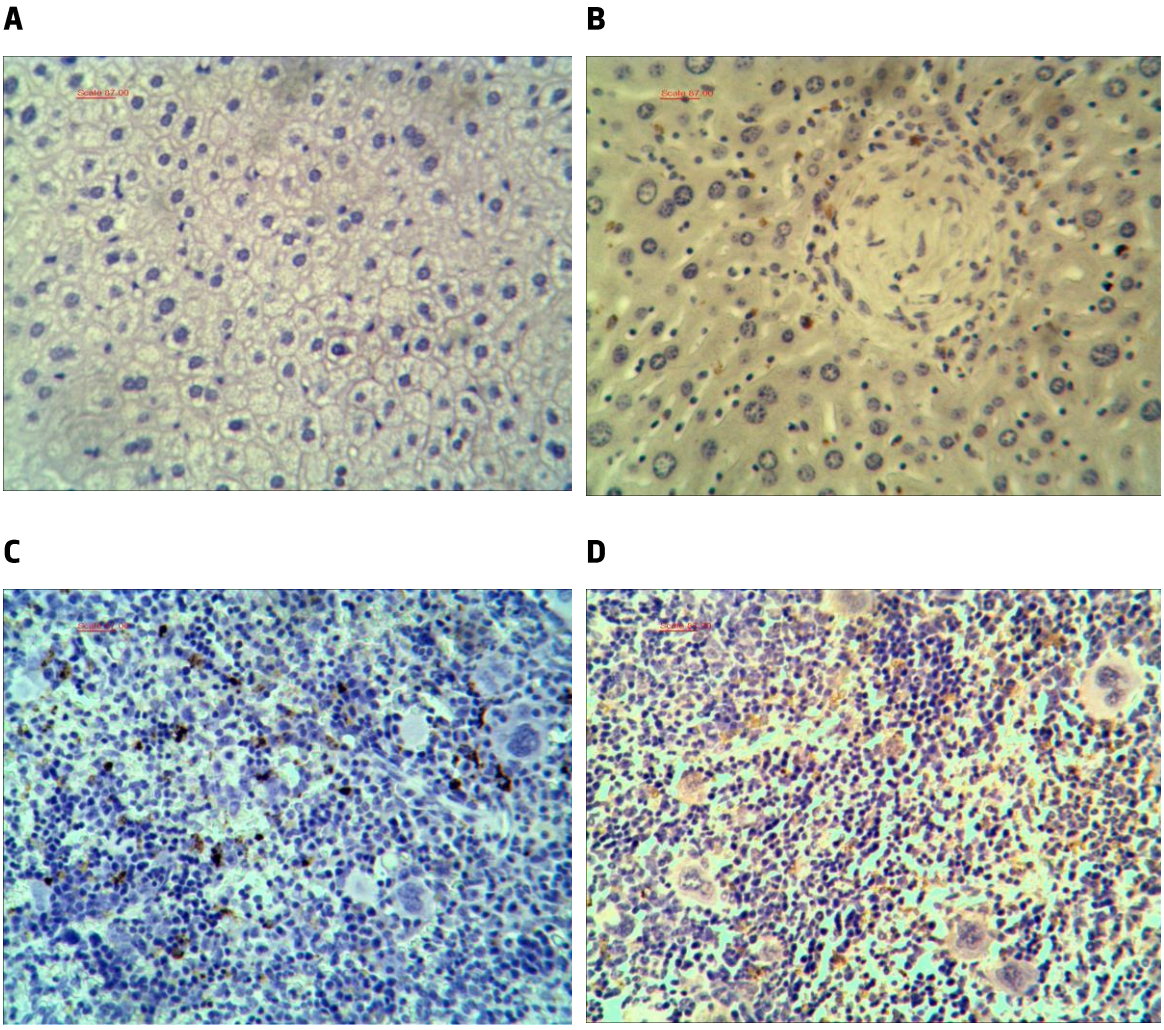


Figure 11. Representative LYVE-1 expression by immunohistochemistry for liver (A and B) and spleen (C and D). A and C are control tissues. B and D are infected tissues. Amplification 400X. Brownish stain denote LYVE-1 expression.

Regarding LYVE-1 expression (Figure 12) the liver presented significantly ($p < 0,05$) lesser expression of marker lymphatic by LYVE-1 in infected mice when compared to controls (1370 ± 119 µm^2 and 1069 ± 7 µm^2 respectively).

Contrariwise, for the spleen, we observed a significant increase ($p < 0,05$) for LYVE-1 expression in infected tissues when compared to the control group (5792 ± 1274 µm^2 and 1069 ± 7 µm^2 respectively).

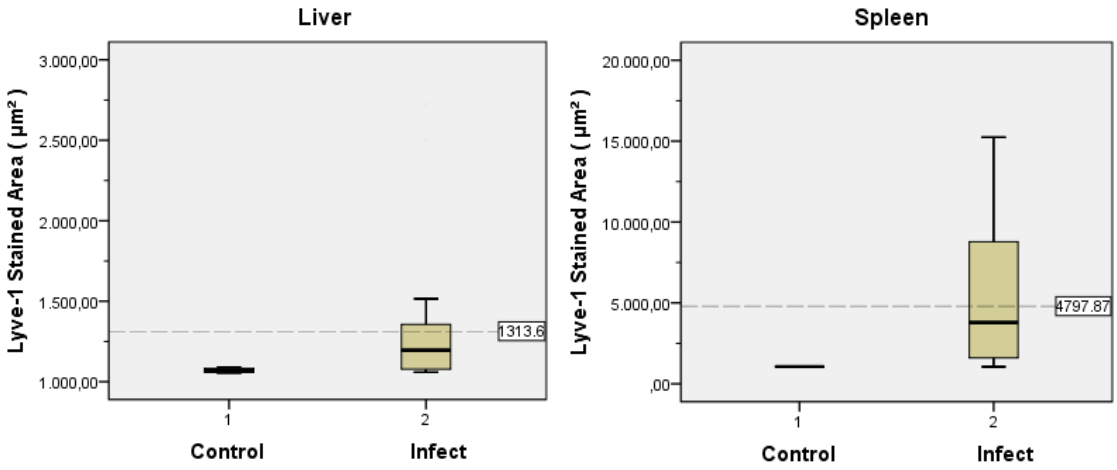


Figure 12. LYVE-1 quantitative immunohistochemistry for liver and spleen. Significant difference ($p < 0.05$) in both liver and spleen. Data obtained by ImageJ bioinformatics (1.49u) and analyzed by SPSS (IBM v. 24) statistic package.

4. Discussion and Conclusion

Schistosomiasis is a tropical disease caused by the flatworm *Schistosoma mansoni*. Immunopathological reactions against Schistosoma eggs trapped in tissues lead to inflammatory and intestinal obstructive disease, hepatosplenic inflammation, and liver fibrosis. Portal hypertension leads ultimately to hypersplenism and cytopenia's, mainly thrombocytopenia and neutropenia (8).

In this work, we measure the fibrous tissue formed around the egg of the parasite by means of Masson's Trichrome. *S. mansoni* infection leads to liver fibrosis and small focal areas of enduring inflammation. Fibrosis immoderations of the extra-cellular matrix are distributed around the periovular granulomas. Liver analysis demonstrates a significant ($p < 0.05$) amount of fibrosis. In endemic areas, liver fibrosis is the outcome of 90% of the infected population. Virtually, the fibrotic response triggers all the chronic-stage liver diseases complications, such as ascites, portal hypertension, synthetic dysfunction, impaired metabolic capacity, and encephalopathy (9).

Schistosomes induce strong Th2 immune response during infection by down-regulation of the Th1 immune response through increased production of Interleukin-6 (IL-6). IL-6 is an immunoregulatory cytokine, secreted by T cells and macrophages to stimulate non-specific immune responses, including fever and acute phase responses in parasitic diseases. The eggs of schistosomes are recognized by host macrophages, which induce the secretion of IL-6 (10). In our study we observed a significant increase in the expression of IL-6 in the liver, it was observed that it presented larger areas of stained IL-6 with a statistically significant result ($p < 0.05$), whereas for the spleen the differences between the two groups were not significant ($p > 0.05$). However, our morphological analysis has demonstrated an elusive lymphocyte infiltration around granulomas (data not shown). These results can be supported by other findings that demonstrate that in this parasitic disease occurs a granulomatous hypersensitivity reaction to parasite eggs entrapped within the intestinal wall and small liver sinusoids that results in an increased inflammation (11).

The shistosome eggs antigens (SEA)-induced neovascularization is promoted due to the resultant inflammatory response. It culminates in the activation of blood

leucocytes, fibroblasts and ECs, whereby the wound healing reaction is coordinated with an earlier angiogenesis and a later fibrogenesis (12).

Through the IHC for CD31 we have study the formation of new vessels by means of the quantitative expression for both tissues in study (liver and spleen) and also have calculate the microvascular density (MVD) in both tissue. As a result, the expression of CD31 in liver was not statistically significant inversely in what occurred in spleen in which the difference between the two experimental groups was significant with an increase of expression in spleen of the infected mice. Nevertheless, the number of blood vessels in infected liver was greater than in the spleen. The result of MVD was significant for both tissues ($p<0.05$) in infected mice supporting the idea of the need for neovascularization as a support for the development or repair of granuloma.

Lymphatic vascularization was measured by LYVE-1 expression. Besides it role in lymphatic hyaluronan transport LYVE-1 also plays a role in tumor metastasis (13) and in infection dissemination by viruses, bacteria and even parasitic worms, such as the HIV, streptococci and filarial parasites among others (14, 15, 16). This a cell surface receptor, on lymphatic endothelial cells can be used as a lymphatic endothelial cell marker. Through the IHC assays, we evaluated the expression of LYVE-1 in lymphatic vessels in liver and spleen of mice. The results obtained for both tissues were significant when compared the experimental mice groups, infected and control. Also, differences in expression were also significant between the two organs (liver and spleen) where the infected spleen presented significant higher CD31 expression than infected liver. Both results point out the need for the immune system to act on infection caused by *S. mansoni* eggs, aiding in the cellular diffusion between tissues – regulating tissue fluid homeostasis, immune cell trafficking and inflammatory responses caused by infection.

In our study, we demonstrated the relationship between *S. mansoni* infection and neovascularization. Understanding the role of the vessels in the dissemination of the shistomese parasites as well as the immune and repair response will enlighten us with to potential therapeutic targets and new insights to better control of schistosomiasis.

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Chapter IV

Central energetic hepatic metabolism in murine schistosomiasis mansoni (Short communication)

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1. Introduction

The liver, plays a crucial role of maintaining the energy potentials in the body, is one of the major target organs affected by schistosomiasis, and therefore infection with schistosomes may result in hepatic disorders and metabolic disturbances.

Schistosomes are facultative anaerobes deriving energy primarily through the degradation of glucose and glycogen. The Cellular glycogen level, is a major source of glucose moieties (1).

In Schistosoma infection, a granulomatous response is observed with active participation of macrophages in response to inflammation triggered by the parasite. Macrophages utilize glutamine, as well as glucose, at high rates. Metabolic pathways essential for macrophage function utilize glucose to generate (i) ATP in the pathways of glycolysis and mitochondrial oxidative phosphorylation, (ii) glycerol 3-phosphate for the synthesis of phospholipids and triacylglycerols, (iii) NADPH for the production of reactive oxygen species (ROS) and (iv) ribose for the synthesis of RNA and subsequently production and secretion of protein mediators (e.g. cytokines) (2). In helminth infections, macrophages are an important source of IL-10; is known to be critical in maintaining the balance between a strong prophylactic immune response and limiting immune-mediated pathology during many diseases caused by parasitic protozoa and helminths (3).

Hepatic stellate cells (HSC) and fibroblasts are considered to be the major source of fibrogenic cells in response to chronic liver injury, while fibroblasts play an important role during cholestatic liver diseases, a complex network of autocrine/paracrine fibrogenic signals promotes the activation, usually called transdifferentiation, of quiescent HSC to a myofibroblastic phenotype. This fibrogenic inputs include cytokines, chemokines, growth factors, lipid mediators and reactive oxygen species (ROS) that are produced by epithelial cells (hepatocytes and cholangiocytes), endothelial cells and cells of the immune system (macrophages, dendritic cells, and B and T lymphocytes) (4).

In Schistosomiasis, an important consequence of liver injury is stimulated glycolysis, which is manifested by a reduction in the levels of plasma glucose, liver glucose, and glycogen. Glycogen degradation and replenishment occur through the body of the parasite, confirming inhibition of aerobic respiration and stimulation of anaerobic glycolysis through hexokinase, a rate-limiting enzyme of glycolysis (5). This metabolism of glucose through glycogen degradation should help in maintaining a low internal free glucose concentration and thus promote sufficient glucose diffusion to deeper tissues. Glucose metabolism leading to ATP synthesis is critical for the survival of schistosomes. The mechanisms required for schistosomes to take up glucose, the major nutritional source exploited by these blood flukes from their mammalian hosts and the subsequent metabolism required to fuel growth and fecundity, can provide new avenues for developing novel interventions for the control of schistosomiasis (6).

This study, aims to demonstrate the relationship of *S. mansoni* eggs infection in the liver of mice, evaluating and quantifying the levels of glycogen presented in tissue by Periodic Acid Schiff technique (PAS).

2. Materials and Methods

2.1. Animal Experiments

This experiment followed the guidelines and recommendations of FELASA (Federation of European Laboratory Animal Science Associations) and the European Directive 2010/63/EU related to animal protection in scientific studies were followed, especially the reduction principle of the 3Rs for animal experiments.

Six-week-old CD-1 mice were provided by Charles River (Barcelona, Spain). Animals spent 1 week being acclimated under routine laboratory conditions before starting the experiments. They did not receive any treatment prior to the study.

They were fed standard balanced food and water ad libitum. All the animals were maintained at the National Institute of Health (Porto, Portugal) in rooms with controlled temperature (22% ± 2°C) and humidity (55% ± 10°C) and continuous air renovation. Animals were housed in a 12 h light/12 h dark cycle (8 am–8 pm). All animal experiments were performed in accordance with the National (DL 129/92; DL 197/96; P 1131/97) and European Convention for the Protection of Animals used for Experimental and Other Scientific Purposes and related European Legislation (OJ L 222, 24.8.1999).

2.2. Experimental infections

Twelve 12 CD-1 mice were experimentally infected with 50 cercariae *S. mansoni*, respectively. Hamsters and mice were infected by member's extremities and tail immersion, respectively. The control animals consisted of 10 littermates. The cercariae were obtained by shedding of snails infected with miracidia.

2.3. Periodic Acid Schiff (PAS)

Sections from liver were stained for PAS, used for detection of glycogen in tissues. The tissue was oxidized in 0.5% periodic acid solution for 5 minutes, placed in Schiff reagent for 15 minutes (until sections become light pink color), washinged in lukewarm tap water for 5 minutes and counterstain in Mayer's hematoxylin for 1 minute. To comparate the level of glycogen in the tissue studied by the PAS technique, in this case, was added 500 µl of ∝-amylase solution in liver before the addition of PA and Schiff reactive. This incubation was performed for 20 min in a 37 °C prewarmed chamber. After staining, the structure is revealed as following: polysaccharides will be stained in purple/dark, purple in a light rose background (cytoplasm). Nuclei are contrasted with hematoxylin (blue).

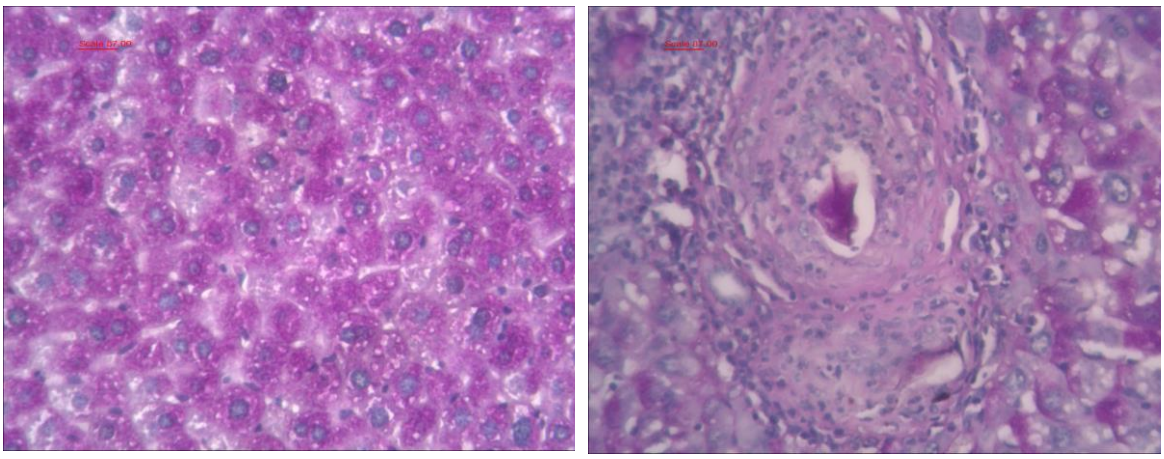
3. Results

3.1. Glycogen depletion

Evaluation of the glycogen level in liver in the context of infection of the parasite eggs was assessed by PAS. The PAS after diastase with salivary ∝-amylase was used to distinguish between glycogen present in liver and other polysaccharides such as mucopolysaccharides present cells. Microscopic observation revealed the presence of staining purple-magenta in great quantity in liver control tissue (Figure 13 A) when compared with infect (Figure 13 B) tissues.

The PAS after diastase demonstrates the ∝-amylase activity was observed on glycogen (Figure 13 C control and D infect).

A **B**



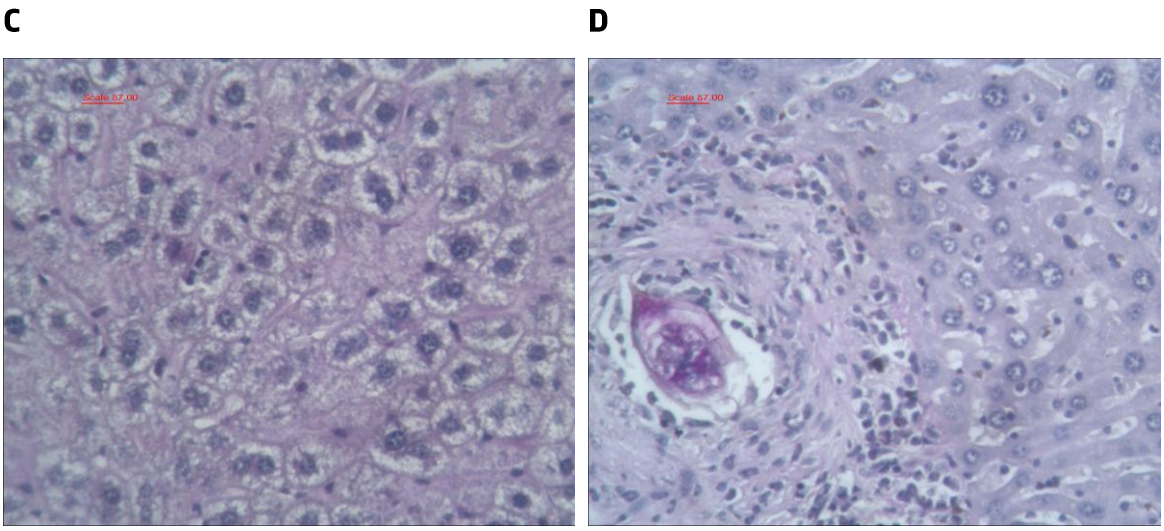


Figure 13. Representative images of Periodic Acid-Schiff and PAS after Histochemistry. Liver control (A and C) and infected (C and D); sections taken at 400X amplification.

The (Figure 14 A) show us a large difference in glycogen levels when compared the control and infected liver. The amount of PAS-positive material for the control group was 161490 ± 12913 μm^2 without diastase and 1295 ± 145 μm^2 after diastase with α-amylase. The decrease of PAS-positive material was significant (p<0,05). The difference between the two values refers to glycogen.

In same manner, the amount of PAS-positive in infected mice was 23312 ± 9660 μm^2 without diastase and 1232 ± 160 μm^2 after diastase. Likewise, the decrease of PAS-positive material was significant (p<0,05). in the infected group besides being so accentuated than in the infected control group.

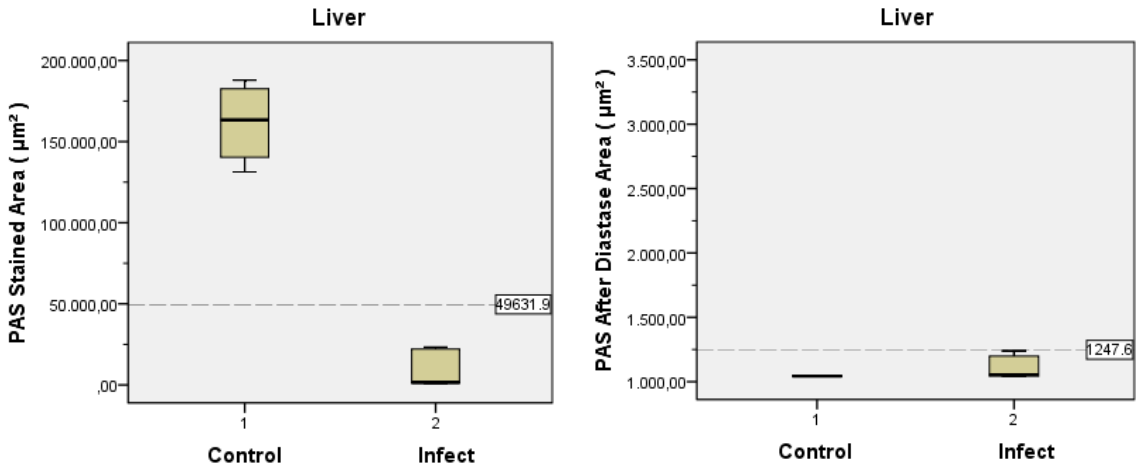


Figure 14. Evaluation of glucose by histochemistry of sections from livers of mice. $p < 0.05$ for technique PAS significance and $p < 0.05$ for application of amylase. The values for control in comparison is $p < 0.05$ and infected when compared $p < 0.05$. The values are represented as the mean ± SEM.

For that reason, we assumed the amount of glycogen is the difference between PAS-positive material before and after diastase with salivary α-amylase. That amount may be calculated by the following expression:

$$\Delta c(\boldsymbol{glycogen}) = \boldsymbol{M1c - M2c} \qquad \Delta \boldsymbol{i}\ (\boldsymbol{glycogen}) = \boldsymbol{M1i - M2i}$$

were,

Δc and Δi refers to the amount of glycogen in both control and infected group respectively, and M1 and M2 refer to amount of PAS positive material before (M1) and after (M2) digestion of α-amylase.

The amount of glycogen content liver between of the control is 160195 ± 12768 µm² and the amount of liver glycogen 22080 ± 9500 µm² (Figure 15). This is a highly distinct amount of glycogen in the liver of both control and infected groups. Within infected mice the amount of glycogen in significantly lower (p<0,05).

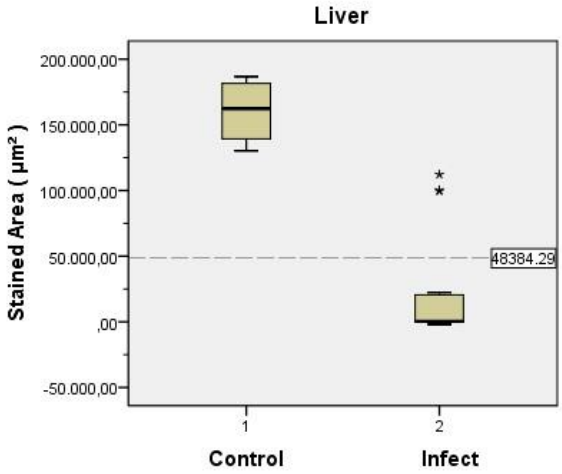


Figure 15: Quantification of glycogen in Liver by technique PAS more PAS diastase in mice, whit significance the of $p < 0.05$ for the amount of glycogen in between the tissues. The values are represented as the mean ± SEM.

4. Discussion and Conclusion

The glucose is cleared from the bloodstream primarily by conversion to glycogen in skeletal muscle and liver. Glycogen is a readily mobilized storage form of glucose it is a very large, branched polymer of glucose residues that can be broken down to yield glucose molecules when energy is needed (7).

Studies to determine the level of serum glucose have revealed significant changes in the glycogen stocks of animals infected by *S. mansoni*, observed accentuated metabolic changes in hamsters co-infected by *Schistosoma japonicum* and *Necator americanus* under experimental conditions, with significant decreases in the levels of glucose, succinate, citrate and amino acids in the plasma of the co-infected animals compared to the control groups (8).

For that reason, we evaluated the levels of glycogen stocks in the liver of mice, where we obtained significant results that support the previous idea. When we compared the infected livers to the uninfected liver, we obtained a significant reduction of the glycogen reserve in hepatocytes in infected tissues, and the result was statistically significant (P <0,05). In schistosomiasis mansoni, hepatic alterations are more important manifestations, animals with infection presented hypoglycemia with values close to malnourished without infection, which demonstrate a reality of infection or malnutrition in plasma glucose levels. Both malnutrition and infection cause depression in blood sugar levels (9).

Decreased hepatic glycogen may be involved in the process of release of substrates (glucose) for the defense cells from the immune system, where macrophages play a key role in combating parasite, or even in the production of fibroblasts involved in the fibrosis process, or even for the parasite itself, in the breeding process and source of energy.

However additional studies are needed to understand the processes that lead to decreased Glycogen in the liver.

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Chapter V

Summarizing Discussion and Concluding Remarks

1. Conclusion and Discussion

Cancers are characterized by uncontrolled growth of abnormal and transformed cells, which can invade adjacent tissues. Cancer may be induced by many environmental and physiological conditions. Infections with viruses, bacteria and parasites have been recognized for years to be associated with human carcinogenicity. The helminth diseases schistosomiasis, are highly carcinogenic (1).

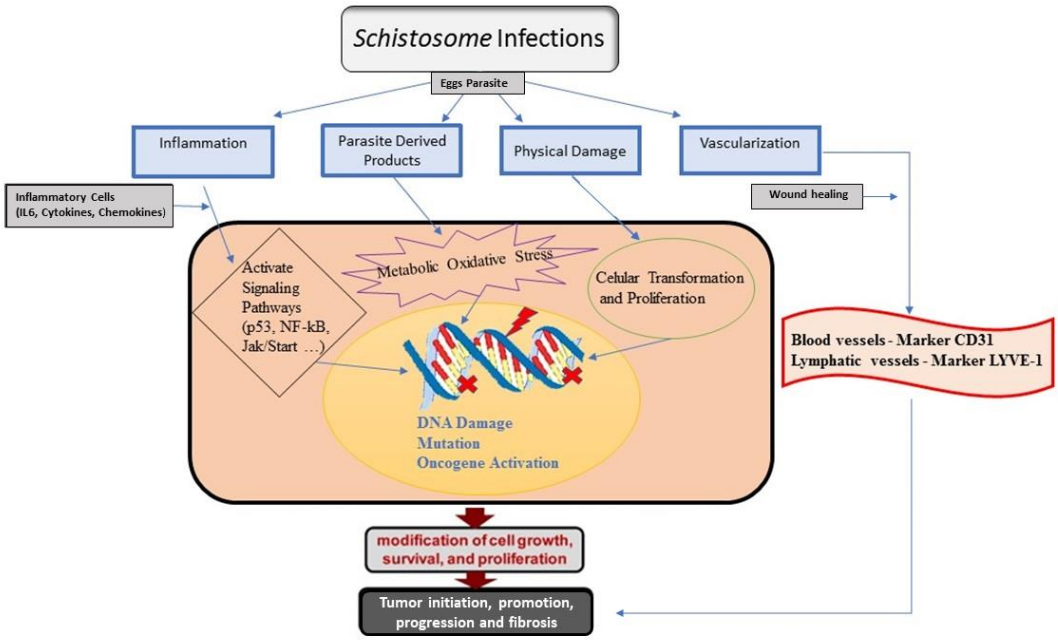

Figure 16. Proposed mechanisms of carcinogenicity and disease induced by infection: *Schistosoma* species.

Infections, in general, can initiate or promote carcinogenesis by three main mechanisms: (a) chronic inflammation due to prolonged persistence of an infectious agent in the host promotes release of reactive oxygen and nitrogen species (ROS and RNOS, respectively), which have the potential to damage DNA, proteins, and cell membranes, and modulates enzyme activities and gene expression, which can cause carcinogenesis; (b) insertion of oncogenes into the host genome, as in hepatitis B and hepatitis C, inhibition of tumor suppressors, or stimulation of mitosis; and (c) induction of immunosuppression and consequently a reduction in immune surveillance. Parasitic infections that initiate or promote neoplasia usually do so by mechanism one (1).

In this study, we evaluated in liver and spleen the inflammatory status, blood and lymphatic microvasculature and the level of glycogen in both tissues of mice. Concerning the inflammatory status, it was found that the liver presented augmented levels of the pro-inflammatory IL-6 in mice which had a large amount of granulomas present in their tissue, when compared to the spleen. This result came in agreement of what happens in the infection, a proinflammatory response is observed both at the transcriptional level and at the protein level by cytokine and chemokine bead assay. Key genes observed elevated transcription in response to the addition of SEA included: IL1-α and IL1-β, IL-6, all associated with inflammation, as described by (2).

In our study, a high blood microvascular density was observed in the liver of mice infected by *S. mansoni* when compared to the control. In addition, we observed in spleen a great expression of CD31 and LYVE-1 in both tissue. In the liver, we observed substantial amounts of eggs in tissue, forming granulomas in the hepatic tissue, Experimental studies have demonstrated the occurrence of angiogenesis, the formation of blood vessels of pre-existing vessels, in the early stage of granuloma formation and during the progression of fibrosis in chronic hepatic schistosomiasis (3).

In the study, it is observed a better expression of LYVE-1 in the spleen when compared to the liver. Other studies have related several lymphangiogenic factors, such as vascular endothelial growth factors (VEGFs), in the development lymphatic (4).

The formation of new vessels from infection becomes important for the control and release of cytokines produced by schistosomiasis eggs.

In infected tissues, it observed a lower availability of glycogen present when compared to controls, which is expected, since glucose in the metabolic process becomes an energetic source.

In conclusion, infection caused by *S. mansoni* involves several processes mediated by the release of cytokines produced by eggs, in our study, we demonstrated the infection ratio in the development of fibrosis and neovascularization caused by *S. mansoni.*

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Chapter VI

Future Perspectives

Future Perspectives

To better understand the mechanisms involved in the infection caused by *S. mansoni* that affect liver and spleen in terms of inflammation and vasculature, it will be necessary to perform new experiments to:

a) To evaluate the expression of molecules associated to inflammation and activation, such as the TNF-α, IL4, and IL-5;

b) To study lymphangiogenic factors as VEGF-C and VEGF-D play a vital role in lymphangiogenesis;

c) To evaluate anti-fibrogenic cytokines such as pro-fibrogenic factors in the formation of fibrosis in periovular granulomas;

Glycogen in the liver is an important energy reserve, where observed a significant reduction in glycogen storage in hepatocytes in infected tissues, further experiments are required to:

d) To establish a relationship between infection and consumption of glycogen in the liver.

e) To study liver pathways involved in glucose catabolic metabolism and uptake by means of PFKFB3 and GLUT2 expression respectively.

In a better perspective, in the future, to better understand the angiogenic and lymphangiogenesis process caused by infection by *S. mansoni*, is necessary so that we can understand how they can influence in the inflammatory and fibrotic process produced by the presence of the egg in the liver and spleen. Furthermore, the need for discovery research will be paramount in order to refine existing and develop new tools and strategies for the control of schistosomiasis.