Schistosoma haematobium infection in the female genital mucosa

Immunohistochemical and clinicopathological analyses with respect to HIV target cells and vascularity in cervicovaginal tissue.
Cross-sectional studies in Malawi and Madagascar.

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Thesis for the degree of Philosophiae Doctor, PhD

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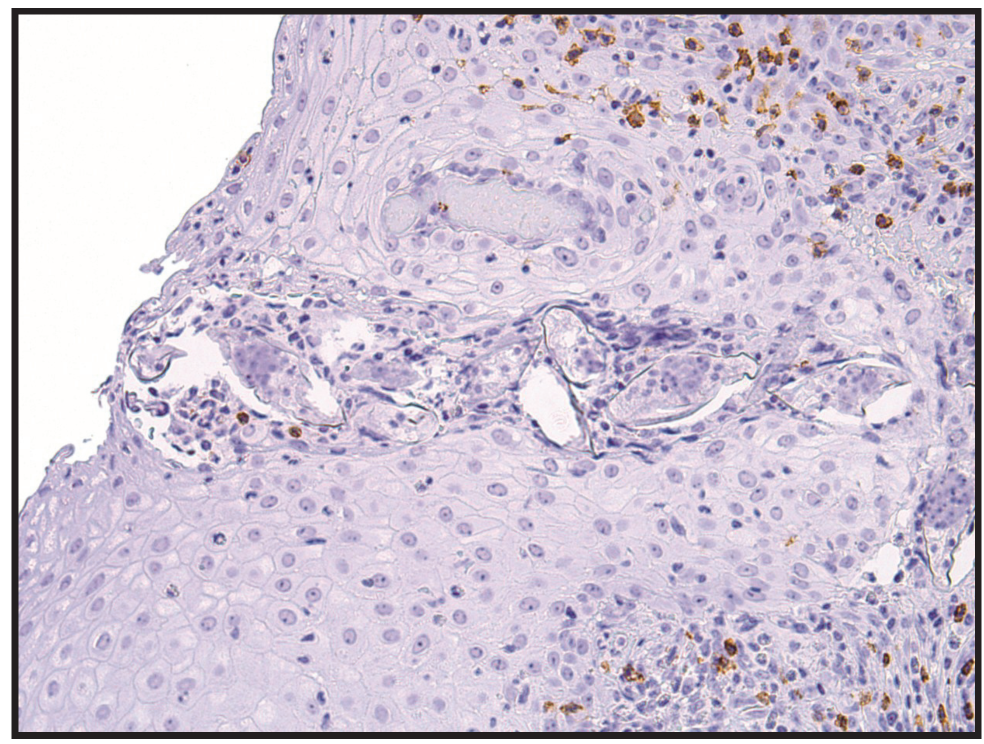

Schistosoma haematobium ova exiting the tissue through the cervical mucosal membrane.

All photographs: Peter M. Jourdan.

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List of publications

Paper I

Increased vascularity in cervicovaginal mucosa with *Schistosoma haematobium* infection

Jourdan PM, Roald B, Poggensee G, Gundersen SG, Kjetland EF.

PLoS Negl Trop Dis. 2011 Jun;5(6):e1170

Paper II

HIV target cells in *Schistosoma haematobium*-infected female genital mucosa

Jourdan PM, Holmen SD, Gundersen SG, Roald B, Kjetland EF.

Am J Trop Med Hyg. 2011; 85(6):1060–1064

Paper III

Pathological mucosal blood vessels in active female genital schistosomiasis

New aspects of a neglected tropical disease

Jourdan PM, Randrianasolo BS, Feldmeier H, Chitsulo L, Ravoniarimbinina P, Roald B, Kjetland EF.

Int J Gynecol Pathol. 2012, in press

Paper IV

A simple method for precise quantification of immunohistochemically stained cells

Jourdan PM, Holmen SD, Kjetland EF, Sandvik L, Roald B.

Submitted

Definitions and abbreviations

Capillary bud	CD31 immunostained endothelial cell structure without a visible lumen or peri-endothelial structures
CCR5	Chemokine receptor, also a co-receptor for HIV
CD	Cluster of differentiation
CD31	Immunohistochemical marker of endothelial cells
CD3	Immunohistochemical marker of T cells
CD4	Immunohistochemical marker of CD4+ T cells, and main receptor for HIV
CD68	Immunohistochemical marker of monocyte-derived cell; monocytes and macrophages
CD8	Immunohistochemical marker of CD8+ T cells
CD4+ T lymphocyte / cell	T cell expressing CD4 receptor on the surface
Computer-assisted image analysis	Digital quantification of image components
Contact bleeding	Mucosal bleeding by only slight contact
CXCR4	Chemokine receptor, also a co-receptor for HIV
Cytotoxic T cell	CD8+ T cell with the capacity to destroy human cells infested with intracellular pathogens
Delayed hypersensitivity (type IV)	A type of T cell-mediated immune response
Dendritic cell (DC)	A “guardian” cell of the innate immune system
Ectocervix	The vaginal portion of the uterine cervix
Endocervix	The canal connecting the vagina with the uterine cavity
Endothelial cell	Cell that lines the inner surface of a blood vessel
Eosinophil granulocyte	One of the main effector cells in the immune response to schistosomiasis
Established blood vessel	vWF-immunostained vessel structures other than buds
Female genital schistosomiasis	Schistosome ova in female genital tissue
Female genital tract	Ovaries, tubes, uterus including the cervix, and vagina
Granulation tissue	Tissue reaction with capillary buds, activated endothelial cells and immature fibroblasts
Granuloma	Histopathological reaction to a foreign body such as ova; clusters of immune cells, especially activated monocytes
HE	Haematoxylin and eosin; standard stain for microscopic examination of human tissue
Histopathology	The scientific study of tissue by light microscopy

HIV	Human immunodeficiency virus
HIV target cells	The (CD4+) cells susceptible to HIV infection
HIV transmission	The transfer of HIV infection between individuals
HPF	High-power field; the microscopic area viewed through objective lens 40
HPV	Human papillomavirus
IL	Interleukin
Immunohistochemistry	Protein detection by use of immunological markers for analysis of cells and tissue structures
Langerhans cell	Dendritic cell in the mucosal membrane and skin
Macrophage	Monocyte-derived phagocytic cell (literally big eater)
Morphological	Here with regard to histopathological findings
Mucosa	The epithelial lining of inner surfaces, such as the alimentary, urinary and genital tracts
Ovum (ova)	Egg(s); here parasite egg(s), which may be viable or non-viable / calcified
Plasma cell	Mature B cell capable of producing antibodies
Pseudotubercle	Term previously used for granuloma (see this term)
SEA	Soluble egg antigen
S100 (protein)	Immunohistochemical marker of several cell types, among others dendritic cells
Sandy patch	Sandy looking mucosal area with or without distinct grains; a typical sign of *S. haematobium* infection
Schistosoma haematobium	Parasite that commonly infects the female genitals
Rubbery nodule	Pustoloid, firm and beige nodule in the mucosa, associated with *S. haematobium* infection
Schistosomiasis	Disease caused by infection with schistosomes
T lymphocyte / cell	Pivotal immune cell of the innate immune system
Th1 / Th2 cell	Subsets of CD4+ T lymphocytes
Transformation zone	The transitional zone between the squamous and columnar epithelium of the cervical portio; the main area of colposcopic examination with regards to cancer
Urogenital	Which affects the urinary and/or genital tract(s)
vWF	von Willebrand Factor; immunohistochemical marker of established blood vessels
WHO	World Health Organization

1.0. Summary

Schistosomiasis is, after malaria, the most important parasite disease in terms of public health impact. *Schistosoma haematobium* infects millions of people through fresh water contact, especially in sub-Saharan Africa. Recent knowledge of the genital manifestations of schistosomiasis and the possible association with human immunodeficiency virus (HIV) infection, has led to increased focus on this common but neglected tropical disease. Anti-schistosomal treatment could potentially become an intervention point against HIV transmission. It is therefore imperative that the possible mechanisms by which female genital *S. haematobium* infection might influence HIV transmission is explored.

S. haematobium infection is a frequent cause of mucosal pathology in the female genital tract. To our knowledge, this is the first study to perform systematic immunohistochemical analyses on *S. haematobium* infected female genital mucosa. This thesis presents the results of up-to-date immunohistochemical and image analyses of periovular mucosal blood vessels and HIV target cells, and of clinicopathological correlates in women with cervicovaginal *S. haematobium* infection in two cross-sectional studies in Malawi and Madagascar.

In line with previous reports, we found a variety of tissue reactions to *S. haematobium* ova. Female genital mucosa infected with *S. haematobium* contained a higher density of HIV target cells, i.e. CD4+ T lymphocytes and CD68+ macrophages than uninfected mucosa. The infected genital mucosa was significantly more vascularised compared to healthy tissue. Clinicopathological analyses suggest that characteristic, abnormal mucosal blood vessels might be suggestive of a persistent tissue reaction to female genital *S. haematobium* ova, and that thrombosis might contribute to the pathogenesis. Finally, we developed a simple model for precise and efficient computer-assisted image analysis of immunostained tissue.

In conclusion, the findings show that *S. haematobium* infected female genital mucosa may contain a higher density of HIV target cells and blood vessels than uninfected mucosa. Pathological changes in both acute and chronic tissue reactions to genital mucosal *S. haematobium* infection could facilitate cervicovaginal HIV transmission in women. Further studies are needed to explore the findings, and to provide clinical advice on the risks and effect of anti-schistosomal treatment on female genital schistosomiasis.

2.0. Background

2.1. Schistosomiasis

2.1.1. History

The symptoms of schistosomiasis had been known for centuries when *Schistosoma haematobium* was first discovered by the German physician Bilharz in Egypt in 1851 [1]. Symptoms and signs of schistosomiasis have been found in Egyptian papyrus fragments as early as 1900 BC [2]. These descriptions have later been confirmed by the detection of *S. haematobium* ova in the kidneys of Egyptian mummies [3], and more recently of *S. mansoni* and *S. japonicum* in Egyptian and Chinese mummies, respectively [4,5].

In the beginning of the 20th century, *S. mansoni* and *S. japonicum* were distinguished from *S. haematobium* [6,7], followed by identification of the intermediate hosts; the fresh water snails, and thus the parasites' life cycles [8]. The parasite and disease were named *Bilharzia* and bilharziasis, respectively, until 1954, when the international nomenclature was changed to *Schistosoma* and schistosomiasis [2]. However, both terms are still commonly used.

2.1.2. Epidemiology

Schistosomiasis is, next to malaria, the second most important cause of parasitic disease burden in terms of public health impact [9,10]. Close to 800 million people are currently exposed to schistosomiasis, and more than 600 million of these live in Africa [9,11]. Estimates indicate that more than 200 million Africans are infected; however, probably less than 15% of these are treated for the disease [12].

Five main schistosome species may cause disease in humans: *S. haematobium*, *S. mansoni*, *S. japonicum*, *S. intercalatum* and *S. mekongi* [13-16]. The geographical distribution of the various schistosome species follows the distribution of the respective intermediate hosts (see 2.1.3. Life cycle) [17,18]. *S. haematobium* is found in Africa and the middle East, *S. mansoni* in Africa, the Caribbean, South America and the middle East, and *S. japonicum* in China, Indonesia and the Philippines (Figure 1) [17]. *S. intercalatum* and *S. mekongi* are found in limited areas of sub-Saharan Africa and Southeast Asia, respectively [17]. Man-

made ecological changes such as irrigation and the migration of infected populations further contribute to the epidemiology of schistosomiasis [18,19].

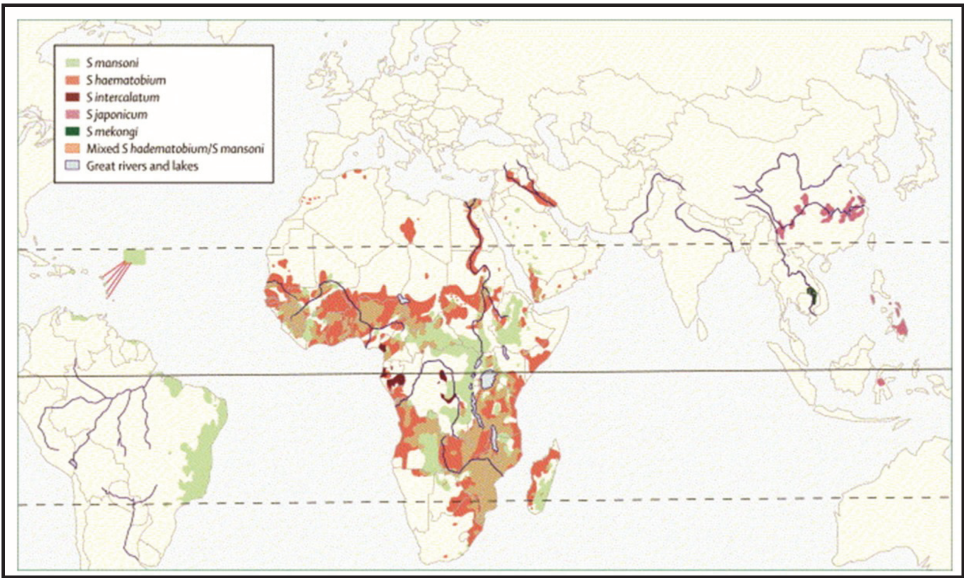


Figure 1. The global distribution of schistosomiasis. **(Reprinted with permission from Elsevier and courtesy of Professor B. Gryseels).**

2.1.3. Life cycle

The main features of the life cycle are similar for the various schistosome species that cause disease in humans (Figure 2). Infection may be initiated when people through leisure or occupational activities, or otherwise come in skin contact with fresh water infested with schistosomal cercariae; the infectious larval stage of the parasite [13]. In case of infection, the cercariae penetrate the skin, develop into schistosomula, gain access to the blood stream and end up in the portal vein of the liver. Following sexual maturation, the pair of female and male schistosomes migrates against the blood stream to the site of ova deposition.

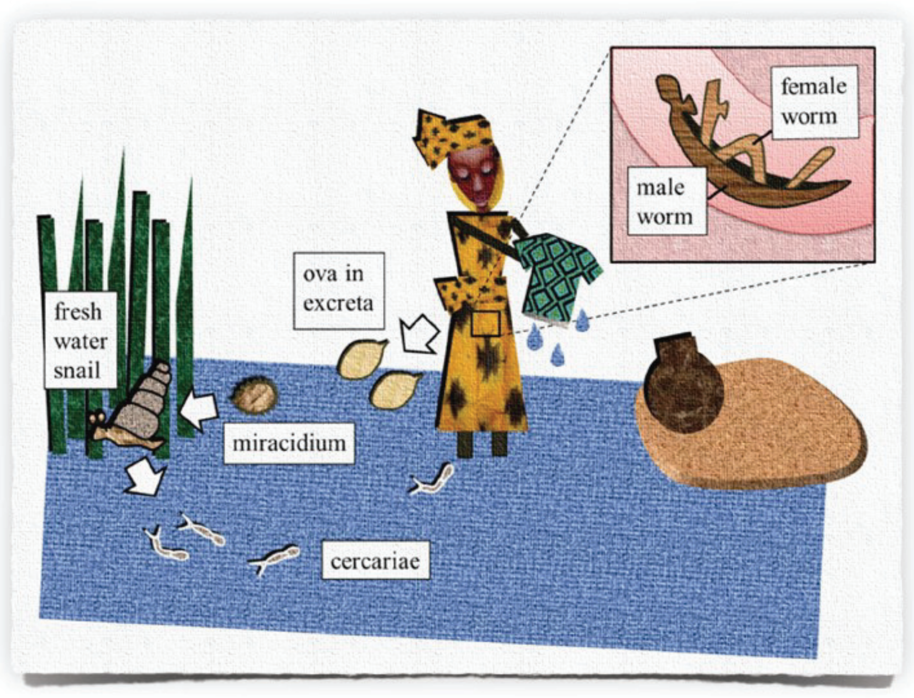


Figure 2. The life cycle of Schistosoma haematobium. The main aspects of the schistosome life cycle are similar for the species that infect humans. The snail, cercariae, ova, miracidium and worms have been enlarged for the sake of clarity.

The female *S. haematobium* and *S. intercalatum* worms lay oval ova with a terminal spine, *S. mansoni* produce ova with a lateral spine, and *S. japonicum* and *S. mekongi* lay round ova with only a minute spine. For reasons still unknown, each species tends to home to a more or less specific venous plexus of the human body; *S. haematobium* worms tend to prefer the urogenital venous plexus, whereas the other species prefer the venous plexus of the intestinal tract [13]. A number of anastomoses exist between the pelvic venous plexuses, possibly affecting the final destination of the schistosome worms and ova [20].

The intravascular ova traverse the wall of the venule to enter the stroma. The ova secrete substances which lead to disintegration of the tissue, allowing them to pass through the stroma and reach the lumen of the organ in which they are deposited; frequently the lumen of the urinary bladder or the colon. A number of ova are frequently retained in the tissue stroma, and are the main cause of pathology in schistosomiasis (see 2.1.5. Histopathology).

Mature ova are excreted from the body mainly through urine (*S. haematobium*) or faeces (*S. mansoni* and *S. japonicum*). It is not yet clear whether the female genital tract also constitutes a route of transmission [21]. The ovum hatches when it comes in contact with water, liberating a miracidium. To complete the cycle, the miracidium must penetrate a fresh water snail (intermediate host) and develop into cercariae that are shed into the fresh water. Each species has its own specific snail host, which explains why the disease follows the distribution of the respective snails [13].

2.1.4. Clinicopathology of urinary schistosomiasis

The World Health Organization (WHO) has recently recommended that disease caused by *S. haematobium* should be called urogenital schistosomiasis [22]. Disease caused by *S. mansoni* or *S. japonicum* is known as intestinal schistosomiasis [12]. This thesis will focus on the description of clinical features of urogenital schistosomiasis (see also 2.2. Female genital schistosomiasis).

Urinary *S. haematobium* infection may cause inflammatory and calcified lesions in the urinary tract, leading to obstructive uropathy such as hydroureter and hydronephrosis, renal lesions such as acute and chronic pyelonephritis and probably a predisposition to squamous carcinoma of the urinary bladder [23-26]. Authors of primate and human autopsy studies have hypothesised that *S. haematobium*-induced lesions develop through three main stages; polypoid patches, fibrous patches and sandy patches [23,27-30]. Occasionally, reports have described lesions surrounded by dilated blood vessels or hyperaemia [31]. Some studies indicate that the degree and outcome of pathology is dependent on the focal location and variable intensity of infection characteristically observed in urinary *S. haematobium* infection [23,29,32,33].

2.1.5. Histopathology of urinary schistosomiasis

The histopathology caused by *S. haematobium* infection in general differs from that caused by other schistosome species [34]. In the following, mainly literature concerning *S. haematobium* infection will be cited. Results of relevant *S. mansoni* studies will be referred to only where no equivalent studies of *S. haematobium* exist. A more detailed account of the histopathology specific to female genital schistosomiasis will be given in chapter 2.2. Female genital schistosomiasis.

Pathogenesis. *S. haematobium*-induced disease in humans is primarily caused by a form of delayed hypersensitivity reaction (type IV) to deposited ova [23,31,35]. The adult parasite worms evade the human immune system by adopting and integrating human antigens on their own surface [36]. Besides ova, dead worms, immature schistosomes and schistosomula may be susceptible to human immune responses [31,37]. There are, however, indications that adult *S. haematobium* worms may undergo immune-mediated clearance over time [38]. *S. haematobium* worms tend to remain in a single location for long periods, which may result in the deposition of large numbers of ova; up to two hundred ova daily [33,39].

More than half of the *S. haematobium* ova laid by the female worm may be retained in the tissue; characteristically found as clusters of calcified ova [33,35,40]. The intensity of urinary infection has been correlated to macroscopic and microscopic pathological findings, especially in children and young adults [23,27,35,41-43]. However, studies indicate that the deposition of *S. haematobium* ova is focal and patchy in humans and primates, although the variation of ova burden decreases with increasing intensity and/or duration of infection [24,29,33]. Moreover, factors other than the duration and density of ova deposition may affect the degree of pathology, such as the site and rate of ova deposition, *in utero* sensitisation to schistosome antigens, nutrition, anti-schistosomal treatment, host immune system, genetic predisposition and co-infections [19,44-49].

Authors of autopsy and surgical specimen studies of *S. haematobium* infection in Egypt have hypothesised that the disease develops through two main stages; 'active' and 'inactive' disease [27,30,42]. The active stage of disease, observed in children and teenagers, was characterised by mature adult worms and mostly viable ova with periovular granulation tissue and infiltrates of mainly lymphoid cells and eosinophils [27,28]. The inactive stage,

observed mostly from the third decade of life, was characterised by calcified ova and healing granulomas with few eosinophils [27,28]. A study of *S. haematobium* ova in mice suggests that calcifying ova may give a persistent granulomatous reaction in the tissue [50]. To our knowledge prospective studies of *S. haematobium* infection have not been performed in man, and thus the understanding of disease stages are hitherto based on hypotheses.

Schistosomal granuloma. Several studies indicate that the periovular granuloma (previously termed 'pseudotubercle') is a common histopathological finding in urinary *S. haematobium* infection [51-56]. The schistosomal granuloma is a T lymphocyte-mediated immune response against miracidial products and consists predominantly of mononucleated cells, i.e. lymphocytes and plasma cells, monocytes, macrophages and fibroblasts, and a few neutrophils, eosinophils and mast cells (Table 1) [51-55].

Studies of granuloma formation in *S. mansoni* infection indicate that the schistosomal granuloma is a dynamic process in which suppressor T lymphocytes drive the granulomatous reaction, closely regulated by mononucleated cells [57,58]. Both T and B lymphocytes have been shown to be a part of the immune response against *S. mansoni* ova [59,60], and plasma cells may produce antibodies targeted at *S. mansoni* antigens [61]. Macrophages surround non-viable *S. haematobium* ova and ova shells are infiltrated by multinucleated giant cells (Table 1) [62]. Although not a dominating feature of the schistosomal granuloma, studies indicate that eosinophils are among the main effector cells in the immune defence against *S. haematobium* and *S. mansoni* [62-67].

Dendritic cells. *In vitro* antigen presentation by dendritic cells (DCs) may overcome T lymphocyte hypo-responsiveness observed in chronic *S. haematobium* infection [68]. In experimental *in vitro* and *in vivo S. mansoni* infections, DCs are competent inducers of T lymphocytes (Table 1) [69]. Binding of antigen to DCs, macrophages and other antigen-binding cells may affect the polarisation of the immune response in schistosomiasis (see below, 2.1.6. Other immunopathological aspects) (reviewed in [70]).

Cell type	Origin	Functions
Lymphocyte	T cells originate from the bone marrow and mature in the thymus, B cells develop in the bone marrow	T lymphocytes (CD4+, CD8+) and natural killer cells: cell-mediated immunity and immune regulation. B lymphocytes: humoral immunity (antibody production).
Plasma cell	Differentiate from B cells	Produce antibodies in the host defence against invading pathogenic agents
Eosinophil	Develop and mature in the bone marrow	Host immune response to multicellular parasites and involved in allergic responses
Macrophage	Monocytes from the bone marrow develop into tissue macrophages	Engulf and digest infectious agents, foreign bodies and cancer cells. Stimulate immune cells.
Giant cell	Fusion of monocytes and/or macrophages	Fusion of monocytes and macrophages in response to a large or refractory foreign body.
Dendritic cell	Originate from immature cells from the bone marrow	Process and present antigens to other immune cells.
Fibroblast	Originate from the primitive mesenchyme	Produce extracellular tissue content, such as collagen. Important for wound healing.

Table 1. The main cells involved in the tissue response to *S. haematobium* ova [71].

Other tissue reactions. Squamous epithelial hyperplasia constitutes a prominent feature of urinary *S. haematobium* infection, and may be found in association with severe urinary schistosomiasis [28,35]. Fibrosis may be characteristic of chronic *S. haematobium* infection, and fibroblasts have been found to adhere to fresh or partially damaged ova and produce extracellular collagen fibres [62]. In *S. mansoni* infection, fibrosis appears to be age- and sex-dependent [72].

2.1.6. Systemic immunopathological aspects

At present, few appropriate experimental study models of *S. haematobium* infection are available, and most of our knowledge of the immunopathogenesis in schistosomiasis has been deduced from studies of murine *S. mansoni* infection [70]. Studies indicate that a closely regulated immune response against schistosomes acts to suppress the parasites' virulent effects, and may further be essential for host survival [73,74].

In *S. mansoni* infection, the first 4-6 weeks of infection are characterised by a moderate T helper 1 cell (Th1) dominated immune response, followed by a shift to a T helper 2 cell (Th2) dominated immune reaction (reviewed in [70]). Th1 cells, a subset of CD4+ T lymphocytes, are recruited in response to schistosomula and immature adult worms, and produce cytokines such as interferon (IFN)-γ involved in macrophage activation [75,76]. Th2 cells, another subset of CD4+ T lymphocytes, are activated in response to ova deposition and produce interleukins such as IL-4, IL-5 and IL-13 involved in establishing eosinophilic and granulomatous reactions and in generating fibrosis (IL-13) [75,76]. Following a peak at around 8 weeks post infection, the Th2 dominant response is down-modulated towards chronic infection [70]. IL-10 regulates the shift from Th1 to Th2 dominant immune responses, and, alongside regulatory T lymphocytes (T regs or CD4+CD25+ T cells) prevents severe pathology due to excessively polarised responses [70].

The immune profile in chronic schistosomiasis, including *S. haematobium* infection, may affect the disease burden of co-infections as well as the capability to adequately respond to vaccines (see chapter 2.3. Schistosomiasis and human immunodeficiency virus (HIV) infection) [77]. On the other hand, data suggest that the immune profile in chronic helminth infections could protect against diseases such as asthma, allergy and autoimmunity [78-80].

2.2. Female genital schistosomiasis

2.2.1. History

The earliest reports of female genital schistosomiasis are found in two case studies published in Egypt in 1899, with descriptions of schistosome ova in cervical and vaginal tissue, respectively [81,82]. In the years that followed, a number of case reports were published, and in 1925, Gibson thought it "probable that the incidence of genital Bilharziasis in the female is much commoner than the published reports of cases would suggest" [83].

Experimental studies have shown that *S. haematobium* infection may lead to various forms of pathology in the female genital tract of infected non-human primates [29,32,55,84,85]. Although animals show individual variations to *S. haematobium* infection, three main reactions have been identified in the genital mucosa: tan and firm polypoid patches, pale fibrous patches, and small, gritty sandy patches [32].

From the middle of the twentieth century a number of cross-sectional autopsy and surgical specimen studies were performed. Table 2 gives an overview over the main characteristics of the histopathological studies of *S. haematobium* infection in the lower female genital tract. Autopsy and surgical specimen studies are often biased to hospitalised and seriously ill patients, and the clinical relevance of the results must be interpreted as such. None of these studies describe the histopathology of the female genital mucosa of the controls [86-89].

Author, year	Material	Number[a]	Country
Gelfand, 1949	Autopsies	30	South Africa
Charlewood, 1949	Surgical specimens	31[b]	South Africa
Badawy, 1962	Surgical specimens	68	Egypt
Berry, 1966	Surgical specimens[c]	161	South Africa
Williams, 1967	Surgical specimens	14	Nigeria
Bland, 1970	Surgical specimens	12	Zimbabwe
Gelfand, 1971	Autopsies	25	Zimbabwe
Renaud, 1971	Surgical specimens	46	Ivory Coast
Diouf, 1973	Surgical specimens	20	Senegal
Coulanges, 1975	Surgical specimens	26	Madagascar
Edington, 1975	Autopsies	34	Nigeria
Koller, 1975	Surgical specimens	21	South Africa
Loubière, 1977	Surgical specimens	51	Ivory Coast
Bayo, 1981	Surgical specimens	51	Mali
van Raalte, 1981	Surgical specimens	145	Tanzania
Wright, 1982	Surgical specimens	139	Malawi
Gouzouv, 1984	Surgical specimens	27	Republic of the Congo
Ricosse, 1985	Surgical specimens	40	France[d]
Renaud, 1989[e]	Community-based	46	Niger
Helling-Giese, 1996	Out-patient department[f]	33	Malawi
Leutscher, 1997[e]	Community-based	12	Madagascar
Poggensee, 2000[e]	Community-based	134	Tanzania

Table 2. Histopathological studies of human *S. haematobium* infection in the lower female genital tract. All the included studies are cross-sectional.

[a]Number of cases with schistosomiasis of the lower female genital tract by histopathological findings and/or digestion technique. Only studies with more than ten cases are included in the table. [b]Additional 66 histopathological slides from other cases than those whose cytological results are presented. [c]Includes 139 cervical smears. [d]Institut de Médicine Tropicale du Service de Santé des Armées, Marseille. [e]No detailed histopathological analyses other than presence or not of schistosome ova. [f]Patients and next of kin.

2.2.2. Clinical manifestations of female genital mucosal schistosomiasis

Women with *S. haematobium* infection of the genital tract may suffer from pelvic pain, genital itch, abnormal vaginal discharge, irregular menstruation and infertility [90-92]. One study showed that women with genital schistosomiasis report significantly more often spontaneous abortions than uninfected controls [90]. *S. haematobium* infection of the female genital mucosa may present as sandy patches, with or without contact bleeding or abnormal, i.e. convoluted, reticular, branched and unevenly calibered mucosal blood vessels [93,94]. The gynaecological manifestations in women with genital *S. haematobium* infection may or may not be found together with urinary *S. haematobium* infection [90,93,95,96].

Female genital schistosomiasis may clinically resemble malignant lesions and/or sexually transmitted infections (STIs), and both may be relevant differential diagnoses [91,93]. Also *S. mansoni* and *S. japonicum* may infect the female genital tract, and some report findings similar to those found in *S. haematobium* infection [20,97-100].

2.2.3. Histopathology of female genital mucosal schistosomiasis

Following the publications by Pétridis and Madden in 1899, a number of cases of *S. haematobium* infection in the female genital organs were reported [81,82,101-112]. The histopathological reactions vary considerably, ranging from periovular granulomas and infiltrates composed of eosinophils, lymphocytes, plasma cells, giant cells and fibroblasts, to predominantly fibrous tissue with little or no apparent periovular cellular reaction. In a review of female genital schistosomiasis published in 1943, Gilbert noted that the varied histopathological manifestations may also be found side by side in the same specimen [113].

The first systematic description of the pathogenesis in female genital *S. haematobium* infection was presented in 1919 [84]. In this study of experimental *S. haematobium* infection in monkeys, a range of tissue reactions were reported; from periovular multinucleated giant cells, eosinophils and small mononucleated cells to calcified ova surrounded by fibrotic tissue [84].

Autopsy and surgical specimen studies. The histopathology of human female genital *S. haematobium* infection has been explored in a number of autopsy and surgical specimen

studies from all regions of Africa (see Table 2) [86-89,114-127]. It must be noted that most studies have not controlled for STIs. The organ distribution of *S. haematobium* ova in infected women varies between studies; however, the lower genital tract is the most frequent detection site in biopsies, commonly taken from the cervix by routine sampling and/or as part of cancer diagnostics [87-89,115,117-120,123-126].

In a study of surgical specimens from South Africa, Charlewood *et al* noted a marked variation in the morphological reaction to schistosome ova; from slight reactive changes to chronic inflammation, often with abscesses of eosinophils [114]. The authors also remarked that the tissue reaction was not necessarily related to the density of ova in tissue, as in some cases "the degree of response appears to be out of all proportion to the number of ova found" [114]. Infected tissue lined by squamous epithelium, such as the vulva, vagina and ectocervix, often showed hyperplastic changes and papilloma formation of the epithelium, whereas tissue lined by columnar epithelium showed little or no epithelial reaction.

In a study in Egypt, Badawy suggested that so-called schistosomal pseudotubercles; consecutive layers of epithelioid cells, eosinophils and lymphocytes surrounding an ovum engulfed by a giant cell, were characteristic for *S. haematobium* infection of the cervix [115]. Squamous epithelial hyperplasia and gradual replacement of 'pseudotubercles' by fibrosis were other characteristics associated with cervical *S. haematobium* infection [115].

A large variation in histopathological findings was also reported by Berry in 1966 [86]. Schistosome ova, most often deposited in the subepithelial tissue of the transformation zone, were commonly surrounded by increased vascularity, 'pseudotubercles', eosinophilic reaction, ulceration, lymphocyte infiltrates, multinucleated histiocytes and epithelial hyperplasia. However, the periovular tissue reaction could also show little or no apparent involvement at all [86]. Comparing the histopathological findings to an analysis of cervical and vaginal smears of other cases, the author "felt that ova (which could often be seen escaping) could have been retrieved in smears" [86]. Others report the findings of *S. haematobium* ova close to denuded or atrophic epithelium and in cytological material [21,105,116,128]. However, one study suggests that cervical smears are unreliable for the diagnosis of female genital schistosomiasis [129].

Categories of histopathogical changes. In a detailed and systematic analysis of surgical specimens from Ivory Coast, Renaud *et al* suggested that tissue reactions to female genital *S. haematobium* infection develop through three main stages: ‘exudative’, ‘productive’ and ‘sclero-cicatricial’ [87]. The exudative stage was characterised by abundant neovascularisation and numerous micro-abscesses containing neutrophils and eosinophils, lymphocytes, histiocytes and red blood cells surrounding an often viable schistosome ovum. The second, productive stage was characterised by the apparition of ‘pseudotubercles’ around both viable and non-viable schistosome ova. Finally, the sclero-cicatricial stage was characterised by dense fibrous tissue with scattered giant cells, few ova remnants without any apparent periovular reaction. The authors also frequently found hyperplastic changes of the squamous epithelium in the *S. haematobium* infected genital mucosa [87].

In line with the findings by Renaud *et al*, a retrospective analyses of surgical specimens in Malawi identified five different tissue reactions to schistosome ova: 1) diffuse infiltration by eosinophils, neutrophils, plasma cells, lymphocytes and macrophages, 2) granulomas or pseudotubercles with epithelioid cells, lymphocytes, eosinophils and fibroblasts, 3) foreign body giant cells surrounding shell fragments, 4) large numbers of shell fragments trapped in fibrous tissue and 5) sparse shell fragments with no apparent tissue reaction [118]. The authors hypthesised that the different categories represented different phases of progress of *S. haematobium*-induced histopathology [118].

The results of the first community-based study were published in 1989 by Renaud *et al* [120]. Vaginal biopsies were sampled from 61 otherwise unselected Nigerien women, identifying a prevalence of vaginal schistosomiasis of 75%. In line with previous studies [89,117], Renaud *et al* found that the intensity of urinary *S. haematobium* infection as measured by urinary ova output and vesical lesions detected by ultrasound, was significantly associated with vaginal *S. haematobium* infection [120].

Most of the studies discussed above describe a diversity of histopathological tissue reactions to schistosome ova, however, in a number of studies, the majority of cases are limited to a moderate to mild tissue reaction [89,117,119,123,124,126]. No study has yet explored the possible pathophysiological mechanisms for this variation of tissue reactions.

Ova detection in tissue specimens. The deposition of *S. haematobium* ova in the female genital mucosal tissue may be highly focal and affect the analyses of the related tissue reactions [89,113,114]. Several studies have explored the ability of tissue digestion with potassium hydroxide as a detection method of schistosome ova [89,113,117,130]. A digestion study in 30 consecutive autopsies in South Africa found *S. haematobium* ova in the genitals of all the cases [130]. In a retrospective autopsy study in Nigeria, complete digestion and histopathological findings were compared in the pelvic organs in 34 females with urinary *S. haematobium* infection, and the two ova detection methods proved to perform well in severe infections, whereas the ability to detect ova by histopathology was reduced compared to digestion technique in milder infections [117]. However, a Zimbabwean study of block dissections of the pelvic organs from 64 consecutive autopsies concluded that neither digestion nor histological examination was sufficient to successfully detect ova in the tissue [89].

2.2.4. Clinicopathological correlates

A clinicopathological, hospital-based study in Malawi from 1994, which correlated histopathological results with clinical findings in female genital *S. haematobium* infection, indicated that sandy patches consist of ova in various stages of disintegration surrounded by a limited cellular reaction, and that polyps consist of a more pronounced immune reaction with a cellular infiltrate [127]. These findings correspond with an experimental study of urogenital *S. haematobium* infection in baboons in which polypoid patches; sharply delimited, resilient patches, were found to consist of large numbers of ova with infiltrates of eosinophils, macrophages, plasma cells and lymphocytes; fibrous patches of fibrosis with some inflammation; and sandy patches of closely packed ova with little inflammation [32].

Although the abovementioned findings may be plausible, no other clinical or experimental evidence definitely supports the hypothesis that acute schistosomal lesions evolve into sandy patches [42]. However, the hypothesis is supported by the findings of what is regarded as acute pathology in children and teenagers with urinary *S. haematobium* infection, and what is thought to be progressive pathology in adults [23,27,28,131].

2.3. Schistosomiasis and human immunodeficiency virus (HIV) infection

Recently, the possible association between schistosomiasis and HIV infection has received increased attention [132-135]. On the one hand, concomitant HIV infection could affect schistosomal morbidity or susceptibility to schistosome infection, and on the other hand, concomitant schistosomiasis could affect HIV-related morbidity or risk of HIV transmission [136,137]. To our knowledge, there are no studies of HIV-2 infection and schistosomiasis. In the following HIV-1 will be referred to as HIV.

2.3.1. The female genital mucosa and HIV transmission

The lower female genital tract is the main site of HIV transmission, and is also one of the most common sites for female genital *S. haematobium* infection [93,138]. Similar to STIs, it has been suggested that genital lesions caused by *S. haematobium* ova may provide points of entry for HIV [133,139-142]. Studies have been demonstrated that *S. haematobium* infection may compromise the barrier function of the female genital mucosa [86,93,94,118,143].

Studies indicate that CD4+ T cells, Langerhans cells and macrophages are among the earliest cervicovaginal cell populations to be infected with HIV [144-149]. Further, HIV co-receptors CCR5 and CXCR4 may be necessary for the propagation of HIV infection [150,151]. It has been hypothesised that *S. haematobium* induced immune reactions in the genital mucosa could increase the density of HIV susceptible immune cells [133,152]. This could potentially facilitate the transmission of HIV in co-endemic areas [153].

2.3.2. Epidemiological data on schistosomiasis and HIV infection

Several aspects of the uneven burden of the HIV epidemic warrant further understanding of the possible factors responsible for driving the epidemic [152]. A disproportionately high HIV prevalence affects Sub-Saharan Africa, and there appears to be a geographical overlap between co-endemic areas for HIV and *S. haematobium* infection [22]. Further, there is an unexplained gender quotient disfavouring women, especially in younger age groups [154].

2.3.3. Immunological data on schistosomiasis and HIV infection

HIV more readily infects and replicates in Th2 lymphocytes, and the Th2-dominated immune response in chronic schistosomiasis might increase HIV-related morbidity in concomitant schistosomal infection (see 2.1.6. Other immunopathological factors) [132,155,156]. *In vitro* peripheral mononuclear blood cells from *S. mansoni* infected individuals were more susceptible to HIV infection than mononuclear blood cells of non-infected individuals [157]. Furthermore, CD4+ cells in patients with *S. mansoni* infection expressed a higher density of HIV co-receptors CCR5 and CCXR4 than did CD4+ cells in patients treated for schistosomiasis [158].

On the other hand, studies indicate that CD4+ T lymphocytes may be essential for the host response to *S. mansoni* infection, and some suggest that a depletion of CD4+ cells in HIV infection could increase schistosomal morbidity and affect susceptibility to schistosomal re-infection [73,74,137]. Moreover, ova excretion may be reduced in schistosomiasis and HIV co-infected, thereby possibly increasing ova-related pathology [159,160]. A cross-sectional study, however, did not find any difference in egg excretion between low-intensity schistosomiasis and HIV co-infected patients and patients with schistosomiasis alone [161]. Studies suggest that Th17 cells may affect the mucosal immune response in HIV-1 infection; however, their role has not yet been explored in schistosomiasis [70,162,163].

2.3.4. Clinical data on a possible association with HIV infection

A cross-sectional, community-based study in Zimbabwe found a nearly three-fold increased odds of HIV infection in women with genital *S. haematobium* infection compared to women without [135]. Women with urinary *S. haematobium* infection were more often co-infected with HIV infection than women without urinary schistosomiasis [164]. These findings are in line with the results from an experimental study of *S. mansoni* infected rhesus monkeys, in which the infectious dose of simian-human immunodeficiency virus was 17-fold lower in parasitised compared to non-parasitised animals [165].

2.4. Computer-assisted image analysis

Quantification of immunostained cells and tissue structures in formalin-fixed, paraffin-embedded tissue sections is a core element of histopathological methodology [166,167]. In many laboratories, immunohistochemical quantification is still mostly performed manually, both in routine and research pathology. This procedure is time-consuming and tiring, and the results may be compromised by intra- and inter-observer variabilities [167].

Computer-assisted image analysis can improve the reliability and validity of quantitative analyses of immunostained tissue sections [168-171]. Costly software has been customised for specific clinical purposes; however, there is a need for simple and comprehensible models for low-cost computer-assisted image analyses [172].

3.0. Study objectives

3.1. Broad objectives

The aim of this study was to explore the histopathological and immunohistochemical factors which might affect cervicovaginal HIV susceptibility, and to explore the histopathological correlates of clinical findings in female genital mucosal *S. haematobium* infection.

3.2. Specific objectives

A. To develop a simple method for precise computer-assisted quantitative analyses of immunostained formalin-fixed and paraffin-embedded tissue sections (**Paper IV**).

B. To study the histopathological and immunohistochemical tissue reactions immediately surrounding *S. haematobium* ova in cervicovaginal mucosal biopsies, by
   1. quantifying and determining the characteristics of periovular endothelial cell structures (**Paper I**).
   2. quantifying the density of periovular CD4+ T lymphocytes, macrophages, Langerhans cells and CCR5 and CXCR4 receptors (**Paper II**).

C. To explore the histopathological correlates of abnormal mucosal blood vessels in cervicovaginal *S. haematobium* infection (**Paper III**).

4.0. Materials and methods

4.1. Study population and design

The analyses were performed on endemic archive biopsies from a study in Malawi and on non-endemic archive biopsies from Norway (Figure 3). An overview of the study groups is shown in Table 3. One of the patients presented in the case study (Paper III) originated from a cross-sectional study in Madagascar which will be described at the end of this section.

Non-endemic normal cervix	**Endemic without FGS[a]**	**Endemic with FGS[a]**	**Non-endemic chronic cervicitis**
No *S. haematobium* ova seen	No *S. haematobium* ova seen	*S. haematobium* ova identified	No *S. haematobium* ova seen
Less than 10 immune cells per HPF[b]			Predominance of plasma cells and/or lymphocytes
No non-specific tissue proliferation			Less than 20 PMNs[c] and less than 5 eosinophils per HPF[b]
No other signs of pathology			No other signs of pathology

Table 3. The study groups. The histopathological characteristics of endemic Malawian and non-endemic Norwegian biopsies included in the study. [a]FGS = Female genital mucosal *S. haematobium* infection. [b]HPF = high-power field. [c]PMN = polymorphonuclear neutrophil.

Endemic cases and controls. The archive biopsies of endemic cases and controls originated from a study of women with urogenital *S. haematobium* infection at Mangochi District Hospital in Malawi in 1994 [173]. All sexually active women between 15 and 49 years of age, irrespective of their complaints or whether they were patients or next of kin, present in the out-patient department before lunch time, were invited to submit urine samples. Women who were willing to provide a urine sample, and who by filtration of the urine were found to have *S. haematobium* ova in the urine, were invited for an interview and gynaecological examinations.

Figure 3. Archive biopsies from an *S. haematobium* endemic study site (Mangochi) were analysed with Norwegian archive biopsies as non-endemic controls.

Women who declined a gynaecological examination or biopsy, virgins, pregnant women (as indicated by the medical history and confirmed by human chorionic gonadotropin (hCG) test in urine) and postmenopausal women were excluded from the study. Excluded women were offered equal medical services and referred to the hospital staff if necessary. Pregnant women with schistosomiasis were asked to come back for treatment after delivery.

Following free and informed consent, a gynaecological examination was performed. Cervical biopsies were taken routinely. In total, biopsies from the cervix and/or vagina were sampled from 61 women. The pathology was photographed using a portable photocolposcope (Leisegang Medical, Berlin, Germany). Results from analyses of HE-stained biopsies and of other specimens collected in 1994 have been published previously [127]. All women with urogenital schistosomiasis were weighed and offered supervised treatment with a single dose of praziquantel 40 mg/kg body weight.

Malawian women with *S. haematobium* ova in genital mucosal biopsies were included as endemic cases, whereas Malawian women without *S. haematobium* ova in genital mucosal biopsies served as endemic negative controls. In order to avoid undetected ova situated just

outside the biopsy borders in presumed negative cases (Figure 4), only women without *S. haematobium* ova in genital mucosal biopsies in both the original and present studies were included as endemic negative controls. A previous study has indicated an association between HPV and urogenital *S. haematobium* infections; however, specific signs of HPV infection were not observed in the endemic biopsies with *S. haematobium* ova [174]. In addition to the cervical biopsies, three vaginal biopsies were included. The histopathological reactions and ova viability were similar in the cervical and vaginal biopsies, and the biopsies were therefore analysed together.

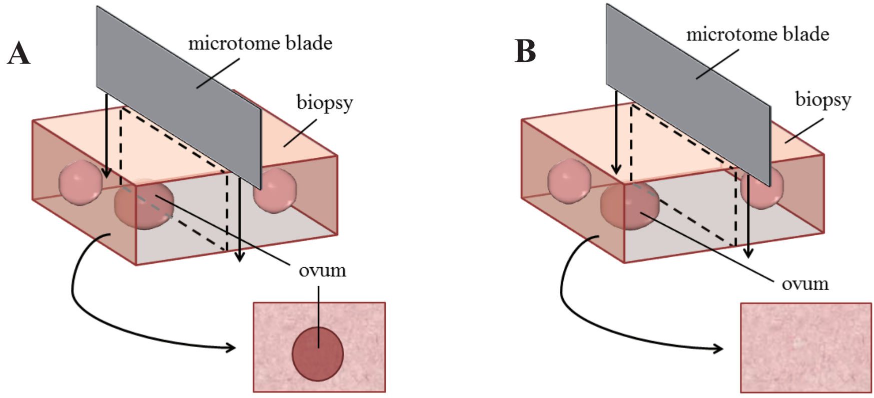


Figure 4. Ova location and tissue sectioning. Due to focal deposition in the tissue, *S. haematobium* ova may be missed by only a few micrometres and the possibility of remaining ova-related histopathology might be misinterpreted.

Non-endemic controls. Non-endemic Norwegian archive biopsies were selected by searching the database at the Department of Pathology at Oslo University Hospital Ulleval from 1994 for combinations of the anatomical site 'cervix uteri' and the morphological diagnoses 'cervicitis' and 'normal morphology'. Included as negative non-endemic controls were women with histologically normal uterine ectocervices with intact tissue architecture (Figure 5). Excluded were all endocervical biopsies, and ectocervical biopsies with more than ten inflammatory cells per high-power field (HPF), non-specific vessel proliferation, epithelial hyperplasia, polyps, cysts, atypical or dysplastic changes, or signs of human papilloma virus (HPV) infection, i.e. clustered koilocytosis and dyskeratosis.

Included as positive non-endemic controls were women with chronic non-specific cervicitis; defined as ectocervices with intact tissue architecture and a distinct, generalised infiltrate dominated by lymphocytes and/or plasma cells (Figure 6). Excluded were all endocervical biopsies, and ectocervical biopsies with more than 20 granulocytes or 5 eosinophils per HPF and specimens with signs of HPV infection, erosion, ulcerations, granulation tissue or extra-vascular erythrocytes (in order to avoid non-specific immunostaining).

For each immunostain, different numbers of biopsies were available in the respective study groups (Table 4). Endemic biopsies were excluded due to insufficient tissue for further analyses and when ova lacked in subsequent sectioning. Non-endemic biopsies were excluded due to technical reasons such as non-specific cross-reactions and insufficient tissue with preserved architecture. The limited number of available biopsies restricted the possibility for stratification by age, and this is addressed during the statistical analyses.

Immunostain (antibodies)	**Non-endemic normal cervix**		**Endemic without FGS**[a]		**Endemic with FGS**		**Non-endemic chronic cervicitis**	
	Incl	**Excl**	**Incl**	**Excl**	**Incl**	**Excl**	**Incl**	**Excl**
vWF[b]	28	1	24	0	20	17	11	7
CD31[c]	29	0	24	0	20	17	15	3
CD3/8	29	0	16	8	13	24	16	2
CD68	29	0	23	1	19	18	18	0
S100	28	1	14	10	17	20	15	3

Table 4. Included and excluded specimens. Biopsies were excluded for technical reasons such as insufficient tissue for further analyses or lack of ova in subsequent sectioning of endemic biopsies with previously detected *S. haematobium* ova. [a]FGS = Female genital mucosal *S. haematobium* infection. [b]vWF = von Willebrand Factor (previously Factor VIII Related Antigen). [c]CD = Cluster of differentiation.

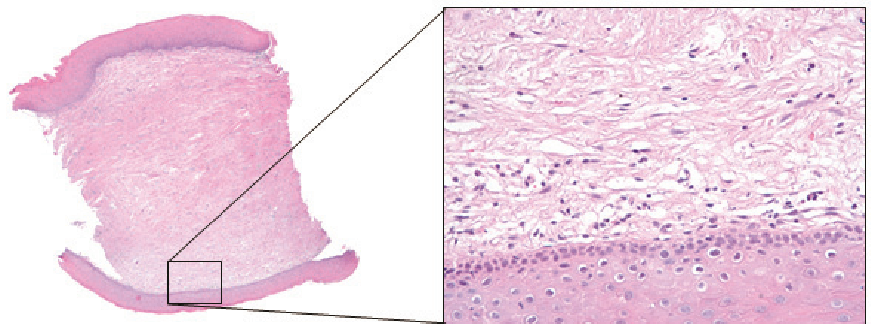


Figure 5. Non-endemic negative control. Archive biopsies of non-endemic Norwegian women with normal cervices were included as negative controls.

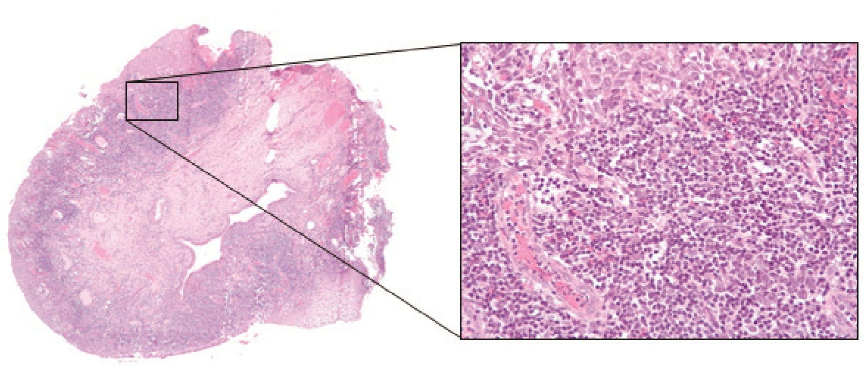


Figure 6. Non-endemic positive control. Archive biopsies of non-endemic Norwegian women with non-specific chronic cervicitis were included as positive controls.

The Madagascan study. A population-based cross-sectional study was carried out in women between 15 and 35 years of age in the district of Miandrivazo in Western Madagascar in June and July of 2010. The surfaces of the lower genital tract were examined in a standardised manner, and accurate biopsies were sampled from observed pathology using biopsy forceps 2.3 mm (ENDO-FLEX GmbH, Voerde, Germany). The precise location of the biopsy was recorded using a pointer and photographed using an Olympus E-420 10 megapixel single lens reflex (SLR) camera (Olympus America Inc., Center Valley, PA, USA) mounted on an Olympus OCS-500 colposcope (Olympus America Inc.) in order to correlate the clinical findings with the histopathological results. Standard histopathological examinations were performed on the Madagascan biopsies.

4.2. Histopathology

All biopsies were fixed in formalin, routinely processed and embedded in paraffin. For histopathological analyses, 3.5 μm serial sections of the included specimens were cut using a Microm HM 355 microtome (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and placed on SuperFrost slides (Menzel-Gläser, Braunschweig, Germany). The sections were deparaffinised and rehydrated prior to standard HE staining.

4.3. Immunohistochemistry

For immunohistochemical analyses of the Malawian biopsies, 3.5 μm thick serial sections of the included specimens were cut using a Microm HM 355 microtome (Thermo Fisher Scientific) and placed on SuperFrost Plus slides (Menzel-Gläser). The immunohistochemical stains were performed using BenchMark XT (Ventana Medical Systems Inc., Tucson, Arizona, USA); an automated immunostain system based on the ABC avidin-biotin-peroxidase method.

The automated slide preparation system performed sequential deparaffinisation, antigen retrieval, and incubation with primary and secondary antibodies, respectively. Enzyme-mediated horseradish peroxidase (HRP) labelled to diaminobenzidine (DAB) reporter molecules was used for colour detection. Amplification was achieved by secondary antibody conjugation to biotin molecules and by avidin protein bound enzymes. All sections were counter-stained with haematoxylin. Optimal antigen retrieval, antibody concentrations

and incubation times were pre-tested on endemic and non-endemic specimens for each immunostain. Table 5 shows the specifications and final dilutions used for each immunostain. Negative and positive controls were included for analyses according to manufacturer recommendations. All slides were examined microscopically by a senior pathologist (BR) for immunohistochemical antigen detection combined with morphological identification.

The immunostains were specifically chosen in order to meet the respective study objectives. For the identification of blood vessels, two endothelial cell markers were used; monoclonal antibodies to CD31 (Dako Denmark A/S, Glostrup, Denmark) (Figure 7) and polyclonal antibodies to von Willebrand Factor (vWF) (Ventana) (Figure 8). In line with previous reports, immunohistochemical antibodies to CD4, CCR5 and CXCR4 cross-reacted non-specifically and could not be interpreted (Figure 9). For identification of CD4+ T lymphocytes, a mature T lymphocyte marker and a cytotoxic T cell marker were applied on two consecutive 3.5 µm thick serial sections of each biopsy; rabbit monoclonal antibodies to CD3 (Ventana) (Figure 10) and mouse monoclonal antibodies to CD8 (Dako), respectively (Figure 11). Macrophages were identified using mouse monoclonal antibodies to CD68 (Dako) (Figure 12) and Langerhans cells were identified using rabbit polyclonal antibodies to S100 protein (Ventana) (Figure 13).

All specimens were originally stored in formalin. Further analyses of receptor expression or activity other than HE and immunohistochemical staining could thus not be performed. Enzyme immunohistochemical analyses were preferred to immunofluorescence techniques, in order to allow for visualisation of tissue architecture and thereby the relative location of the respective tissue reactions to *S. haematobium* ova.

Antigen[a]	Antibody[b]	Clone[c]	Manufacturer	Dilution	Incubation[d]
CD31[e]	Monoclonal, mouse	JC70A	Dako[f]	1/80	32'
vWF[g]	Polyclonal, rabbit	-	Ventana[h]	Ready to use[i]	20'
CD4	Monoclonal, mouse	1F6	Leica[j]	1/10	36'
CD3	Monoclonal, rabbit	2GV6	Ventana	Ready to use[i]	32'
CD8	Monoclonal, mouse	C8/144B	Dako	1/150	30'
CD68	Monoclonal, mouse	KP1	Dako	1/3000	32'
CD14	Monoclonal, mouse	7	Leica	1/15	40'
CCR5[k]	Polyclonal, rabbit	-	AbD Serotec[l]	1/60	32'
CXCR4[m]	Polyclonal, rabbit	-	AbD Serotec	1/50	32'
S100	Polyclonal, rabbit	-	Ventana	Ready to use[i]	16'

Table 5. Antigen markers used for immunohistochemical staining. [a]Target antigen. [b]Antibody characteristics. [c]Clone number of monoclonal antibodies. [d]Incubation time (minutes) of primary antibody. [e]CD = Cluster of differentiation. [f]Dako Denmark A/S, Glostrup, Denmark. [g]vWF = von Willebrand Factor (previously Factor VIII Related Antigen). [h]Ventana Medical Systems Inc., Tucson, Arizona, USA. [i]No dilution needed prior to application. [j]Leica Biosystems Newcastle Ltd., Newcastle Upon Tyne, UK. [k]Also named CD195. [l]AbD Serotec, Oxford, UK. [m]Also named CD184.

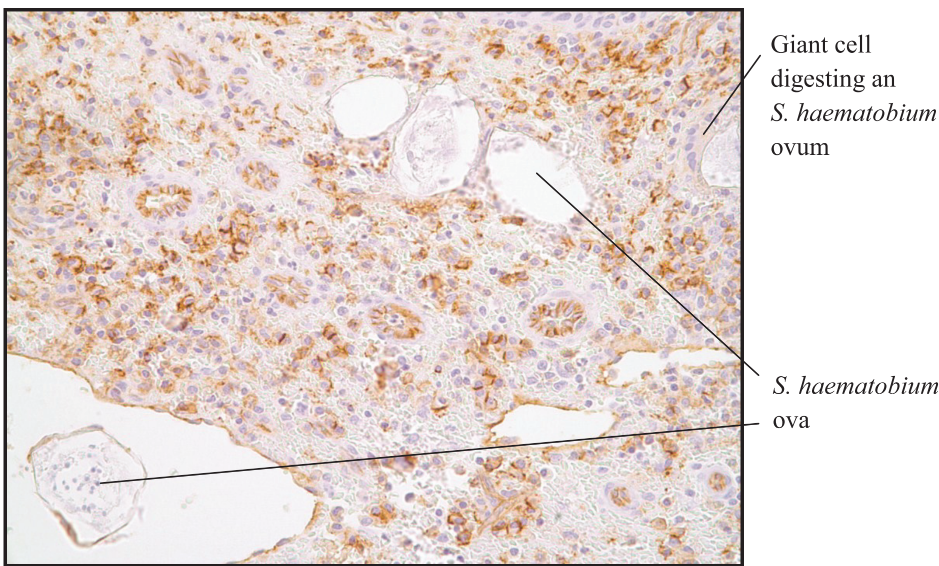


Figure 7. Capillary budding in tissue with *S. haematobium* ova. Immunostain to CD31 of sprouting of capillary buds (brown) surrounding several calcifying ova. Note the viable, intravascular ovum (left) and the ovum undergoing digestion by a multinucleated giant cell.

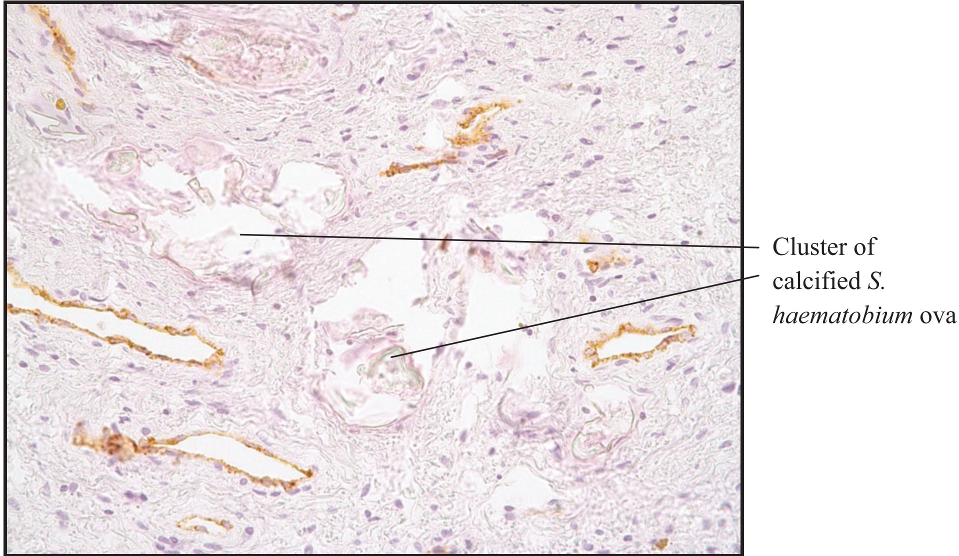
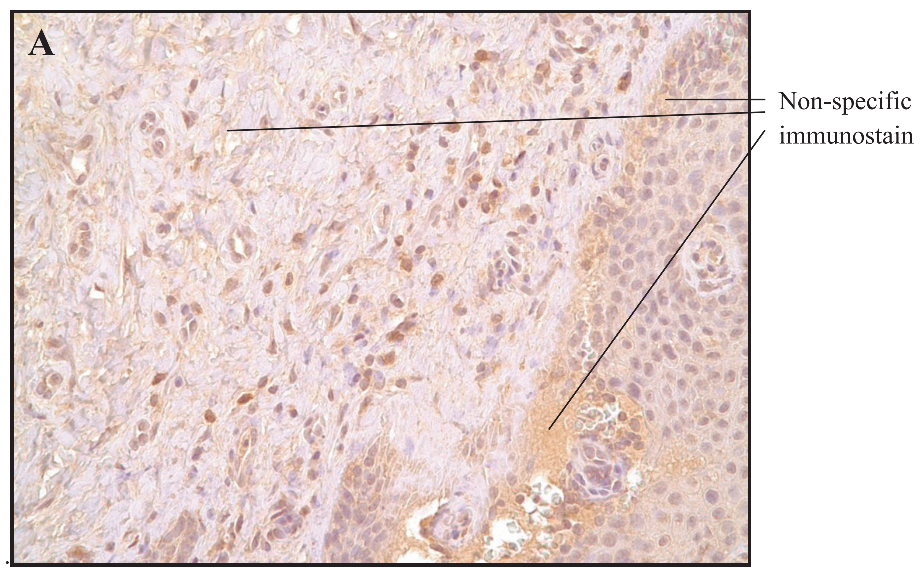


Figure 8. Established blood vessels in tissue with calcified *S. haematobium* ova. Immunostain to vWF clearly demarcates established blood vessels (brown) surrounding a cluster of calcified ova



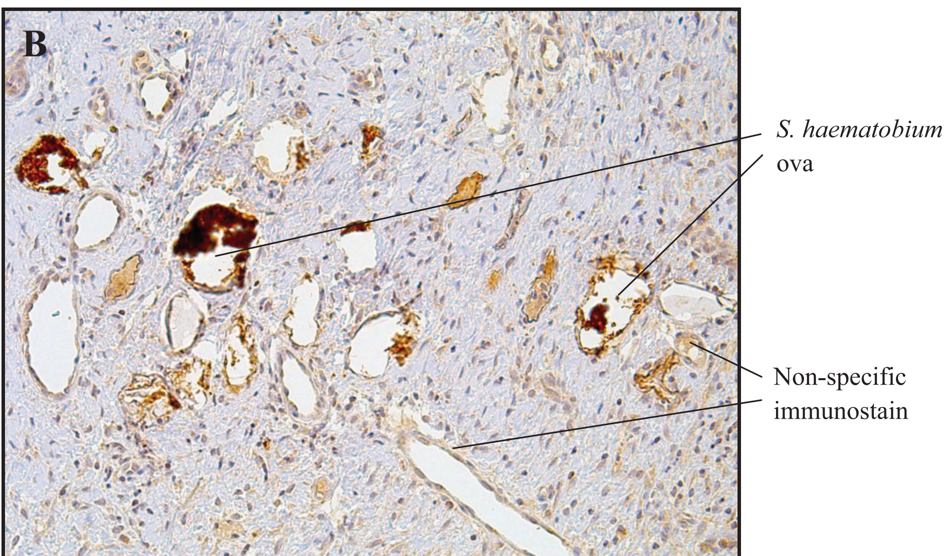


Figure 9. Non-specific protein detection. Immunostain of endemic biopsy with antibodies to CD4 (**A**) and CCR5 (**B**) stain non-specific epitopes on ova and other structures and could therefore not be interpreted. Similar results were seen for antibodies to CXCR4 (not shown).

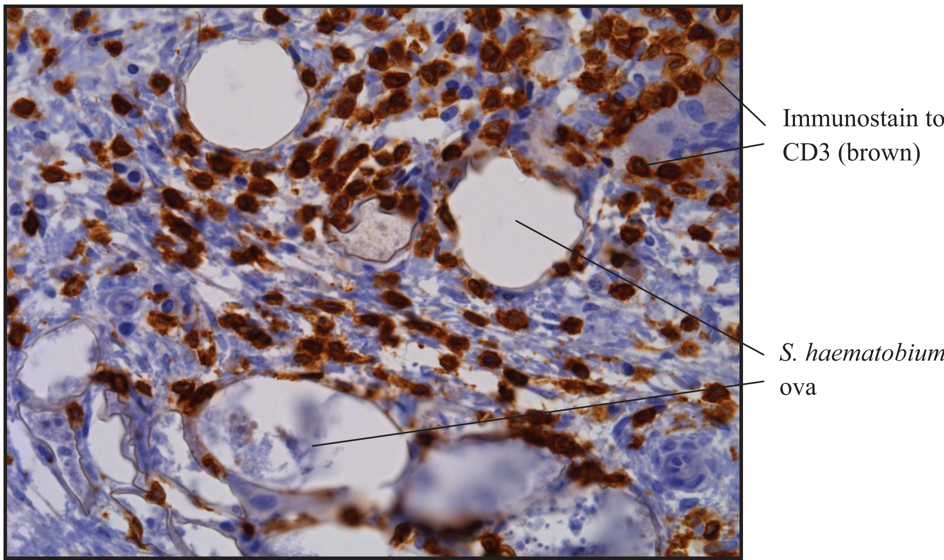


Figure 10. CD3 immunostain. Section of biopsy with CD3+ T lymphocytes (brown) surrounding *S. haematobium* ova (3.5 µm above the next serial section (Figure 11)).

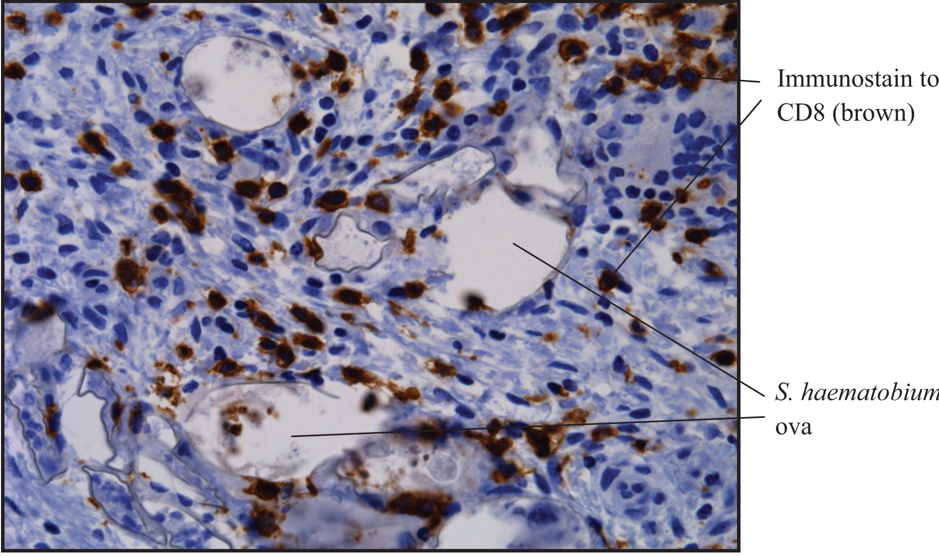


Figure 11. CD8 immunostain. The consecutive section of the same biopsy as in Figure 10 (3.5 µm below the previous section).

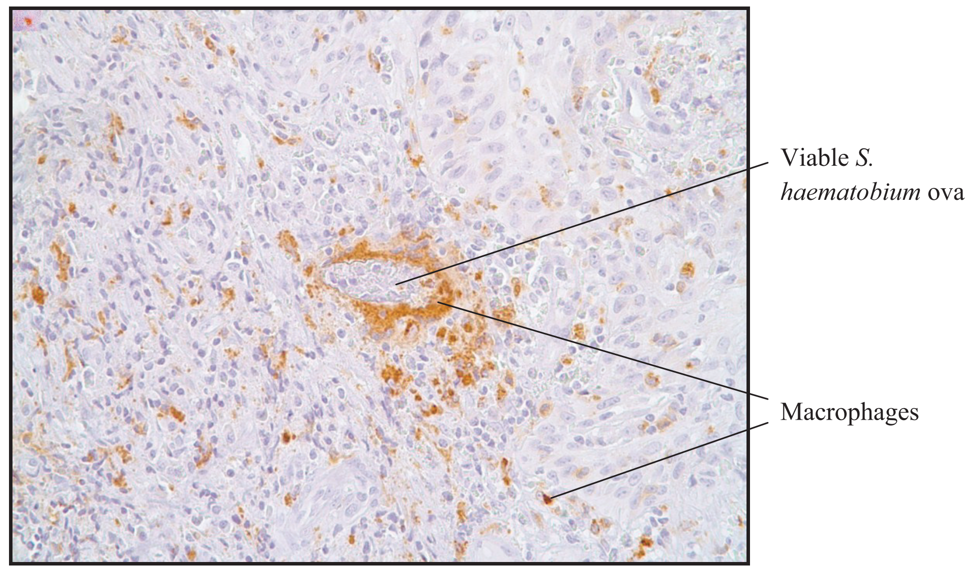


Figure 12. Macrophages in *S. haematobium* infected cervical mucosa. Immunostain to CD68 demonstrates tissue macrophages (brown). Note the fusion of macrophages (giant cell) surrounding a viable *S. haematobium* ovum.

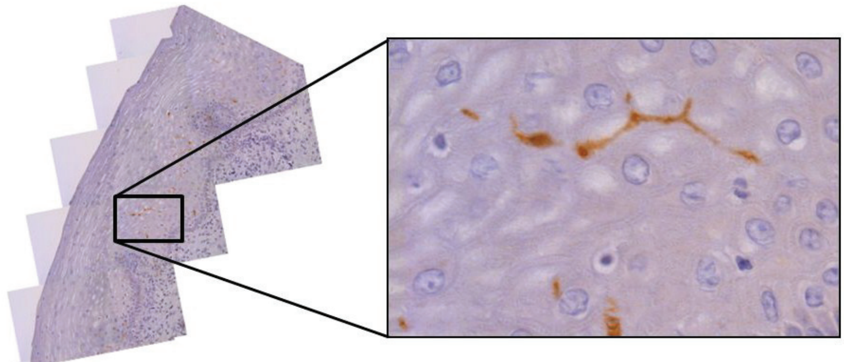


Figure 13. Dendritic cells in female genital mucosa. Immunostain to S100 protein visualises dendrites of Langerhans cells (brown). *S. haematobium* ova are located immediately beneath the analysed epithelium (other sections, not shown).

Identification of ova spines is deemed insufficient for determination of schistosome species in tissue specimens [86]. A modified Ziehl-Neelsen stain (VWR International, LLC, Radnor, Pennsylvania, USA) was therefore used to distinguish *S. haematobium* type ova from *S. mansoni* or *S. intercalatum* ova (Figure 14) [175]. In addition, characteristic features such as ova clustering and calcification contributed to the identification of *S. haematobium* type of schistosome ova [86].

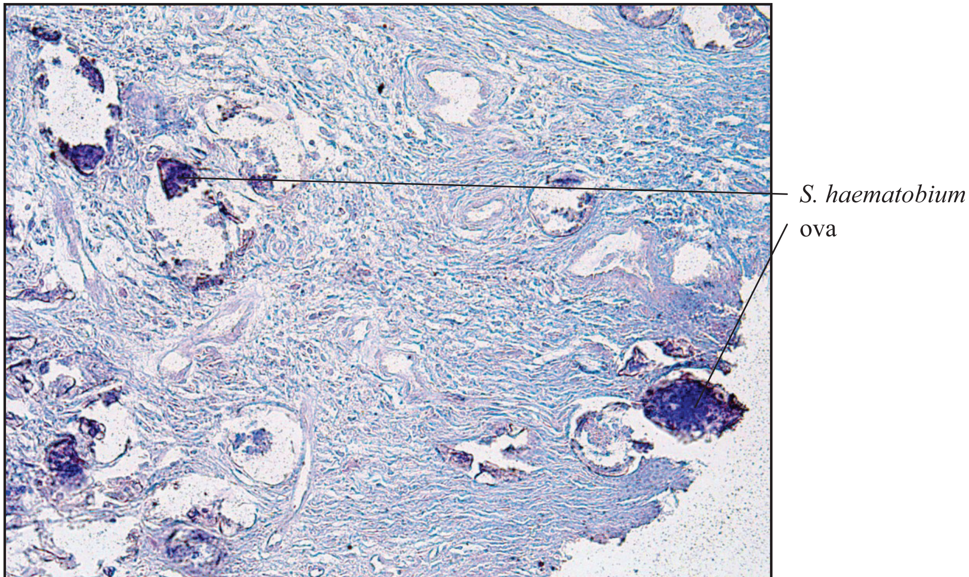


Figure 14. Species identification of *S. haematobium* ova. A modified Ziehl-Neelsen stain developed by Rousset *et al* distinguishes *S. haematobium* ova (dark blue) from other schistosome ova (none identified in these study populations).

4.4. Microscopy and photomicrography

The sections were examined using a Nikon Eclipse 80i and a Leica DM3000 microscope with standard illumination. The former was used for histopathological analyses and immunohistochemical analyses of blood vessels, whereas the latter was used for immunohistochemical analyses of immune cells. The change of microscopes was due to logistics at the department where the analyses took place. The sections were photographed at 40x objective magnification using a SPOT Insight 2 Megapixel Firewire Color 3-shot and

a Leica DFC420 digital camera digital camera, respectively. The cameras were attached to the respective microscope and a Hewlett-Packard Compaq stationary computer. Colour photomicrographs of 1600 by 1200 pixels and 2592 by 1944 pixels, respectively were obtained.

The morphological and immunohistochemical analyses and photomicrographs were performed in a standardised manner. Blinded for the immunostain results, the candidate evaluated the HE-stained sections. An HPF of the HE-stained sections of the endemic biopsies with *S. haematobium* ova was photographed with the ovum or ova placed centrally. The area of tissue surrounding the ovum or ova in one such photograph was defined as 'periovular'. To study the histopathology directly adjacent to the ova, and to avoid the possible influence of undetected ova just outside the biopsy borders, the sections were photographed only in areas containing ova (see Figure 4). In biopsies with more than one cluster of ova, the most representative area for the respective biopsy's tissue reaction was included. The control biopsies were photographed in a subepithelial area representative for the pathologist's overall diagnosis of the biopsy. The subepithelium was chosen since this is the most common location for female genital mucosal *S. haematobium* ova [86].

Once the HE-stained sections had been analysed and photographed, the near-exact same areas of the consecutive immunostained serial sections were photographed, identified by histological anatomical structures. All sections were examined microscopically and all photographs evaluated by an experienced pathologist (BR). The area of *S. haematobium* ova and by extra-mucosal space was estimated for the respective photomicrographs by superimposing a centimetre grid on the computer screen.

S. haematobium ova were defined as 'viable' if miracidia with eosinophilic glands or germinal cells were seen [86], whereas ova containing dark purple stain identified histologically as calcification were defined as 'calcified'. The histopathological tissue reaction was defined as 'granulation tissue' if dominated by capillary buds with activation of endothelial cells and immature fibroblasts. The tissue reaction was defined as 'fibrosis' if dominated by collagen rich stroma with scant mature fibroblasts.

4.5. Manual immunohistochemical quantification

The heterogeneous immunostaining and diverse topographical distribution of endothelial cell structures and Langerhans cells required manual analyses for accurate quantification. Although the specimens were evaluated several times by two investigators who attempted to be objective, blinding was not possible due to the presence of schistosome ova and the respective immunostains.

Immunostained blood vessels (by vWF and CD31 (Figures 7 and 8)) were counted in accordance with pre-established criteria [176,177]. 'Capillary buds' were defined as immunostained vessel structures without an identifiable lumen or peri-endothelial structures, whereas all other stained vessel structures were counted as 'established blood vessels'. The density of capillary buds and vessels were calculated per mm^2 cervicovaginal tissue, correcting for the space made up by ova and extra-mucosal space. In sections with more than 300 capillary buds per mm^2, the numbers were truncated to 300.

For quantification of Langerhans cells (Figure 13), the epithelium of the immunohistochemically stained sections was photographed at 40 times objective magnification and reconstructed using Adobe® Illustrator® CS5 software (Adobe Systems Inc., San Jose, CA, USA). Langerhans cell structures were counted manually in an area equivalent to one HPF to each side of the epithelium overlying the original photograph of the HE-stained section (Figure 13). Counted were only immunohistochemically stained epidermal structures with distinct demarcation and immunostained cells with distinct dendritic processes located in the basal and parabasal strata (Ventana, USA).

The immunostained structures in each photograph were counted manually by the candidate, and one in ten randomly selected photographs of immunostains to endothelial cell structures were quality controlled by a senior pathologist (BR). The immunostain to Langerhans cells showed a larger variation and therefore every third photograph was counted by the senior pathologist. Discrepancies were resolved by consensus, if necessary after consulting a second senior pathologist. Finally, each photograph was recounted.

4.6. Computer-assisted immunohistochemical quantification

Stromal immune cells were distinctly immunostained with antibodies to CD3, CD8, and CD68. Following pre-defined criteria, two independent investigators counted manually a random selection of ten photomicrographs of each immunostain. Immunostained cytoplasma or membrane structures of at least the average diameter of a lymphocyte and/or immunostained perinuclear cell structures were counted. For the quantification of T lymphocytes, non-intact cell structures histopathologically identified as apoptotic cell fragments were registered separately.

Macros (computer algorithms) were developed for computer-assisted image analysis using ImageJ version 1.44p; a Java image processing application available at the website of the National Institutes of Health (NIH) [168,172,178,179]. A macro was developed for the respective immunostain to quantify the number of immunostained cells in each series of photomicrographs, based on the average manual counts as described above. Results were given as the density of separate cells for T lymphocytes and as the area of macrophages; correcting for the area made up of ova and extra-mucosal space. An outline of the final structures was superimposed on the original photographs and manually validated. The results were verified by a senior pathologist, and a second senior pathologist was consulted if necessary.

A three step algorithm for computer-assisted image analysis of immunostained tissue structures was developed. In step one, the immunostained structures are identified by selecting the colour properties which correspond best to the structures of interest. This is achieved using a perceptive colour model in which a specific colour is defined by its respective hue, saturation and brightness (HSB). The structures of interest are isolated based on the intersection of selected threshold intervals in each colour channel. Such a perceptive colour model has the advantage of being more intuitive to the human eye than a purely technical colour model such as red, green and blue (RGB) or cyan, magenta, yellow and key black (CMYK). The selected structures are measured (step two) and the results verified (step three). A macro may then be recorded based on the average threshold values and applied for analysis of any number of immunostained tissue sections.

4.7. Data analyses

4.7.1. Sample size calculation

The effect of *S. haematobium* on genital cell receptor expression has not previously been explored. Based on previous studies of HIV co-receptors in sexually transmitted genital ulcers [139,150], we assumed that the true average percentage of HIV target cells would be 11% in the endemic cases with genital schistosomiasis, and that the standard deviation (SD) of percentage of HIV target cells would be 2.5% in both endemic cases and controls. We assumed that the true average percentage of HIV target cells would be 5% and the SD 3% in the non-endemic controls. The available study samples consisted of between 18 and 29 subjects in each group. It may be shown that the study will have 80% probability (test power) to detect a significant difference in percentage of HIV target cells between the groups and the test power will be sufficient.

4.7.2. Statistical analyses

The statistical analyses and sample size estimation were performed with SPSS version 16.0 and PS Power and sample size calculations version 2.1.31. Most variables were not normally distributed and non-parametric tests were therefore used when studying associations. Medians and interquartile ranges (IQR) were used to describe the results. The Mann-Whitney U and Kruskal-Wallis H tests were applied where appropriate. For the calculation of odds ratio (OR), it was necessary to tertilise the not normally distributed variables. Spearman's rank correlation coefficient was used when studying associations between continuous variables. Intra-observer variability was determined by calculating the intra-class correlation coefficient (ICC) after log-transformation of the data. A 5% significance level was used throughout.

4.8. Ethical considerations

Permissions for the histopathological and immunohistochemical investigations of anonymised archival Malawian and Norwegian biopsies, without additional consent from the study subjects, were granted by the National Health Science and Research Committee of Malawi (2009/NHSRC #620) and the Norwegian Regional Ethics Committee (2009/1250a). The permissions were based on the fact that the proposed analyses did not require identifiable information or history, did not have any direct relevance to the physical, mental or social well-being, and did not have any direct diagnostic or therapeutic implications for the study women. Permissions for the study in Madagascar were obtained from the Ministère de la Santé, Comité d'Ethique N° 031-CE/MINSAN. The histopathological examinations of the Madagascan biopsies were performed by the Department of Pathology, Rigshospitalet, Copenhagen.

Study information was provided to the study populations in the local languages. Participation in the studies was voluntary, and free informed consent was obtained. Each study participant went through an information session with a female nurse or medical doctor about the purpose of the study, the procedures, the benefits and negative consequences of participation, confidentiality and the possibility to withdraw at any point during the investigations. To assess the participants' understanding, the clinician posed control questions to the women. The women were allowed to ask questions at any time. All investigations were performed after further oral permission re-ascertained by the physician before each step of the investigations and sampling.

Women who withdrew from study participation received the same possibility of health support. All women, including those who declined further investigations, were offered treatment with praziquantel. Treatment and follow-up for schistosomiasis, STIs, cancer and other conditions were given in collaboration with the local health care professionals. All non-endemic controls were followed up by the referring clinician in Norway.

There are some concerns associated with sampling of biopsies from women with genital mucosal *S. haematobium* infection. A biopsy of the female genital mucosa will disrupt the mucosal barrier and might increase the risk of HIV infection and other STIs. In these studies, only superficial biopsies were taken and vascular lesions were avoided (Dr. Kjetland,

personal communication). The participating women were not asked for HIV testing. Participating women were asked if they could abstain from sex or would consistently use condoms for two weeks following a possible biopsy. Biopsies were performed in women with lesions only and were confined to a few millimetres of the observed pathology. Occasionally, more than one biopsy was taken if the procedure did not discomfort the woman. Each biopsied woman was offered condoms in the examination room. All data, specimens, paper and digital documentation were coded. The connection list between the data and personal identities was stored securely.

5.0. Summary of articles

Paper I

Increased vascularity in cervicovaginal mucosa with *Schistosoma haematobium* infection
Jourdan PM, Roald B, Poggensee G, Gundersen SG, Kjetland EF.
PLoS Negl Trop Dis. 2011 Jun;5(6):e1170

Background. Female genital mucosa infected with *S. haematobium* may appear inflamed with abnormal mucosal blood vessels, and may provide mucosal points of entry for HIV. The aim of this study was to quantify and analyse the characteristics of the blood vessels surrounding *S. haematobium* ova in mucosa of the lower female genital tract.

Methods. Cervicovaginal biopsies with *S. haematobium* ova (n=20) and control biopsies (n=69) were immunostained with CD31 and von Willebrand Factor (vWF), which stain endothelial cells in capillary buds and established blood vessels, respectively.

Results. *S. haematobium* ova were found in 44% (20/45) of the Malawian women, with a median of 3 (IQR = 1-8) ova per HPF (Figures 14-16). We found a variety of tissue reactions surrounding *S. haematobium* ova, ranging from marked periovular granulation tissue to fibrosis. Genital mucosal tissue surrounding *S. haematobium* ova had a higher density of established blood vessels (vWF) compared to non-endemic healthy controls ($p = 0.017$). There was no significant difference in the density of capillary buds between women with cervicovaginal *S. haematobium* infection and non-endemic or endemic negative controls ($p = 0.44$ and $p = 0.95$, respectively). However, immunostain to CD31 identified significantly more granulation tissue surrounding viable compared to calcified ova ($p = 0.032$), and a tendency to increased density of capillary buds in tissue surrounding viable ova compared to healthy cervical mucosa ($p = 0.052$).

Conclusion. Female genital mucosa with *S. haematobium* ova was significantly more vascularised compared to the density of established blood vessels in healthy cervical tissue. These findings might add to the understanding of the pathophysiological mechanisms in female genital schistosomiasis and of the postulated association with HIV infection.

Paper II

HIV target cells in *Schistosoma haematobium*-infected female genital mucosa

Jourdan PM, Holmen SD, Gundersen SG, Roald B, Kjetland EF.

Am J Trop Med Hyg. 2011 85(6):1060–1064

Background. *S. haematobium* frequently causes genital lesions in women and, similar to sexually transmitted infections, might increase the risk of HIV transmission. The aim of this study was to quantify the HIV target cells in schistosome infected female genital mucosa.

Methods. The same cohort of cervicovaginal biopsies as in Paper I were immunostained for quantification of CD4+ T lymphocytes (CD3, CD8), macrophages (CD68) and dendritic Langerhans cells (S100 protein).

Results. The density of periovular CD4+ T lymphocytes and macrophages were significantly higher in women with than in women without *S. haematobium* infected genital mucosa (p = 0.034 and p = 0.018, respectively). The highest density of macrophages was found in genital mucosa with viable ova and in non-endemic chronic cervicitis; between which there was no significant difference (p = 0.32). The highest densities of CD4+ T lymphocytes were found in cervicovaginal tissue with calcified *S. haematobium* ova; however, the median densities of CD4+ T lymphocytes were similar in tissue with viable ova (n = 2) and in tissue with calcified ova (n = 11, significance testing not performed due to low sample sizes). There was no increased density of Langerhans cells in *S. haematobium* infected female genital mucosa in this study (p = 0.25). There was no significant association between age and the density of CD4+ T lymphocytes or macrophages in the two groups (p = 0.51 and p = 0.65, respectively). The median ages were similar in women with viable ova (n = 2) and in women with calcified ova (n = 11, significance testing not performed).

Conclusion. The findings indicate that *S. haematobium* may significantly increase the density of HIV target cells (CD4+ T lymphocytes and macrophages) in the female genitals, possibly affecting HIV susceptibility in infected women.

Paper III

Pathological mucosal blood vessels in active female genital schistosomiasis

New aspects of a neglected tropical disease

Jourdan PM, Randrianasolo BS, Feldmeier H, Chitsulo L, Ravoniarimbinina P, Roald B, Kjetland EF.

Int J Gynecol Pathol. 2012, in press

Background. *S. haematobium* infection is a prevalent, but neglected cause of gynaecological pathology in African women, and precise clinical descriptions are rarely available alongside histological sections. The aim of this study was to explore the histopathology of the mucosal vascular findings in female genital schistosomiasis.

Methods. This case study presents previously unreported histopathological correlates of colposcopic images of abnormal mucosal blood vessels in two women with genital mucosal *S. haematobium* infection.

Results. Colposcopic inspection showed schistosomal lesions surrounded by dilated, unevenly calibered and convoluted blood vessels in the cervicovaginal mucosa. In one case, histopathology revealed dilated and tortuous mucosal venules containing viable *Schistosoma haematobium* eggs surrounded by a thrombus (Figure 17). In another case, the histopathology showed a viable ovum penetrating the venule wall and the surrounding stroma was characterised by an intense host response with sprouting capillary buds (CD31) and endothelial cell activation.

Conclusion. Thrombosis might in part explain the pathogenesis of the abnormal mucosal blood vessels visible in female genital schistosomiasis. The characteristic blood vessels could be an indication of a persistent tissue reaction to female genital *S. haematobium* ova.

Paper IV

A simple method for precise quantification of immunohistochemically stained cells

Jourdan PM, Holmen SD, Kjetland EF, Sandvik L, Roald B.

Submitted

Background. Computer-assisted image analysis may facilitate and even improve the quantitative analyses of immunostained tissue sections. However, access to simple, valid and low-cost models is limited. The aim of this study was to explore the validity and efficiency of a user-friendly and low-cost method developed in Papers I and II.

Methods. Immunostained immune cells (CD3, CD8, CD68) and endothelial cell structures (vWF, CD31) in cervicovaginal tissue sections (n = 414) were quantified manually and by computer-assisted image analysis using ImageJ (National Institutes of Health, USA).

Results. Automated counts corresponded very well with manual counts of CD3, CD8 and CD68 immunostained cells (intra-class correlation coefficients (ICCs) = 0.93-0.98), but less well with manual counts of vWF and CD31 immunostained endothelial cell structures (ICCs = 0.62-0.68). Significant ICCs between manual and computer-assisted counts of immunostained cells were achieved following inclusion of a limited number of images, and inclusion of more than ten images did not significantly improve the correlation coefficients. Computer-assisted image analysis would, according to this model, be faster and more valid and reliable than cell manual counting for analyses of approximately 50 images or more.

Conclusion. The low-cost model for computer-assisted image analysis presented in this study is an efficient method for precise quantification of immunostained cells. The method should, however, be used with caution for more unevenly stained structures, such as endothelial cell structures. Further studies are warranted to explore other comprehensible and low-cost alternatives of computer-assisted image analysis.

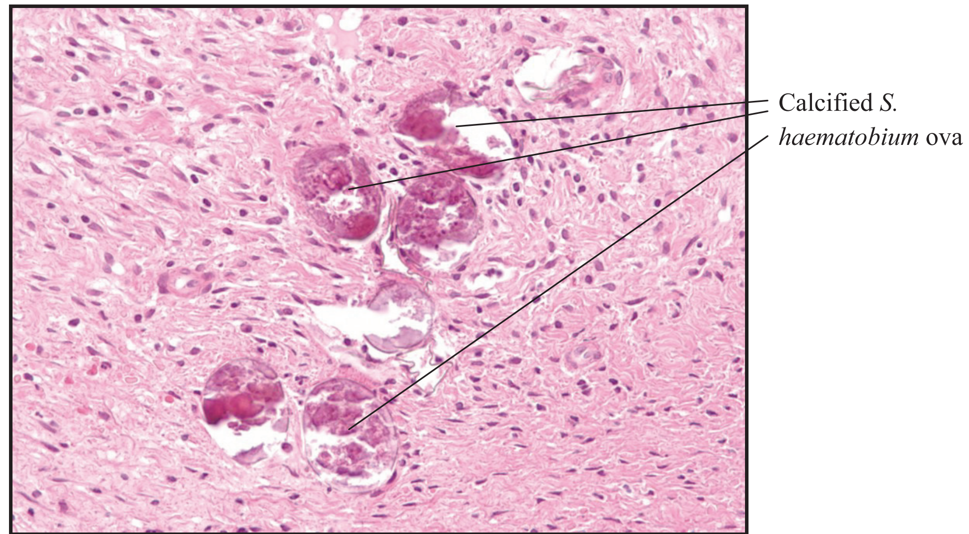


Figure 14. Moderate tissue reaction to *S. haematobium* ova in the cervical mucosa. A moderate tissue reaction to *S. haematobium* ova was seen in most cases. Note the cluster of calcified ova, characteristic of *S. haematobium* infection.

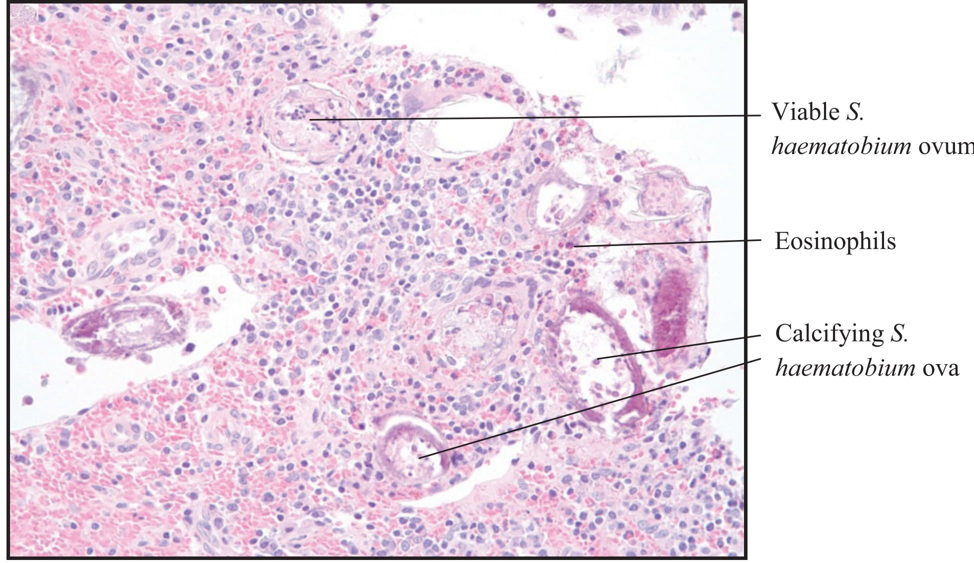


Figure 15. Granulation tissue and cellular infiltrate surrounding *S. haematobium* ova. In a few cases a heavy infiltrate of lymphocytes, plasma cells, eosinophils and macrophages dominated the periovular tissue reaction.

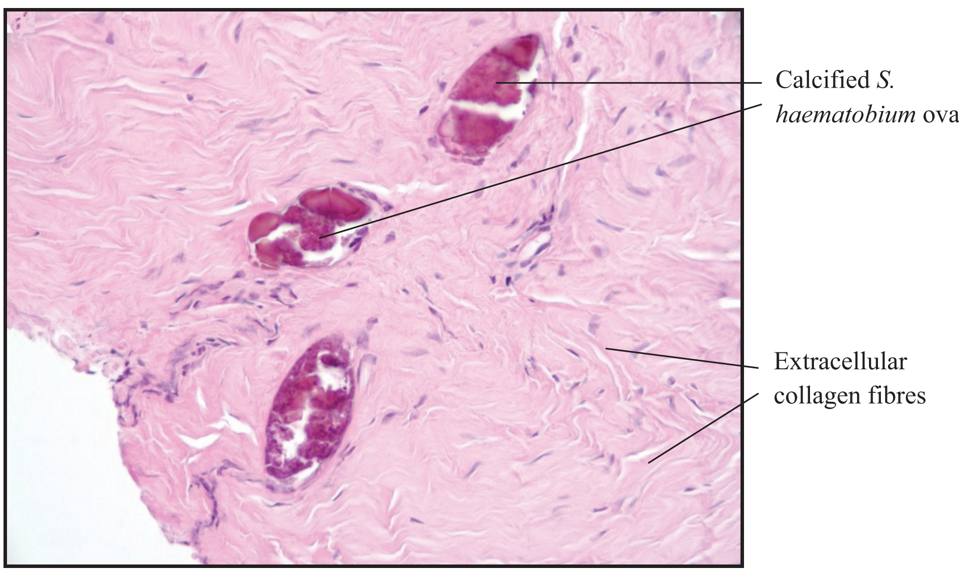


Figure 16. Fibrous tissue surrounding *S. haematobium* ova in the genital mucosa. Pronounced fibrosis with extracellular collagen fibres was seen in a few cases. Note the calcified *S. haematobium* ova and lack of cellular tissue response.

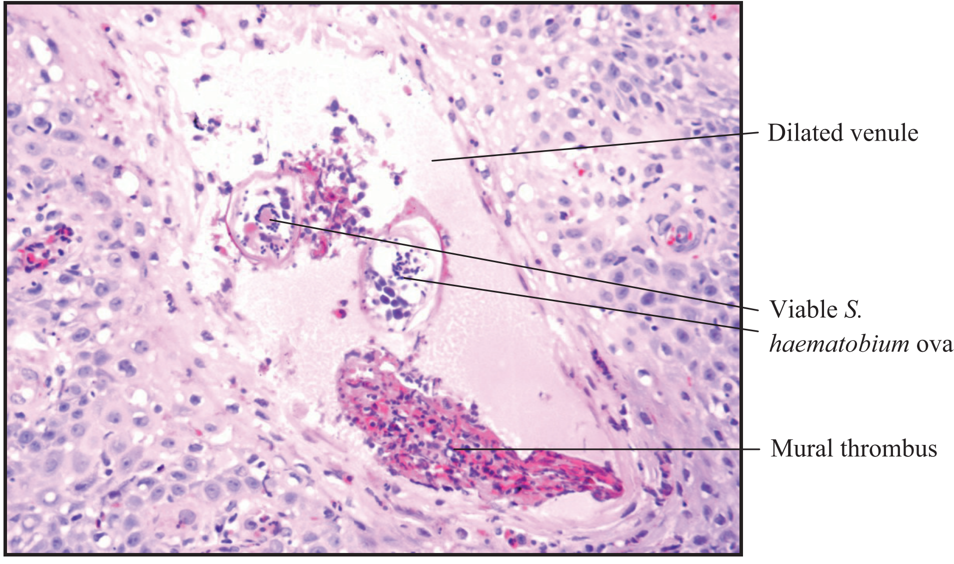


Figure 17. Thrombus surrounding viable *S. haematobium* ova in the cervical mucosa. Note the dilated venule and presence of eosinophils.

6.0. Discussion

This study demonstrates an increased density of blood vessels and HIV target cells in *S. haematobium* infected female genital mucosa. The clinically observed abnormal mucosal blood vessels could be an indication of a persistent tissue reaction to *S. haematobium* ova, and ova-associated thrombosis might in part contribute to the pathogenesis. Moreover, low-cost computer software may provide valid and reliable analyses of tissue specimens. *S. haematobium* infection is a frequent cause of pathology in the female genital mucosa [90,93,94,180]. Studies indicate that female genital schistosomiasis could increase the risk of HIV transmission; however, the potential pathophysiological mechanisms for such an association have hitherto not been sufficiently explored [133,135,136].

Periovular density of blood vessels

This study found a significantly higher blood vessel density in *S. haematobium* infected genital mucosa compared with healthy cervical tissue from non-endemic controls. The denser vasculature consisted mainly of established blood vessels, whereas cases with viable ova contained granulation tissue rich in sprouting capillary buds significantly more often than cases with calcified ova. Similar changes were seen in positive controls.

Some histopathological studies of female genital *S. haematobium* infection have described localised, increased vascularity in association with *S. haematobium* ova [86,87,119]. Studies of blood vessel proliferation in *S. mansoni* infection have suggested that soluble egg antigens (SEA) from viable ova induce neovascularisation by stimulating endothelial cell activation and proliferation [181,182]. Recent experimental studies indicate that blood vessel proliferation in *S. mansoni* not only accompanies hepatic fibrogenesis, but possibly also regression of fibrosis after treatment [183]. Wound healing is a variegated process depending on cause and extent of damage, but the formation of new blood vessels is not a commonly reported feature of the end stages [184,185].

Increased vascular density may impair the cervicovaginal tissue structure and lead to disruption of the genital mucosal barrier [143,186]. In clinical studies, cervical *S. haematobium* infection has been associated with bleeding tendency of the mucosal

membrane [93]. Further, endothelial cell surface proteoglycans have been reported to serve as HIV-1 receptors, and endothelial cells have been suggested to harbour HIV [187-190].

Periovular density of HIV target cells

Human histopathological studies have demonstrated a range of periovular immune cells in standard HE stained tissue specimens of female genital *S. haematobium* infection [86,87,114,118,127]. The present study indicates that *S. haematobium* infected mucosa may contain a higher density of HIV target cells; CD4 + T lymphocytes and macrophages than in uninfected mucosa. On the one hand, this may create conditions conducive to HIV transmission *to* women. On the other hand, in *S. haematobium* and HIV co-infected women, the findings could imply an increased risk of HIV transmission *from* women [136,191].

Deposition of viable *S. haematobium* ova may recruit HIV target cells to the genital mucosa, similar to STIs [140,142,133,139,144,148,152,192]. In this study, an increased density of macrophages was found in association with viable *S. haematobium* ova. Studies indicate that eosinophils are among the main effector cells in the host response to the deposition of *S. haematobium* ova [62,65]. Some *in vitro* studies suggest that eosinophils may be susceptible to HIV infection [193-197], however this could not be assessed in our study due to the limited sample size.

A moderate tissue reaction against *S. haematobium* has been seen in several histopathological studies [89,117,119,123,124,126,136,144,191]. The increased density of CD4+ T lymphocytes surrounding calcified ova in the present study suggests that chronic *S. haematobium* infection may sustain a long-lasting immune reaction in the tissue. Furthermore, the CD4+ T lymphocytes were associated with CD4+ cell apoptosis, indicating a persistent immune activation in the tissue [70]. Clinical data indicate that *S. haematobium* infection may cause chronic damage to the genital mucosa [93,96,198]. Moreover, the findings are in line with one study of *S. haematobium* infected mice, in which calcifying *S. haematobium* ova were capable of sustaining a persistent granulomatous reaction in the tissue [50].

We did not identify any association between the density of epithelial Langerhans cells and *S. haematobium* ova in the stroma. Recently, a study indicated that the function of dendritic

cells might be impaired in human *S. haematobium* infection [199]. Results from experimental studies indicate that *S. mansoni* infection activates dendritic cells by a modulated rather than a conventional pathway [200]. It was not possible to explore dendritic cell function or activation, or alternative dendritic cell markers in these tissue specimens due to formalin fixation and the limited biopsy sizes.

A recent experimental study on rhesus monkeys found an increased mucosal susceptibility to *de novo* HIV infection in acute *S. mansoni* infection [201]. Taken together, this suggests that both acute and chronic schistosomiasis could stimulate immunological tissue responses in the female genital mucosa facilitating HIV transmission. The findings might add to the understanding of potential mechanisms for the epidemiologically observed increased odds of HIV infection in two *S. haematobium* endemic areas of Africa [135,191,202].

Clinicopathological correlates

Gynaecological examinations of *S. haematobium* infected women may reveal dilated, tortuous and unevenly calibered blood vessels in the cervicovaginal mucosa [93]. These abnormal blood vessels are poorly described in pathological specimens. Post mortem and experimental studies of *S. mansoni* and *S. haematobium* infections suggest that abnormal, dilated blood vessels in schistosomiasis may be caused by the parasite worms or ova themselves, or by perivascular ova-induced granulomas and oedema [84,203-205]. In one of the study cases with largely dilated venules we demonstrated viable *S. haematobium* ova surrounded by a thrombus. The clinically observed abnormal mucosal blood vessels might be a result of endothelial damage, abnormal blood flow or thrombosis associated with ova deposition [84,203-206]. The clinical implications of these findings need to be further explored.

Computer-assisted image analyses

This study describes an efficient method for precise quantification of immunostained cells using low-cost software for computer-assisted image analysis. The method should, however, be used with caution for more unevenly stained structures such as endothelial cell structures. Our results correspond with a number of reports which suggest that computer-assisted quantitative analyses may be a valid and reliable way to quantify immunohistochemically

stained structures [166,169-172]. To our knowledge, this is one of few studies to have validated a simple and user-friendly model for computer-assisted image analyses of immunostained tissue.

The method described in this study was run on a highly endemic, but neglected disease in Africa and would be particularly useful for pathologists with access to large series of tissue samples but with limited resources for addressing important research issues. Further studies are warranted to confirm the findings and to explore other comprehensible and low-cost alternatives for computer-assisted image analysis.

Study limitations

The sample size of the Malawian population is small and the data are not normally distributed. Age could therefore not be corrected for in regression analyses. Age was not significantly associated with the density of established blood vessels, CD4+ T lymphocytes or macrophages when tested with non-parametric tests. A small sample size makes the findings prone to type 1 and type 2 errors. This complicates the interpretation of the results, especially those related to viable ova, and particularly when findings are non-significant.

The endemic biopsies originated from a study in rural Malawi and were 14 years old at the time of analyses, whereas most of the control biopsies were 10 to 20 years old. We cannot preclude that the process of fixation and duration of storage might have impaired the quality of antigen detection [207,208]. Furthermore, variations in the cervicovaginal tissue reactions between endemic and non-endemic populations may have affected the results [209]. However, the findings indicate that computer-assisted immunohistochemical quantification method may be valid for use on archival biopsies.

Although the tissue areas selected for analysis were considered representative for the respective biopsies, only a limited area of the entire biopsy was analysed. Recently, one study demonstrated that the analysis of small biopsies may be comparable to that of larger specimens; however, the small area selections may have affected the representativity of our data [210]. Furthermore, analyses were performed by two investigators, and, although a second senior pathologist was consulted when necessary, the interpretations may have differed from that of other pathologists. Although initially blinded for the immunostains, the

investigators might have been influenced by the tissue reactions apparent in the HE-stained sections in the selection process of the regions of interest.

In presumed negative cases, schistosome ova might have been located just outside the studied tissue sections, possibly affecting the histopathological results. Moreover, cervicovaginal biopsy procedures are cumbersome and the identification of pathology is dependent on adequate optical magnification and precisely sampled biopsies [93]. There was no true schistosomiasis negative endemic control group, as is the case for most studies on schistosomiasis [86,89,114,117-119,126], and the observed differences between cases and controls may therefore have been underestimated.

The density of CD4+ T lymphocytes was estimated by subtracting the number of CD8+ cells from the number of CD3+ cells on two consecutive 3.5 μm thick sections. Although the thickness of the sections were less than half the average diameter of a lymphocyte, the reported CD4+ cell density may to some extent have differed from its true value [178]. Our calculations of CD4+ T lymphocytes might have been slightly underestimated since antibodies to CD8 also recognise CD8 antigen on γδ T cells and natural killer cells (Dako).

We were not able to apply computer-assisted image analyses on endothelial cell structures in this sample due to inconsistent immunostaining. Previous studies indicate that CD31 and vWF may show heterogeneous immunostaining [211-214]. One study found increased endothelial cell activation and vWF expression in HIV infected individuals [215]. Moreover, endothelial properties may be affected by the menstrual cycle [216]; however, this could not be assessed in this study due to a limited sample size.

The HIV prevalence in Malawi is estimated to have been approximately 10% of the population at the time of sampling (1994) [217]. Infection with HIV may have affected the genital mucosal levels of CD4+ T lymphocytes [218]. HIV diagnosis was not a part of the original project and could therefore not be performed. Moreover, STIs may affect the pathogenesis in *S. haematobium* infected genital mucosa [93]. Apart from histopathologically apparent HPV infection, STIs could not be corrected for in the original study. Other factors that could have affected the immunological tissue processes, such as microtrauma and menstrual cycle, could not be adequately corrected for in this study [219].

Pathogenesis in female genital *S. haematobium* infection

In line with previous reports, this study found a variety of tissue reactions surrounding *S. haematobium* ova in the genital mucosa ranging from marked granulation tissue with potent eosinophils surrounding viable ova, to waning inflammation, calcified ova and fibrosis [86,87,89,114,117-119,123,124,126,127]. Fibrosis has by some been regarded as the end-stage pathology in schistosome infected tissue [31]. In our study, there was an association between calcified ova and HIV target cells, whereas we found no significant association between calcified ova and fibrosis. The latter must be interpreted with caution as biopsies were directed at clinical pathology, and as the small sample size may lead to type 2 errors.

The pathogenesis of female genital *S. haematobium* infection is still not fully understood. Prospective studies of human infection have not been performed and the sequence of pathological processes has not been adequately explored [220]. A clinicopathological study of *S. haematobium*-infected chimpanzees suggested that pathology in *S. haematobium* infection develops through polypoid patches to fibrous patches and sandy patches in the urinary and gastrointestinal tract [29]. A number of terms have been used to describe the various disease stages observed in human autopsy and surgical specimen studies; such as 'exudative', 'productive' and 'sclero-cicatricial' stages [87] and 'active' and 'inactive' stages of disease [23,27,28,30,118].

Our findings suggest that any tissue reaction to *S. haematobium* ova, viable or calcified, should be regarded as schistosomal disease. Thus, we believe that terms such as 'active' and 'inactive' do not adequately represent the potential clinical implications for the patient. In spite of the apparent lack of tissue reaction in some cases of female genital mucosal *S. haematobium* infection, our findings suggest that the histopathological changes could affect HIV susceptibility and morbidity in co-endemic areas [89,117,119,123,124,126,136,191].

The immunological host response to schistosome infection is complex and may be affected by a number of factors, including *in utero* sensitisation, the site of ova deposition, the frequency and degree of exposure, the duration and intensity of infection, and host factors, including genetic predisposition, nutrition, anti-schistosomal treatment and co-infections with other infectious agents [19,44-49]. Some studies have demonstrated increased urinary *S. haematobium* ova excretion in young children; a peak in teenagers and young adults and a

fall in older age groups; suggesting that the respective pathological processes may be age-dependent [27,28,221-224]. However, others have reported a constant presence of *S. haematobium*-associated lesions across different adult age groups [93,94]. Although urinary ova excretion may decrease following anti-schistosomal treatment, *S. haematobium* induced urinary or genital mucosal lesions may remain refractory to such treatment [225-229].

Although a disputed matter, epidemiological and clinical data suggest that individuals in endemic areas may acquire immunological resistance to re-infection with *S. haematobium* [47,48,230-232]. Cheever *et al* suggested that "the pathologic effect of a given number of eggs per gram tissue varies greatly with the degree of host reaction to the eggs" [33]. The systemic immunological status may affect the local tissue reaction, and we hypothesise that the various tissue reactions to *S. haematobium* ova depend on the current immune status of the infected individual and the respective stage of immune adaptation to *S. haematobium* ova (Table 6 and Figure 18) [42]. The initially viable ova may thus be destroyed by macrophages and eosinophils (acute reaction), remain and calcify in the tissue, or possibly pass through and out of the tissue (chronic reaction). We would argue that the histopathological reactions to *S. haematobium* ova are pivotal in understanding the clinical implications of infection for the individual patient and the appropriate timing of treatment.

	First infections prior to immune modulation	**Re-infections and gradual immune modulation**
Main immune cells involved	Macrophages Eosinophils	Lymphocytes
Clinical manifestations	Polypoid patches, rubbery nodules Abnormal mucosal blood vessels and contact bleeding	Sandy patches Fibrous patches? Abnormal mucosal blood vessels and contact bleeding
Reversibility?	Possible	Chronic changes probable

Table 6. The suggested clinicopathological reactions to *S. haematobium* ova in the female genital mucosa.

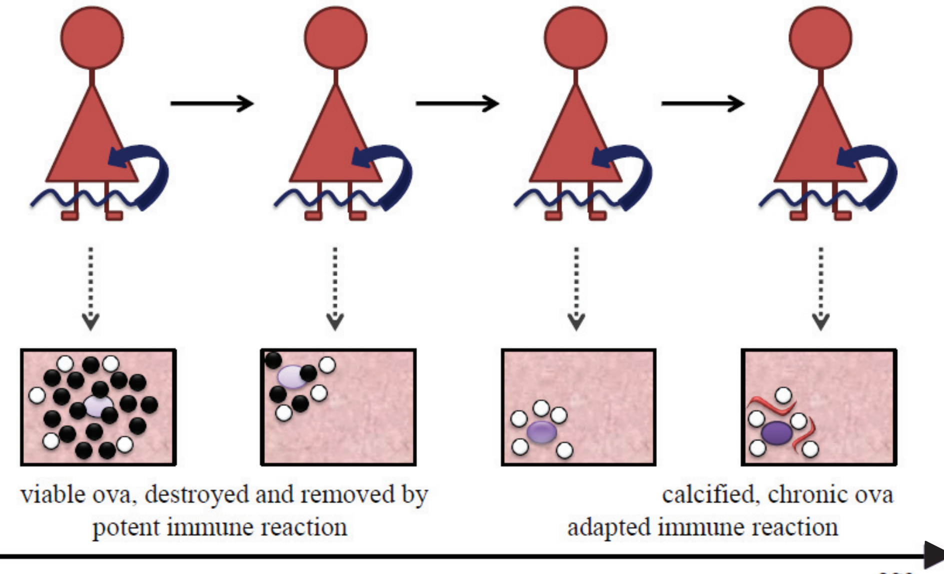


Figure 18. A model for immunopathogenesis in *S. haematobium* female genital tissue. Immune adaption to *S. haematobium* infection might result in various histopathological reactions, depending on a number of factors including age, number of re-infections, anti-schistosomal treatment and the individual genetic profile. Effector cells (black) such as macrophages and eosinophils, and chronic immune cells (white) such as lymphocytes may influence the clinical implications and susceptibility to HIV infection. Established blood vessels (far right) may be found in association with calcified *S. haematobium* ova.

Male genital schistosomiasis and HIV susceptibility

Male genital schistosomiasis is a highly neglected tropical disease and is poorly understood [126,233]. *S. haematobium* infection may cause lesions in all parts of the male genital tract, and may have a role in HIV transmission in co-endemic areas [84,126,191,234]. Genital ulcer disease may put men at high risk of HIV infection [235], and further understanding of the possible implications of male genital schistosomiasis is urgently needed.

7.0. Conclusions and perspectives for future research

In conclusion, this study indicates that acute and chronic *S. haematobium* infection of the female genital mucosa might facilitate HIV transmission. Increased vascularity and density of CD4+ T lymphocytes surrounding calcified ova suggest that *S. haematobium* infection could create a long-lasting HIV susceptibility in chronically infected women. Characteristic, abnormal mucosal blood vessels might be a sign of persistent tissue reactions to ova. Histopathological and clinicopathological studies are needed to validate the findings and to further understand the pathogenetic mechanisms in female genital *S. haematobium* infection.

Biopsy studies, including autopsy digestion and analyses of surgical specimens, should be performed in order to further explore the pathophysiological mechanisms in female genital *S. haematobium* infection. A better understanding of genital schistosomal disease staging and identification is warranted. In order to make recommendations regarding treatment, the effect of patient age and duration of infection should be explored. Furthermore, features associated with HIV susceptibility and infection should be explored, such as the role of immunological factors; immune activation, HIV co-receptors and Th17 cells, and the barrier function of the genital mucosal membrane.

Clinicians working in *S. haematobium* and HIV co-endemic areas need to be aware of the clinical implications of female genital schistosomiasis in order to give appropriate advice and treatment. Present mass treatment campaigns in schistosomiasis endemic areas should be followed by targeted clinical investigations of sexually active females to explore whether the clinical findings could be used to monitor the effect of intervention.

Finally, appropriate studies of male genital schistosomiasis, as well as studies of genital schistosomiasis due to *S. mansoni*, should be included in the targeted efforts to explore the possible HIV susceptibility in schistosomiasis endemic areas.

8.0. References

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Paper I

Increased Vascularity in Cervicovaginal Mucosa with *Schistosoma haematobium* Infection

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Abstract

Background: Close to 800 million people in the world are at risk of schistosomiasis, 85 per cent of whom live in Africa. Recent studies have indicated that female genital schistosomiasis might increase the risk of human immunodeficiency virus (HIV) infection. The aim of this study is to quantify and analyse the characteristics of the vasculature surrounding *Schistosoma haematobium* ova in the female genital mucosa.

Methodology/Principal Findings: Cervicovaginal biopsies with *S. haematobium* ova (n = 20) and control biopsies (n = 69) were stained with immunohistochemical blood vessel markers CD31 and von Willebrand Factor (vWF), which stain endothelial cells in capillary buds and established blood vessels respectively. Haematoxylin and eosin (HE) were applied for histopathological assessment. The tissue surrounding *S. haematobium* ova had a higher density of established blood vessels stained by vWF compared to healthy controls ($p = 0.017$). Immunostain to CD31 identified significantly more granulation tissue surrounding viable compared to calcified ova ($p = 0.032$), and a tendency to neovascularisation in the tissue surrounding viable ova compared to healthy cervical mucosa ($p = 0.052$).

Conclusions/Significance: In this study female genital mucosa with *S. haematobium* ova was significantly more vascularised compared to healthy cervical tissue. Viable parasite ova were associated with granulation tissue rich in sprouting blood vessels. Although the findings of blood vessel proliferation in this study may be a step to better understand the implications of *S. haematobium* infection, further studies are needed to explore the biological, clinical and epidemiological features of female genital schistosomiasis and its possible influence on HIV susceptibility.

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Introduction

Schistosomiasis is the most important parasite infection in the world after malaria [1]. Of the estimated 780 million people exposed to this fresh water parasite, 85 per cent live in Africa. *Schistosoma haematobium* and human immunodeficiency virus (HIV) are co-endemic in large parts of this area [2,3]. In a recent cross-sectional study from Zimbabwe a near 3-fold increased odds for HIV infection was found in women with genital schistosomiasis [4].

The lower female reproductive tract is a major entry site in HIV transmission, and is also a common site for *S. haematobium* oviposition [5–7]. The *S. haematobium* infected cervix appears inflamed with abnormal mucosal blood vessels, contact bleeding and pathognomonic lesions named sandy patches [7–9]. It has been suggested that products from schistosome ova (*S. mansoni*) may induce endothelial cell proliferation and activation [10,11]. Similar to sexually transmitted infections, it has been postulated that female genital schistosomiasis may provide mucosal points of entry for HIV [12,13].

The aim of this study is to combine immunohistochemical protein detection of endothelial cells with histopathological evaluation to quantify and analyse the characteristics of the blood vessels surrounding *S. haematobium* ova in biopsies of the lower female genital tract.

Methods

Ethics statement

Permissions for the histopathological and immunohistochemical investigations of anonymised archival Malawian and Norwegian biopsies, without additional consent from the study subjects, were granted by the National Health Science and Research Committee of Malawi (2009/NHSRC #620) and the Norwegian Regional Ethics Committee (2009/1250a). The permissions were based on the fact that the proposed analyses did not require identifiable

Author Summary

Schistosomiasis is a fresh water parasite infection that affects millions of people, especially in Africa. Recent knowledge about the genital manifestations of schistosomiasis; especially its possible association with human immunodeficiency virus (HIV) infection, has led to increased focus on this neglected tropical disease. Millions of women remain undiagnosed for genital schistosomiasis, and may suffer from abnormal mucosal blood vessels, contact bleeding and lesions named sandy patches. This study analyses a unique selection of female genital biopsies containing parasite eggs. Protein detection and standard histopathological assessment are combined to quantify and study the characteristics of the mucosal blood vessels surrounding the eggs. Our results show that the genital mucosa with parasite eggs is more vascularised compared to healthy tissue, and that viable eggs tend to be surrounded by proliferating blood vessels. These findings have not yet been correlated directly to clinical manifestations. Further studies are needed in order to provide clinical advice on the risks and consequences of mucosal lesions particular to female genital schistosomiasis.

information or history, have any direct relevance to the physical, mental or social well-being, or have any direct diagnostic or therapeutic implications for the study women.

The majority of the Malawian women who volunteered in the 1994 study were illiterate [8]. Study information was provided in Yao and Chichewa (the local languages) and free informed oral consent was obtained. Following consent, all women who had urinary schistosomiasis were offered gynaecological examination. Consent was reascertained orally by the physician before each step of sampling and investigations. Treatment and follow-up for sexually transmitted infections, cancers and other complaints were done in collaboration with the physicians in Mangochi District Hospital [8]. The women were not asked for HIV testing. All women, including those who declined further investigations, were offered treatment with praziquantel. All non-endemic controls were followed up by the referring clinician in Norway.

Study subjects

As described previously [8], biopsies from the cervix and/or vagina were sampled from 61 women with urogenital *S. haematobium* infection in Mangochi District Hospital in Malawi in 1994. In short, sexually active women between 15 and 49 years of age present in the out-patient department, irrespective of whether they were patients or next of kin, were invited to submit urine samples. Women with *S. haematobium* ova in the urine were invited for further interviews and gynaecological examinations. Samples including biopsies were taken from observed lesions or at random if no lesions were present. Women without urinary schistosomiasis were excluded.

An overview of the study groups is given in Figure 1. The cases with *S. haematobium* ova in genital tissue included 17 cervical and three vaginal biopsies. The histopathological changes and ova viability were similar in the cervical and vaginal biopsies, and the biopsies were therefore analysed together. Malawian women with urinary schistosomiasis who were found not to have *S. haematobium* ova in the biopsy specimen served as endemic negative controls.

Non-endemic Norwegian control biopsies were selected by searching the database from 1998 for key word combinations of the anatomical site 'cervix uteri' and the morphological diagnoses 'cervicitis' and 'normal morphology'. Women included in the negative non-endemic control group had histologically normal uterine ectocervices with intact tissue architecture. Excluded were endocervical biopsies and biopsies showing signs of pathology, i.e. more than 10 inflammatory cells per high-power field, non-specific vessel proliferation, epithelial hyperplasia, polyps, cysts, atypical or dysplastic changes, or signs of human papilloma virus (HPV) infection (i.e. clustered koilocytosis and dyskeratosis).

Women included in the positive non-endemic control group had chronic non-specific cervicitis; i.e. biopsies with a distinct, generalised infiltrate dominated by lymphocytes and/or plasma cells. Excluded were endocervical biopsies with more than 20 granulocytes or 5 eosinophils per high-power field and specimens with signs of HPV infection, erosion, ulcerations, granulation tissue or extra-vascular erythrocytes.

Immunohistochemistry and histopathology

The biopsies were fixed in formalin, routinely processed, embedded in paraffin, sectioned and stained. Findings from the histological examination of haematoxylin and eosin (HE)-stained sections have been published previously [14].

For the analyses in this study, 3.5 µm thick serial sections of the included specimens were cut and placed on SuperFrost slides (Menzel-Gläser, Braunschweig, Germany) for HE-stain and on SuperFrost Plus slides (Menzel-Gläser) for immunohistochemical stains. The immunohistochemical stains were performed using Benchmark XT, Antibody diluent (251-018) and Detection Kit Ventana ultraView Universal DAB (760-500) (Ventana Medical Systems, Inc., Tucson, Arizona, USA), an automated immunostain system based on the ABC avidin-biotin-peroxidase method, including negative and positive controls. For identification of blood vessels, two endothelial cell markers were used; mouse monoclonal antibody CD31 (clone JC70A, Dako Denmark AS, Glostrup, Denmark) and rabbit polyclonal antibody von Willebrand Factor (vWF) (Ventana). All slides were examined microscopically for immunohistochemical antigen detection combined with histological identification.

Microscopy and image analysis

The sections were examined using a Nikon Eclipse 80i microscope and photographed at 40× objective magnification, obtaining 1600 by 1200 pixels colour images using a SPOT Insight 2 Megapixel Firewire Color 3-shot digital camera attached to the microscope and a Hewlett-Packard Compaq stationary computer.

The morphological and immunohistochemical analyses were performed in a standardised manner. First, the HE-stained sections were evaluated. Ova were defined as 'viable' if miracidia with eosinophilic glands or germinal cells were identified [15], whereas ova containing dark purple stain identified histologically as calcification were defined as 'calcified'. The histopathological tissue reaction was defined as 'granulation tissue' if dominated by neovascularisation with activation of endothelial cells and by immature fibroblasts. The tissue reaction was defined as 'fibrosis' if dominated by collagen rich stroma with scant mature fibroblasts.

A high-power field (40× objective magnification) of each HE-stained section containing *S. haematobium* ova was photographed with the ovum or ova located centrally. The area of tissue surrounding the ovum or ova in one such photograph was defined as 'periovular'. In the controls, one high-power field of a subepithelial area was photographed, as this is the most common location for oviposition [15,16]. This area was representative for the pathologist's overall diagnosis of the section. In order to analyse the same area in the consecutive immunohistochemically stained serial sections of each biopsy, the near-exact same areas,

Original Malawian study population with urinary schistosomiasis

Endemic controls: no ova found in genital tissue (n=25)

Cases: *Schistosoma haematobium* ova in genital tissue (n=20)

Excluded:
- insufficient tissue for further analyses (n=1)
- ova found in previous but not present study (n=15)

Non-endemic Norwegian controls

Negative controls: healthy cervical tissue (n=29)

Positive controls: chronic non-specific cervicitis (n=15)

Figure 1. Overview of the study groups.
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identified by histological anatomical structures, were photographed. In areas where the morphology of the immunohistochemically stained section differed from the HE-stained section, the closest approximation was photographed. All selections were controlled by an experienced pathologist (BR).

Immunostained blood vessels (by vWF and CD31) were counted in accordance with pre-established criteria [17,18]. 'Capillary buds' were defined as vessel structures without an identifiable lumen or periendothelial structures, whereas all other stained vessel structures were counted as 'established blood vessels'. The density of capillary buds and vessels were calculated per mm^2. In sections with more than 300 capillary buds per mm^2, the numbers were truncated to 300. Each photograph was counted manually and one in ten randomly selected photographs were quality controlled. Discrepancies were resolved by consensus, if necessary after consulting a second senior pathologist. Finally, each photograph was recounted.

Statistical analyses

The statistical analyses and sample size estimation were performed with SPSS version 16.0 and PS Power and sample size calculations version 2.1.31. Most variables were not normally distributed and non-parametric tests were therefore used when studying associations. Medians and interquartile ranges were used to describe the results. The Mann-Whitney U and Kruskal-Wallis H tests were applied where appropriate. For the calculation of odds ratio (OR), it was necessary to tertilise the not normally distributed variables. Spearman's rank correlation coefficient was used when studying associations between continuous variables. Intra-observer variability was determined by calculating the intra-class correlation coefficient (ICC) after log-transformation of the data. A 5% significance level was used throughout.

Results

The median age of the Malawian patients was 23.5 years (interquartile range (IQR) = 20.0–31.5), and the median age of the Norwegian control patients was 44.0 years (IQR = 30.5–55.5). In the biopsies with *S. haematobium* ova, the median number of ova per high-power field was 3 (IQR = 1–8), of which 80 per cent were calcified. Most ova were localised in the subepithelial tissue, whereas only a few ova were found deeper in the stroma. Calcified and viable ova were found together in the same biopsy in one patient only. No schistosome worms were seen.

Figures 2 and 3A show the distributions of established blood vessels and capillary buds respectively per mm^2 genital tissue in the four study groups. The periovular tissue contained significantly

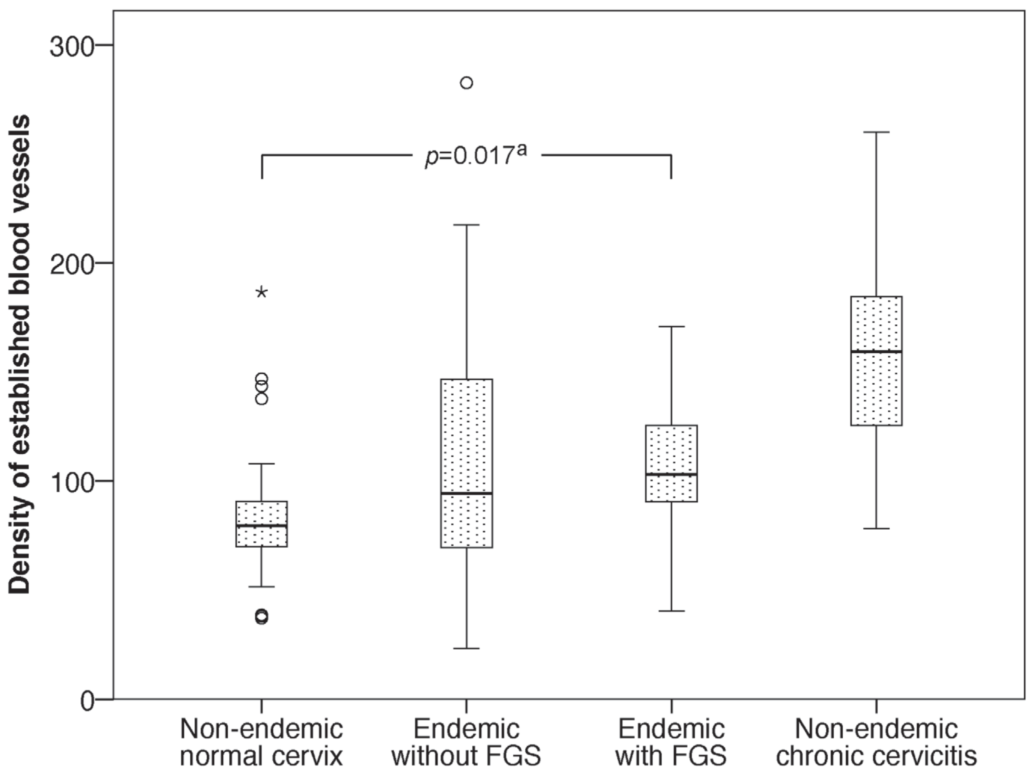

Figure 2. Density of established blood vessels per mm^2 in the four study groups. Established blood vessels stained with von Willebrand Factor. FGS = cervicovaginal tissue with *S. haematobium* ova. [a]Comparison of women with FGS and non-endemic women with normal cervical tissue.
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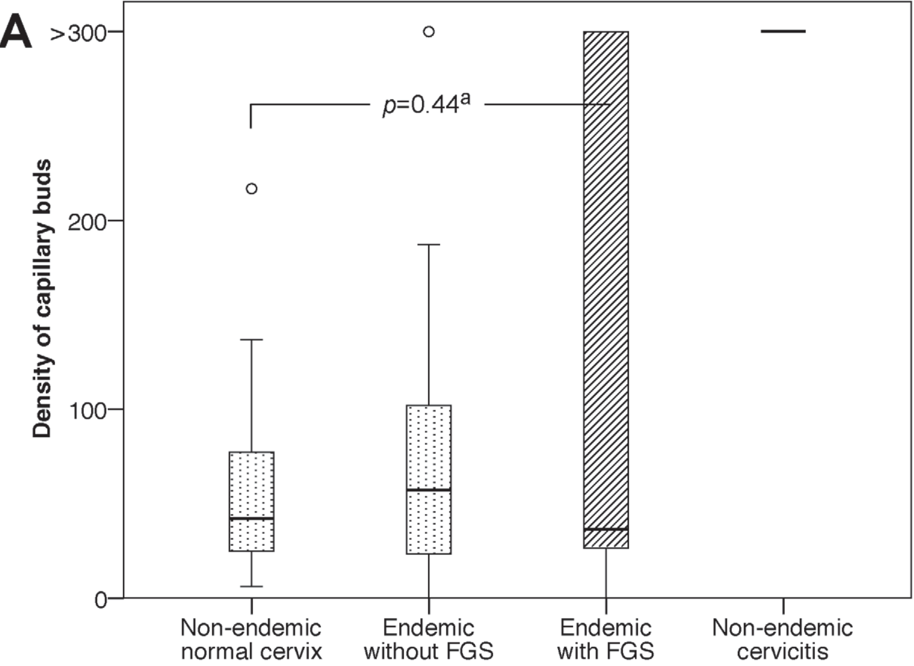
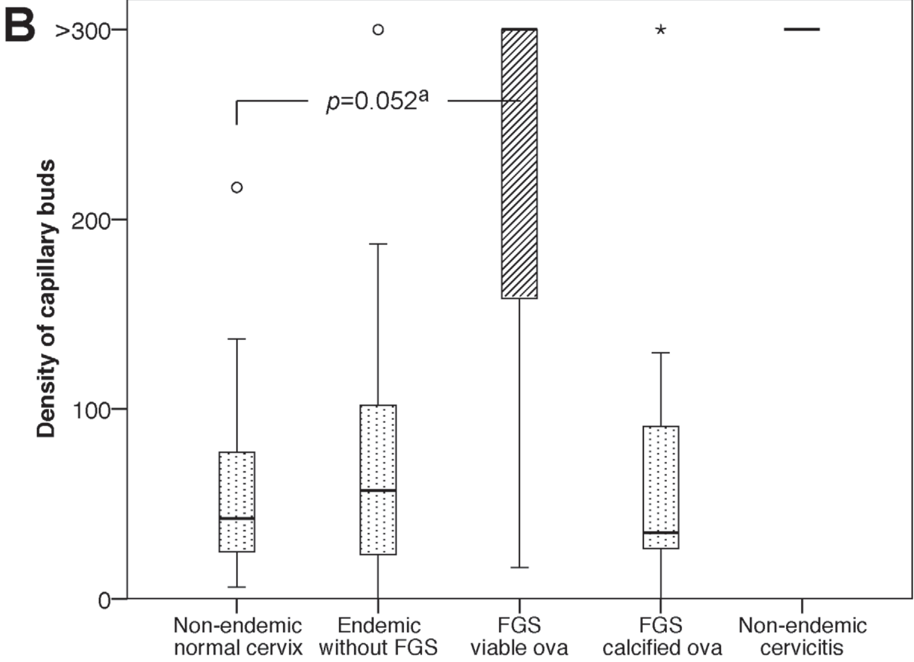

A
>300
200
100
0
p=0.44a
Density of capillary buds
Non-endemic normal cervix
Endemic without FGS
Endemic with FGS
Non-endemic cervicitis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0
p=0.052a
Density of capillary buds
Non-endemic normal cervix
Endemic without FGS
FGS viable ova
FGS calcified ova
Non-endemic cervicitis

Figure 3. Density of capillary buds. A. Density of capillary buds per mm^2 in the four study groups. Capillary buds stained with CD31; counts were truncated at 300 buds per mm^2 for technical reasons. All non-endemic women with chronic cervicitis had capillary bud densities above 300 per mm^2. FGS = cervicovaginal tissue with *S. haematobium* ova. [a]Comparison of women with FGS and non-endemic women with normal cervical tissue. **B. Density of capillary buds per mm^2 tissue with viable versus calcified ova.**
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more established blood vessels compared with non-endemic healthy cervices ($p = 0.017$). However, ova viability was not significantly associated with the density of established blood vessels ($p = 0.40$). There was no significant difference in the density of established blood vessels between endemic biopsies with and without *S. haematobium* ova ($p = 0.51$).

As shown in Figure 3A, there were no significant differences in the density of capillary buds between the Malawian cases and the non-endemic or endemic negative controls ($p = 0.44$ and $p = 0.95$ respectively). However, there was a large variation in periovular capillary bud density. As shown in Figure 3B, there was a tendency towards a higher density of capillary buds surrounding viable ova compared with non-endemic healthy cervical tissue (OR = 13.0, Confidence Interval (CI) = 1.0–172.9, $p = 0.052$). Furthermore, viable ova were more often found in granulation tissue compared to calcified ova ($p = 0.032$). There was no association between calcified ova and fibrous tissue ($p = 0.53$). The main histopathological periovular tissue reactions are shown in Figures S1 and S2.

In the endemic cases with cervicovaginal *S. haematobium* ova, age was neither associated with the number of ova ($p = 0.45$) nor the viability status of the ova ($p = 0.50$). There was no significant difference in density of established blood vessels or capillary buds between tissue with viable ova and non-endemic tissue with chronic cervicitis ($p = 0.20$ and $p = 0.053$ respectively). All other study groups had significantly lower densities of established blood vessels and capillary buds than tissue with chronic cervicitis (data not shown).

The intra-observer reliability of counting established blood vessels and capillary buds were 0.93 (95% Confidence Interval (CI) = 0.90–0.95, $p<0.001$) and 0.88 (95% CI = 0.82–0.91, $p<0.001$), respectively.

Discussion

To our knowledge, this is the first study to analyse the quantity and characteristics of the mucosal vasculature surrounding female genital *S. haematobium* ova. Similar to non-specific chronic cervicitis, the mucosa of the Malawian women diagnosed with genital schistosomiasis was significantly more vascularised than healthy cervical tissue of non-endemic controls. The denser vasculature consisted of established blood vessels as opposed to currently active neovascularisation. However, cases with viable ova contained granulation tissue rich in sprouting blood vessels significantly more often than cases with calcified ova.

Similar to previous reports of genital and urinary schistosomiasis, we found a variety of tissue reactions surrounding *S. haematobium* ova; ranging from marked periovular granulation tissue to fibrosis [14–16,19–25]. Although fibrosis is regarded as the end-stage pathology in schistosome infected tissue [26], there was no significant association between calcified ova and fibrosis in our study.

This study has several limitations. Firstly, the sample size is small and the findings are prone to type 1 and 2 errors. Secondly, there may have been schistosome ova just outside the biopsy borders in presumed negative cases, which might explain why there was no significant difference in capillary density between endemic cases and controls in this study. As in most schistosomiasis studies, there was no true schistosomiasis negative endemic control group [27]. The differences between women with and without genital *S. haematobium* ova may therefore have been underestimated. Thirdly, the analyses may have been affected by the age of the endemic biopsies, and by endothelial cell activation or vWF which may be elevated in HIV positive individuals [28]. The HIV prevalence has subsequently been estimated to be approximately 10 per cent of the population in 1994 [2], but individual HIV diagnosis was not a part of the original project and could therefore not be done. Lastly, although the specimens were evaluated several times by two investigators who attempted to be objective, blinding was not possible due to the presence of schistosome ova in the tissue.

Previous studies on blood vessel proliferation and schistosomiasis have suggested that soluble egg antigens (SEA) from viable *S. mansoni* ova induce neovascularisation by stimulating endothelial cell activation and proliferation [10,11]. Recent experimental studies indicate that blood vessel proliferation in *S. mansoni* not only accompanies hepatic fibrogenesis, but possibly also regression of fibrosis after treatment [29]. To our knowledge, there have been no studies on *S. haematobium* and blood vessel proliferation.

Female genital schistosomiasis is associated with genital mucosal bleeding tendency; a clinical finding that might support the suggested HIV susceptibility in these women [30]. Endothelial cell surface proteoglycans have been reported to serve as HIV-1 receptors, and endothelial cells have even been suggested to harbour HIV [31–34]. Finally, an increase in microvessel density may impair the original tissue structure and lead to easy disruption of the genital mucosal barrier [35,36].

Women living in endemic areas are prone to high burdens of infection and frequent reinfections with *S. haematobium* [37]. The histopathological correlates of the clinical signs in female genital schistosomiasis, e.g. abnormal blood vessel proliferation and contact bleeding, have not yet been studied in detail [38]. It is therefore not known which clinical manifestations may increase the risk of reproductive tract morbidity or possibly HIV acquisition. Hence it is not yet possible to give clinical advice related to the risks and consequences of the typical lesions in female genital schistosomiasis. In order to determine the implications for the patients, further studies are needed to correlate the clinical signs to morphological and immunohistochemical findings.

In conclusion, this study shows that female genital mucosa infected with *S. haematobium* is significantly more vascularised than healthy cervical tissue. Viable schistosome ova are more often surrounded by highly vascularised granulation tissue compared with calcified ova. These results might contribute to improve the understanding of the pathophysiological mechanisms in female genital schistosomiasis and of the postulated association with HIV infection. However, further studies are needed to validate these findings, to study the immunological tissue reaction and to explore the clinical correlates of the various histopathological manifestations in female genital schistosomiasis.

Supporting Information

Figure S1 Section of uterine cervix with calcified *S. haematobium* ova. A. Histopathology Calcified schistosome ova (white arrows) with periovular collagenised fibrous tissue (long,

thin black arrows), scant mature fibroblasts (short, thin black arrows) and established blood vessels (thick black arrow). Haematoxylin and eosin (HE), 40× objective magnification. **B. Immunohistochemical detection of established mucosal blood vessels** Calcified schistosome ova (white arrows) with periovular established blood vessels (black arrows). von Willebrand Factor, 40× objective magnification.
(TIF)

Figure S2 Section of vagina with viable *S. haematobium* ova. A. Histopathology Viable schistosome ova and shell fragments (white arrows) surrounded by giant cells (long, thin black arrows), granulation tissue, i.e. endothelial cell proliferation and activation (E) and proliferation of immature fibroblasts (short, thin black arrows), and inflammation with marked eosinophilia (Eos). A tissue artifact (Art) traverses the lower half of the image. Haematoxylin and eosin (HE), 40× objective magnification. **B. Immunohistochemical detection of mucosal blood vessel budding** Viable schistosome ova (white arrows) with abundant periovular capillary blood vessel budding (black arrows). CD31, 40× objective magnification.
(TIF)

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Author Contributions

Conceived and designed the experiments: PMJ BR GP SGG EFK. Performed the experiments: PMJ BR. Analyzed the data: PMJ BR GP SGG EFK. Wrote the paper: PMJ BR GP SGG EFK.

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Paper II

Paper III

Paper IV